PROTEINASE ACTION

Symposia Biologica Hungarica 25

PROTEINASE ACTION

25

Akadémiai Kiadó, Budapest

PROTEINASE ACTION

Edited by

P. ELŐDI

(Symposia Biologica Hungarica 25)

This volume contains the material of the international conference held in August 1983 in Debrecen. It comprises 11 papers of researchers—mostly from European countries—engaged in the study of different proteolytic enzymes.

The first part discusses the mechanism of proteolytic enzymes with special regard to the plasmin action and its regulation. In the second part the regulating role of these enzymes as well as the intracellular proteinases are dealt with. In the next section the lectures are to be found which examine the significance of the natural and artificial substrate of small molecule inhibiting proteinase action. Finally, the isolation and mechanism of the proteolytic enzymes of plant and microbial origin are surveyed.

The papers include lively discussions rendering the book even more valuable from the scientific point of view.

AKADÉMIAI KIADÓ
BUDAPEST

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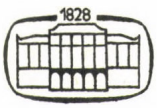

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Proceedings of the International Workshop August 29–31 1983 Debrecen, Hungary

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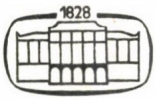

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INTRODUCTORY REMARKS

Ladies and Gentlemen,

Let me first express our sincere thanks to you for accepting our invitation and for participating in the present meeting in this small, quiet university town which some of you may have never heard of before. Our colleagues are often complaining that the huge international congresses and even the smaller national gatherings tend to be more and more costly and formal day by day. Therefore, the participants have less and less opportunity for intimate discussions.

One of our motives for organizing this meeting was to create quiet conditions and a calm atmosphere for exchanging ideas of mutual interest. This explains why we decided to invite only a small number of participants and to cover at least the expenses of their stay. The latter became possible with the generous help of the Hungarian Biochemical Society and some Hungarian pharmaceutical factories (REANAL and CHINOIN, Budapest; BIOGAL, Debrecen) to whom thanks are due.

Proteinases can be listed among the classical objects of biochemistry or rather among those of enzymology. Some of them, particularly the extracellular pancreatic enzymes had been described in detai' by the late 1960s. The intracellular enzymes, however, were investigated with much less intensity. As regards the importance of the latter enzymes, this situation seems to be inconsistent because Schoenheimer reported in 1935 already that a very intensive protein metabolism is continuously going on in the cells. Since then Schoenheimer's discovery has been treated in textbooks and manuals as a principle of biochemistry. It is obvious that protein turnover

can hardly go on without the participation of proteolytic enzymes.

The renaissance of the study of proteinase action began some 15 years ago. Nowadays proteolytic enzymes are investigated with a different aspect in view. They are known, for example, to play an important role in biological regulation either in a direct way by activating inactive proteins and inactivating active ones, or in an indirect manner by releasing biologically active peptides. The role played by proteolytic enzymes in the release of secretory proteins from the cells is also commonly known. The keen interest excited by the subject is therefore obvious.

The rapid growth in the knowledge of proteolytic enzymes has been promoted greatly by the development of research techniques. In addition, the integration of techniques applied in other fields of research, e.g., in physics, mathematics, chemistry, etc., into biochemical studies also enriched the technical inventory of protein research in the last decades. The new methods have opened up new territories and made research more fruitful. This enabled us to attempt to elucidate such delicate issues that could not even be thought of 10-15 years ago.

The topics covered by the papers presented at this meeting will also indicate this situation. We were lucky enough to bring together a colourful spectrum of papers covering a wide field of proteinase research. The speakers will sum up their most recent data on the mechanism of proteinase action in viruses, plants and in man in health and disease as well. In the days ahead we will have a chance to discuss topics ranging from the submolecular mechanism of proteinase action to the application of these results in the everyday practice, e.g., in diagnostics and treatment of patients.

I wish you a pleasant time at our meeting and a useful exchange of ideas, opinions which, hopefully, will stimulate our future research activities.

Thank you for your attention.

P. Elődi

CONTENTS

PROTEINASE INHIBITORS

GENERAL ASPECTS

Symposia Biologica Hungarica 25 (1984)

GENERAL PROBLEMS OF PROTEOLYTIC MECHANISM

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Proteolytic enzymes constitute one of the largest classes of biocatalysts. Despite their great diversity, these enzymes can be classified according to the structure of their catalytic apparatus, into four main groups, namely, serine-, thiol-, carboxylic- and metal-dependent proteases. Latest advances in X-ray crystallography have shed much light on the mechanism of action of proteolytic enzymes. However, some aspects common for all of them still need thorough investigation.

In this report I would like to bring your attention to the following questions:

1./ What is the function of catalytic groups of proteases, that is, do they act as covalent or as general base catalysts at the stage of nucleophilic attack of the substrate carbonyl?

2./ Does enzymatic hydrolysis of amides proceed through the tetrahedral intermediates?

3./ Is the formation of a productive enzyme-substrate complex and the dissociation of the reaction products a one- or a multistep process?

4./ What is the structure of the substrate in a productive complex?

All these problems are directly related to the efficiency and specificity of proteolysis.

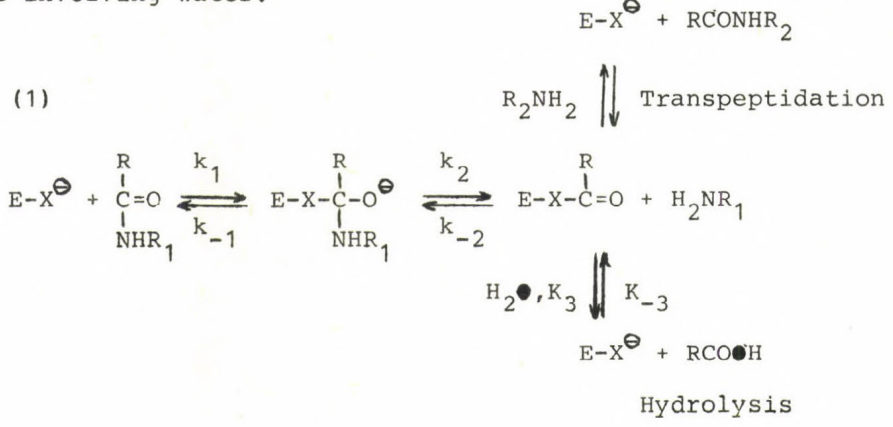
The first problem concerning the function of catalytic groups of proteases can be formulated otherwise, namely does the enzyme form covalent intermediate with the substrate fragments? There is no unequivocal answer to this question yet,

at least as far as amide hydrolysis is concerned, since in this case the intermediates are not accumulated in the system.

Recently a number of approaches have been developed in our laboratory which are aimed at identification of covalent intermediates and which allow to establish the type of catalytic action of various proteases (Antonov et al., 1978, 1979, 1981).

Both the nucleophilic catalysis (1) giving rise to a covalent intermediate and the general base catalysis (2) that is not completed by this intermediate formation, differ by a stage involving water.

(1)



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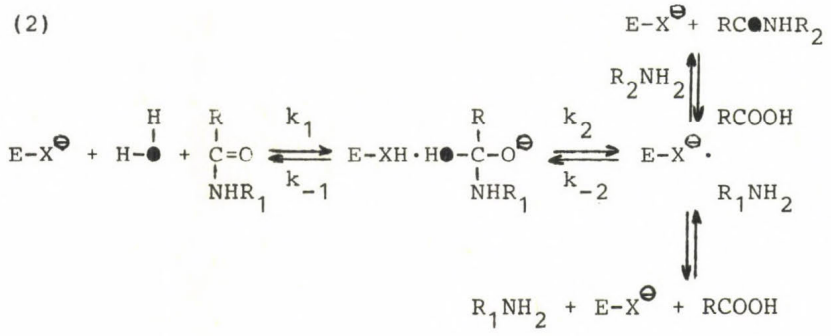
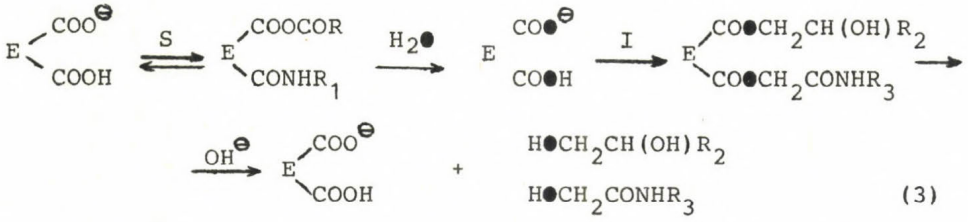


When the reaction is performed with heavy oxygen-labelled water it becomes possible to differentiate between these two mechanisms. During general base catalysis, when the rate of formation of the tetrahedral intermediate is higher than that

of its degradation, the heavy oxygen label will be incorporated into the substrate. When the reaction is in a thermodynamic equilibrium, the rate of the heavy oxygen incorporation into the substrate should be close to that of the amine product incorporation, irrespective of the rates ratio of the tetrahedral intermediate formation and degradation (Lyakisheva et al., 1973). Only in case of general base catalysis will the ^{18}O label be incorporated into the amide group of the transpeptidation or acyl transfer product. And, finally, the rate of the heavy oxygen exchange at the carboxyl of the acyl product would be increased by the amine product due to the synthesis and hydrolysis of the corresponding peptide, provided that the reaction occurs via a general base mechanism.

It should be mentioned that when the nucleophilic group is represented by a carboxyl ion of the enzyme, the cleavage of the "acyl" or "amino" enzyme at protein carbonyl moiety will result in heavy oxygen incorporation into the enzyme. To test this assumption we have elaborated a special procedure (Antonov, 1977; Antonov et al., 1980). After completion of the reaction in the heavy oxygen-labelled water the enzyme is subjected to the action of specific inhibitors (3) which form ester bonds with the active center carboxyls. The next step is mild alkaline hydrolysis and separation of the hydroxy-compounds where the heavy oxygen content is determined. In experiments with pepsin no label was detected in the enzyme.



(3)

Data from ^{18}O incorporation into the substrates and transpeptidation products, for all the four protease groups tested are shown in Table 1. It can be seen that the amide bond hydrolysis catalyzed by serine and thiol proteases occurs via a nucleophilic mechanism. However, carboxylic proteases as well

Table 1

^{18}O Content of the transpeptidation products and non-hydrolyzed substrates

Enzyme	Reaction studied	^{18}O Content in atom. %	
		sub-strate	trans-peptid. product
Chymotrypsin	$AcPheGlyNH_2 \rightleftarrows AcPheOH+GlyNH_2$	0	
	$2LeuLeuNH_2 \rightleftarrows LeuLeuLeu+LeuNH_2+NH_3$	0	0
Papain	$ZGlyNH_2+LeuGly \rightleftarrows ZGlyLeuGly+NH_3$		0
	$ZGlyOMe+LeuGly \rightleftarrows ZGlyLeuGly+MeOH$		0
Pepsin	$2LeuTyrNH_2 \rightleftarrows LeuLeu+2TyrNH_2$	13±4	65±12
Leucine amino-peptidase	$2LeuNH_2 \rightleftarrows LeuLeuNH_2+NH_3$	46±5	48±3
	$2[^{18}O]LeuNH_2 \rightleftarrows [^{18}O]LeuLeuNH_2+NH_3$		55±5
Thermolysin	$2LeuLeuNH_2 \rightleftarrows LeuLeu+2LeuNH_2$	52±5	47±5

Table 2

Rates of oxygen exchange

Enzyme	Substrate and additions	^{18}O content in atom. %	Maximal rate of exchange $M^{-1}min^{-1}$	$\frac{V_s}{V_{s+a}}$
Chymotrypsin	AcPheOH	35±3	$1.8 \cdot 10^{-5}$	
	$AcPheOH+AlaNH_2$	22±3	$0.8 \cdot 10^{-5}$	0.45
	$AcPheOH+GlyNH_2$	16±2	$1.0 \cdot 10^{-5}$	0.63
Pepsin	AcPheOH	13±2	$2.5 \cdot 10^{-5}$	
	AcPheOH+PheAlaAlaOMe	29±3	$5.6 \cdot 10^{-5}$	2.2
Leucine amino-Peptidase	LeuOH	21±1	$1.6 \cdot 10^{-3}$	
	$LeuOH+NH_4Cl$	19±1	$1.5 \cdot 10^{-3}$	0.9
	$LeuOH+LeuNH(CH_2)_2OH$	40±2	$3.5 \cdot 10^{-3}$	1.95
Carboxypep-tidase A	AcPheOH	18±2	$7.9 \cdot 10^{-6}$	
	AcPheOH+PheOH	40±3	$1.8 \cdot 10^{-5}$	2.3

as metal-dependent endo- and exopeptidases act as general base catalysts and do not form covalent intermediates with the substrate fragments. The same results have been obtained in the study of the rates of heavy oxygen exchange in the acyl products in the presence and absence of amines (Table 2).

Thus, according to their mechanism of action the enzymes uncer study and, probably, other enzymes hydrolyzing carboxylic acid derivatives, may be subdivided into two groups. To the first group we may relate serine and thiol proteases which act as nucleophilic or covalent catalysts, to the second one - carboxylic and metal-dependent enzymes functioning as general base catalysts.

It might be expected that in the case of the second group enzymes the heavy oxygen incorporation into the non-hydrolyzed substrate will point to the formation of a tetrahedral intermediate. However, a detailed analysis of the kinetics of this process corroborated our expectations (Kapitannikov, 1983). Indeed, it was found that in case of leucine aminopeptidase (Fig. 1) and pepsin the heavy oxygen incorporation occurs simultaneously and in a comparable degree with incorporation of labelled amine reaction product into the substrate. Presumably, the oxygen exchange is due to the acyl transfer to the amino product formed during hydrolysis. These data suggest that the commonly accepted viewpoint that the heavy oxygen incorporation into the substrate is inevitably associated with the formation of a tetrahedral intermediate cannot be applied to enzymatic hydrolysis. However, these results do not exclude the possibility of such formation.

Using quantum-chemical approaches, S. Alexandrov in our laboratory calculated the pK_a values for the leaving amino group at different stages of the planar amide conversion into a tetrahedron. It appeared that this value becomes comparable to that of the proton acceptor, imidazole, long before the completion of the tetrahedron formation (Fig. 2). Therefore, the reaction of amide hydrolysis is likely to occur, at least partly, via a S_N2 mechanism (Komiyama and Bender, 1979).

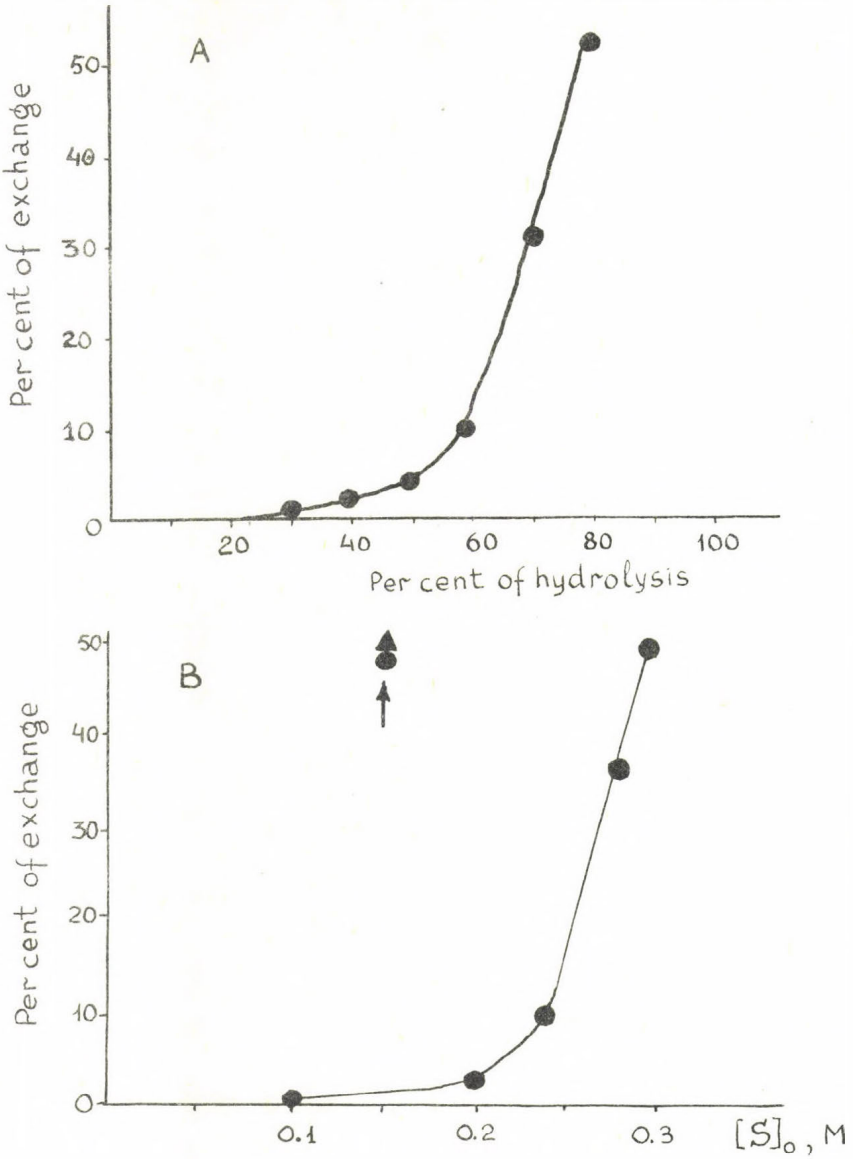


Fig. 1: Kinetic of ^{18}O incorporation into the leucine amide catalyzed by leucine aminopeptidase. A: Dependence of the exchange on the degree of the substrate hydrolysis [S]:0.3 M. B : Dependence of the exchange on the substrate concentration at 80 % of hydrolysis. Arrow indicates the exchange in the presence of 0.15 M of $^{15}NH_3$ (Circle - oxygen; triangle - nitrogen).

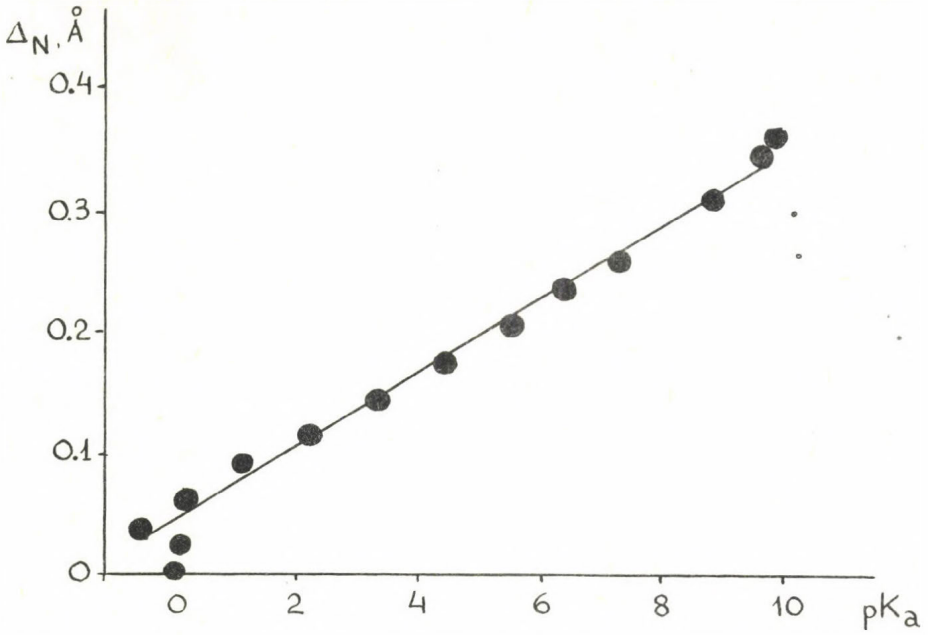


Fig. 2: Relation between the degree of pyramidalyzation of the amide bond and pK_a of the amino-leaving group. Calculated by S. Alexandrov (CNDO-2 method, programme GEOMO).

The observation that the enzymes incapable to form covalent intermediates can catalyze the transfer and transpeptidation reactions provides evidence for some important conclusions.

First, the nucleophilic "entrapment", an approach commonly employed in enzymology as a proof of the covalent nature of the reaction intermediates, seems to be inadequate in this case, because the transfer to the nucleophilic moiety may take place in the absence of covalent bond between the enzyme and the substrate fragment.

Second, here it is the nature of the enzyme complexes with the reaction products that comes to the foreground. The stability of the enzyme complexes with the reaction products whose dissociation constants can easily and directly be measured by routine methods, is incompatible with the high rates

observed during transpeptidation reaction (Antonov, 1980). The only possible explanation is that the dissociation of the reaction product occurs stepwise and that transpeptidation affects the "internal" (E_pP) enzyme-product complex (4).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_oS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_pS \overset{k_3}{\rightleftharpoons} E_pP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_oP \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + P \qquad (4)$$

In such a system the equilibrium is usually shifted toward the "external" complex (E_oP) and the apparent K_d value for the reaction product coincides with the true K_d of the external complex. The formation of the internal complex responsible for transpeptidation will be effective only during substrate conversion into the reaction product, while its formation from the free enzyme and the reaction product will be limited by the low rate of the external complex conversion into the internal one (k_{-4}). This hypothesis provides satisfactory explanation for the basic differences between transpeptidation and resynthesis.

Naturally, the internal enzyme-product complex is formed from the corresponding enzyme-substrate complex (E_pS), i.e. in this case the formation of the enzyme-substrate complex occurs in at least two stages, a situation similar to that observed during dissociation of the reaction products. This finding can be confirmed by numerous data from literature (for references see Antonov, 1980).

Now a question arises as what the structural differences between the internal and external complexes are. It is hardly likely that a complementarity between the enzyme and its substrate can be achieved in a one-step diffusion-controlled reaction. It seems more reasonable to assume that the substrate is at first "anchored" by the enzyme which thus reveals its primary specificity and only thereafter interacts with the secondary sites of the substrate to form a productive complex (Fig. 3).

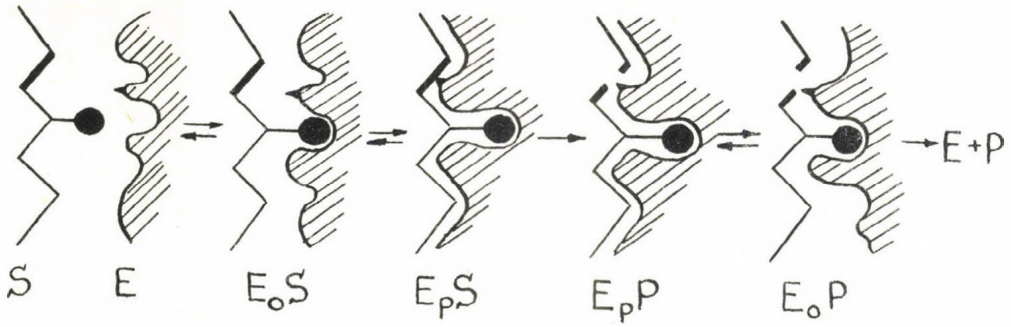


Fig. 3: Schematic representation of the stepwise binding of a substrate and dissociation of a product in the course of enzymic hydrolysis.

These suggestions allow to understand why the specificity of some enzymes is manifested either in the maximal rates or in the values of the binding constants. Both these parameters depend on the equilibrium between the external and internal enzyme-substrate complexes (see Scheme 4):

$$V = \frac{k_3 K_p}{1+K_p} [E]_o \quad \text{and} \quad K_{m(app)} = \frac{K_s}{1+K_p} \quad \text{where} \quad K_s = \frac{k_{-1}}{k_1}, \; K_p = \frac{k_2}{k_{-2}+k_3}.$$

When this equilibrium is shifted toward the external complex ($K_p<1$), the value of catalytic constant is changed while that of Michaelis constant is not, provided that the primary specificity of the enzymes (K_s) for all the substrate tested is the same. When the equilibrium is shifted toward the internal complex ($K_p>1$), the specificity is reflected on the values of the Michaelis constants. This assumption is in good agreement with the data obtained by comparing the apparent K_m and k_{cat} values for the structurally similar substrates of three different proteases (Fig. 4).

Although the data mentioned above clearly demonstrate the specificity of proteases, they fail to provide sufficient explanation of their efficiency. As the hypothesis on "charge

relay system" is not valid for serine proteases, and in case of carboxylic or metal-dependent proteases the substrate-attacking molecule is water, the nucleophility of catalytic groups of proteases is too low to explain the high rates of the reactions.

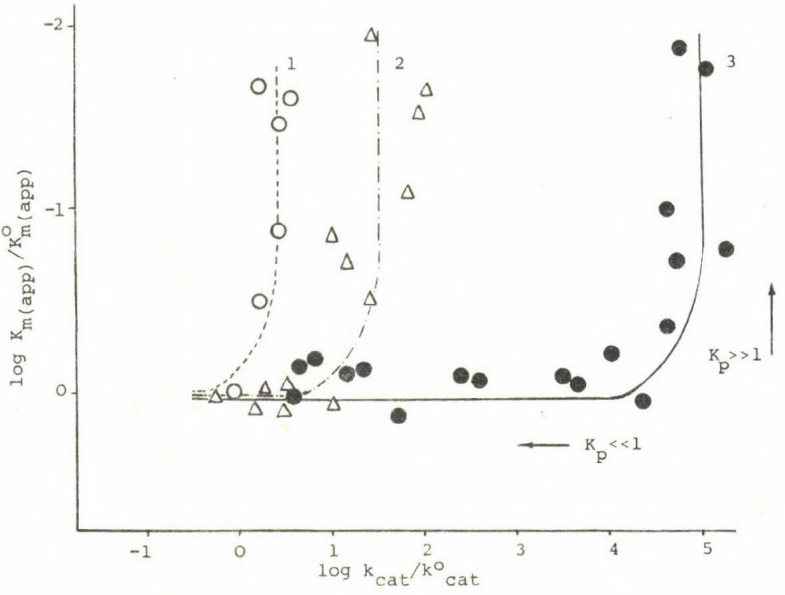


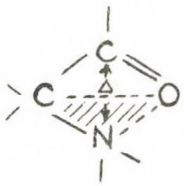
Fig. 4: Plots of log K_m (rel.) vs. log k_{cat} (rel.) for series of substrates of carboxypeptidase A (1), chymotrypsin (2) and pepsin (3). Data and references see Antonov (1980).

It is known (cf. Fastrez, 1977) that the main cause of the amide resistance to nucleophilic attack is the resonance stabilization. Evidence from X-ray crystallography obtained in the last few years suggest that in some protease-inhibitor complexes the potentially hydrolyzable amide bond is destabilized due to pyramidalization (Table 3). It might be assumed that partial destabilization of the hydrolyzable bond also takes place in productive enzyme-substrate complexes. The results of quantum-chemical analysis show that the degree of amide pyramidalization depends on the nature of the nucleophilic group and can rather be prominent, even when the nucleo-

phil is a long distance off the substrate carbonyl carbon (Fig. 5).

Table 3

Pyramidalization of the amide bond



Enzyme	Ligand	Bond	Δ, Å*	References**
Chymotrypsin	BPTI	Lys^{15}-Ala^{16}	0.38	a
Trypsin	BPTI	Lys^{15}-Ala^{16}	0.28	b
-	BPTI (free)	Lys^{15}-Ala^{16}	0.008	c
Elastase	$CF_3COLysAlaNHC_6H_4CF_3$-p	CF_3CO-NH	0.042	d
Carboxypeptidase A	GlyTyr	CO-NH	0.012	e
Thermolysin	$C_6H_5(CH_2)_2COPheOH$	CO-NH	0.06	f

* Calculated from the atomic coordinates of the complexes.

** Source of the atomic coordinates: a.) D. Blow, personal communication; b.) R. Huber et al. (1974); c.) R. Huber, personal communication; d.) D. Hughes et al. (1982); e.) F. Quiocho et al. (1971); f.) W. Kester, B. Matthews (1977).

If the resonance destabilization of the amide group in the enzyme-substrate complex does take place, any further nucleophilic attack will occur at very low activation barrier (Fig. 6).

In conclusion, I would like to emphasize that the concept presented here explains the efficiency of catalysis in terms other than strain or transition state stabilization. If we consider chemical transformation of a substrate as an elementary act, we will need to select the same structural forms of the

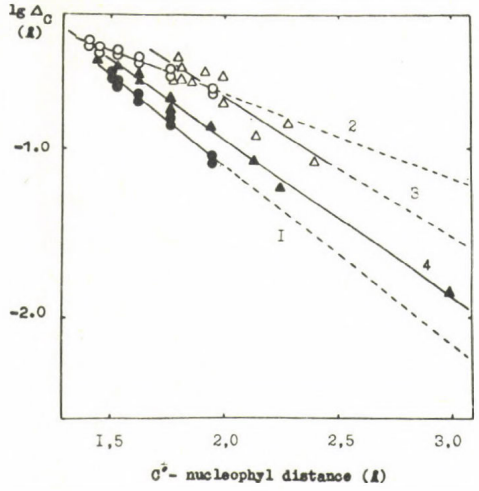


Fig. 5: Dependence of the degree of pyramidalyzation of the amide bond on the distance between the carbonyl carbon of the substrate and the nucleophyl for CH_3OH (1), CH_3O^- (2), CH_3S^- (3) and H_2O-$HCOO^-$ system (4). Calculated by CNDO-2 method.

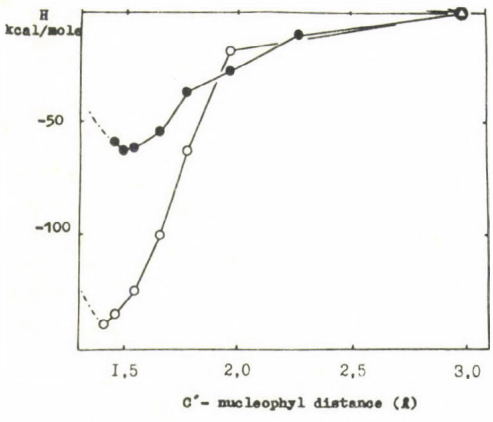


Fig. 6: Change of the energy at the nucleophilic attack of MeCONHMe by MeOH (1) and MeO^- (2). The initial state of the CONH corresponds to that shown in Fig. 5. for 3 Å distance. Calculated by S. Alexandrov (CNDO-2 method, programme GEOMO).

substrate in the ground state of the enzymatic and non-enzymatic reactions. In a non-enzymatic reaction the resonance-destabilized form of the substrate exists in solution at very low concentration. The enzyme can stabilize a highly reactive but energetically unfavourable form of the substrate. The activation barrier of the elementary step of chemical transformation for an enzymatic reaction may be the same as the barrier of transformation of the reactive form of substrate in solution. Hence, the reactive form of the substrate is not subjected to any strain when binding to the enzyme; nor does the enzyme stabilize the transition state of substrate (cf. Karpeiskii et al., 1980).

We believe that experimental verification of these ideas will be a step forward in establishing a general theory of enzymatic hydrolysis of carboxylic acid derivatives.

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STABILIZATION OF THE TETRAHEDRAL INTERMEDIATE IN THE CATALYSIS BY SERINE AND CYSTEINE PROTEINASES

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Budapest, Hungary

INTRODUCTION

The basic features of the mechanism of action of serine proteinases, like chymotrypsin and subtilisin, are illustrated in Fig. 1. It is seen that the nucleophilic attack by the

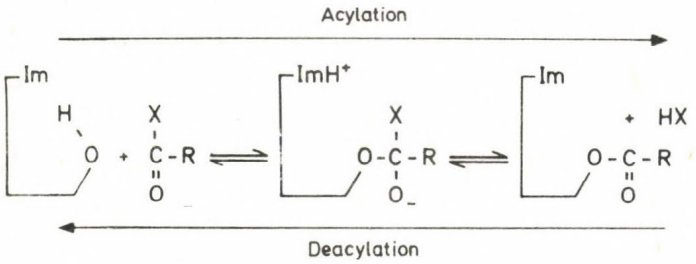

Fig. 1. X stands for OR' or NHR' group in acylation and for OH group in deacylation

serine hydroxyl group on the carbonyl carbon atom of the substrate is catalyzed by a histidine residue as a general base. This leads to the formation of a tetrahedral intermediate and an imidazolium ion. The intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base and alcohol or amine. The acyl-enzyme is hydrolyzed through the reverse reaction pathway of acylation. The tetrahedral intermediates formed during acylation and deacylation are transition-state-

Abbreviation: ATTE: N-acetyl-L-tyrosine ethyl ester

like species (Robertus et al., 1972; Polgár, 1972). The stabilization of the negatively charged oxygen by hydrogen bonds from the protein was postulated on the basis of low Hammett ρ values (Williams, 1970) and of X-ray diffraction studies (Henderson, 1970; Robertus et al., 1972). The oxyanion binding site was observed in all serine proteinases whose three-dimensional structure has been determined (Kraut, 1977; James, 1980).

Cysteine proteinases, like papain and chymopapain, form an acyl-enzyme intermediate, a thiol ester, which builds up and breaks down through a tetrahedral adduct. On the basis of X-ray diffraction studies, an oxyanion binding site was proposed to be involved also in the catalysis by cysteine proteinases (Drenth et al., 1976).

To obtain experimental evidence on the importance of the oxyanion binding site in the catalysis by both serine and cysteine proteinases, we have synthesized thiono ester substrates containing a sulfur in place of the carbonyl oxygen atom, and compared the catalytic constants with those found with the corresponding oxygen esters. It was assumed that replacement of the carbonyl oxygen by the sulfur atom, which is larger and has reduced hydrogen bonding ability, should seriously affect the enzymatic reaction if oxyanion binding is indeed important in the catalysis.

RESULTS AND DISCUSSION

Comparison of the alkaline hydrolysis of thiono and the corresponding oxygen esters. To interpret the effect of oxygen-sulfur exchange in the enzymatic reactions, first the reactivity change in the substrates should be delineated. Therefore, alkaline hydrolysis of esters and thiono esters used in this study were examined. The rate constants obtained are shown in Table 1. It is seen that the ester and the corresponding thiono ester bonds are hydrolyzed at similar rates. Thus large differences in the enzymatic reactions cannot arise from the different chemical reactivities of the substrates but rather from their different interactions with the oxyanion binding site.

Table 1

Base-catalyzed hydrolysis of oxygen and corresponding thiono esters[a]

	Oxygen ester $k\ (M^{-1}s^{-1})$	Thiono ester $k\ (M^{-1}s^{-1})$
Methyl N-benzoylglycinate	1.9 ± 0.2	2.7 ± 0.4
Ethyl N-acetylphenylalaninate	0.4 ± 0.1	0.4 ± 0.1
Ethyl N-Z-phenylalanylglycinate	0.3 ± 0.1	0.4 ± 0.1

[a] In 0.2 M KCl, 3 % acetonitrile at 25°C; determined at least at three different pH-values between 10.0 and 11.5; ester concentration range: 0.3-3 mM

Reaction of oxygen esters and thiono esters with serine proteinases. The second-order rate constants for acylation of chymotrypsin and subtilisin are shown in Table 2 and Table 3, respectively. It is seen that the thiono esters are not hydrolyzed by either serine proteinase at a measurable rate (the data represent upper limits). Particularly in the case of the better substrate (the phenylalanine derivative), the differences in the rate constants for the oxygen and thiono compounds are considerable, more than four orders of magnitude, with both chymotrypsin and subtilisin. Since the acylation rate constant ($k = k_2/K_s$) is a complex constant which involves binding, the binding constants (K_s or K_i) were also determined as seen in Tables 2 and 3. Their values clearly show that binding is not significantly affected by the oxygen to sulfur change, as the respective constants are within a factor of 2. Accordingly, the substantial difference in the acylation rate constants may be attributed to poor catalysis with thiono esters rather than to the impediment of the formation of the enzyme-substrate com-

Table 2

Kinetic parameters of the reaction of chymotrypsin with oxygen and corresponding thiono esters[a]

	Oxygen ester		Thiono ester	
	k ($M^{-1}s^{-1}$)	K (mM)	k ($M^{-1}s^{-1}$)	K (mM)
Methyl N-benzoylglycinate	31±1[b,d]	4±0.5[f,g,h]	< 1[b,e]	4±0.5[f,g,h]
Ethyl N-acetyl-phenylalaninate	15 000±1000[b,f]	7.4[j]	< 1[c,e]	4±0.5[f,g,i]

a: in 0.2 M KCl, 3 % acetonitrile at pH 7.5, at 25°C; the second-order rate constants were determined under pseudo first-order conditions by dividing the first-order rate constants by the enzyme concentration; b: substrate concentration $(0.5\text{-}5)\times10^{-4}$ M; c: substrate concentration $(1\text{-}3)\times10^{-3}$ M; d: enzyme concentration 10^{-4} M; e: enzyme concentration 10^{-3} M; f: enzyme concentration 10^{-7} M; g: K_i values on N-acetyl-L-tyrosine ethyl ester (ATEE) hydrolysis, ATEE concentration $(0.5\text{-}2)\times10^{-3}$ M; h: substrate concentration $(1\text{-}10)\times10^{-3}$ M; i: substrate concentration $(0.2\text{-}2)\times10^{-3}$ M; j: K_S value from Zerner et al. (1964).

plex. The similar binding constants of esters and thiono esters with their extremely different behavior in enzymatic reactions strongly suggest that substrate binding in the oxyanion binding site is critical in the transition state (tetrahedral adduct) while it is less important in the ground state (Michaelis complex) of the reaction.

Table 3

Kinetic parameters of the reaction of subtilisin with oxygen and corresponding thiono esters[a]

	Oxygen ester		Thiono ester	
	k ($M^{-1}s^{-1}$)	K (mM)	k ($M^{-1}s^{-1}$)	K (mM)
Methyl N-benzoylglycinate	18±0.5[c,j]	9±3[e,f,g]	<0.6[d,j]	7±3[e,f,g]
Ethyl N-acetyl-phenylalaninate	25 000±1000[e,j]	20±4[e,i]	<2[d,k]	11±4[e,f,h]

a: in 0.2 M KCl, at pH 8.5, at 25°C, in 3 % acetonitrile; b: in 8 % acetonitrile; c: enzyme concentration 10^{-4} M; d: enzyme concentration 10^{-3} M; e: enzyme concentration 10^{-7} M; f: K_i values on ATEE hydrolysis, ATEE concentration $(0.5\text{-}2)\times10^{-3}$ M; g: substrate concentration $(2\text{-}15)\times10^{-3}$ M; h: substrate concentration $(1\text{-}6)\times10^{-3}$ M; i: K_s value determined by the added nucleophile method (Bender et al., 1964; Berezin et al., 1971); substrate concentration $(5\text{-}15)\times10^{-3}$ M, 1,4-butanediol concentration 0.11 and 0.22 M; j: substrate concentration $(0.5\text{-}5)\times10^{-4}$ M; k: substrate concentration $(1\text{-}3)\times10^{-3}$ M.

Hydrolysis of oxygen and thiono esters by cysteine proteinases. A stereochemical mechanism for the action of papain, which was based on X-ray diffraction (Wolthers et al., 1970) and kinetic investigations (cf. Lowe and Yuthavong, 1971), suggested that the oxyanion of the tetrahedral intermediate was in a sterically non-restricted environment, i.e. without hydrogen bonding to papain (Lowe and Yuthavong, 1971). In contrast, from the three-dimensional structure of papain derivative formed with a specific chloromethyl ketone inhibitor, the existence of an oxyanion binding site was deduced (Drenth et al., 1976). In order to clarify this contradiction, we measured the second-order acylation rate constants for the papain reactions ($k = k_{cat}/K_m$) with the oxygen and thiono pairs of methyl N-benzoyl-glycinate and the more specific ethyl N-benzyloxycarbonyl-phenylalanyl-glycinate. It is seen from Table 4 that with the

Table 4

Kinetic parameters of the reaction of papain with oxygen and corresponding thiono esters[a]

	Oxygen			Thiono ester		
	k ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)
Methyl N-benzoylglycinate	120[d]	8.5[d]	72[d]	100[e]	0.15[e]	1.5[e]
Ethyl N-Z-phenylalanylglycinate	360000[f]	11[f]	0.03[f]	15000[g]	1.5[g]	0.1[g]

a: in 0.2 M KCl, pH 6.0, at 25°C, the second-order rate constants were calculated from the k_{cat} and K_m values; b: in 16 % acetonitrile; c: in 0.5 % acetonitrile; d: substrate concentration 0.01-0.13 M, enzyme concentration $6x10^{-6}$ M; e: substrate concentration $(0.5-10)x10^{-3}$ M, enzyme concentration $2x10^{-5}$ M; f: substrate concentration $(1-10)x10^{-5}$ M, enzyme concentration $5x10^{-8}$ M; g: substrate concentration $(1-10)x10^{-5}$ M, enzyme concentration 10^{-6} M.

less specific substrate the oxygen and thiono derivatives exhibit similar rate constants, whereas with the specific substrate the thiono derivative is less reactive than the oxygen one, but the difference is much less (24-fold) than in the case of the specific substrates of serine proteinases (more than four orders or magnitude).

Table 4 also shows the k_{cat} values, which in the case of the dipeptide derivatives is practically equal to the first-order deacylation rate constant, since the added nucleophile method (Bender et al., 1964) showed that the first-order acylation rate constant is at least 10 times as high as the deacylation constant. Thus, deacylation being rate-determining, its rate constant approaches the overall rate constant, k_{cat}. Since the tetrahedral intermediate is formed also in deacylation, its stabilization at the oxyanion binding site is expected to be comparable to the stabilization of the tetrahedral adduct formed in the acylation step. In fact, the 7-fold dif-

ference between the rate constants for the oxygen and thiono substrates is consistent with this assumption. Because the deacylation rate constant is a simple kinetic constant not complicated by binding, it is more meaningful with respect to the transition state stabilization than the composite acylation rate constant. In the case of the serine proteinases the deacylation could not be estimated, because the acyl-enzyme was not formed with the thiono substrates.

Table 5

Acylation and deacylation of thiol proteinases by the oxygen and thiono esters of N-benzyloxycarbonyl-phenylalanylglucine[a]

	Oxygen ester			Thiono ester		
	k ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	$K_m x10^5$ (M)	k ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	$K_m x10^5$ (M)
Chymopapain-S[b]	3 400[c]	0.25[c]	7.3[c]	540[d]	0.066[d]	12[d]
Papaya peptidase A[e]	127 000[f]	6.3[f]	5[f]	15 500[g]	0.59[g]	3.8[g]
Ficin	53 000[f]	5.6[f]	10.5[f]	10 000[g]	1.1[g]	11[g]

a: in 0.2 M KCl, at pH 6.0, at 25°C, substrate concentration (1-10)x10^{-5} M; b: enzyme purified according to Khan and Polgár (1983); c: enzyme concentration 5x10^{-7} M; d: enzyme concentration 5x10^{-6} M; e: enzyme isolated according to Polgár (1981); f: enzyme concentration 2x10^{-7} M; g: enzyme concentration 2x10^{-6} M.

By the same method we also tested cysteine proteinases other than papain. The results obtained with chymopapain, papaya peptidase and ficin are shown in Table 5. From the data one can conclude that replacement of sulfur for the oxygen atom does not seriously affect either the acylation or the deacylation with either cysteine proteinase.

A comparison of our results obtained with the serine and cysteine proteinases is presented in Table 6 showing the ratios of the second-order rate constants for the oxygen and the corresponding thiono substrates.

Table 6

Acylation of serine and cysteine proteinases: ratios of the second-order rate constants of the oxygen and the corresponding thiono esters

	Serine Proteinases		Cysteine Proteinases			
	Chymo-trypsin	Subti-lisin	Papain	Chymo-papain-S	Papaya pepti-dase-A	Ficin
Non-specific substrate						
Methyl N-benzoyl-glycinate	>30	>30	1.2	0.4	-	-
Specific substrate						
Ethyl N-acetyl-phenylalaninate	>10 000	>10 000	-	-	-	-
Ethyl N-Z-phenyl-alanylglycinate	-		24	6.3	8.2	5.3

It is seen that with the specific substrates of serine proteinases the ratios are more than four orders of magnitude whereas with cysteine proteinases they are only one order of magnitude. This implies the importance of oxyanion binding in the catalysis by serine proteinases but not in the catalysis by cysteine proteinases, which appears to be an important difference in the mechanisms of the two types of proteinases. Since most of the mechanistic features of the serine and cysteine proteinases are common, it is usual to assume that they operate via the same mechanism. We have already pointed out a significant difference, i.e., with serine proteinases the nucleophilic attack on the substrate is a general-base catalyzed process whereas with cysteine proteinases it is not catalyzed (Polgár, 1973). By comparing the reactions of oxygen and thiono esters, we presented here another important difference in oxyanion binding.

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DISCUSSION

BARRETT:

The serine and cysteine groups of proteinases each consists of more than one homologous group. You have shown that the serine proteinases of the trypsin/chymotrypsin and subtilisin families are similar, but all the cysteine proteinases were from the papain superfamily. Would it be worth trying some others, like clostripain or streptococcal cysteine proteinase?

POLGÁR:

We didn't study thiol proteinases evolved in a different way as those of the papain family. However, since the active site appears to be alike in the most important respects, it seems probable that all cystein proteinases lack oxyanion binding.

STEPANOV:

Have you tested the thionoesters as inhibitors of serine proteinases?

POLGÁR:

Yes, thionoesters depending on their binding constant can be good inhibitors. They may become a new type of proteinase inhibitors.

ELŐDI:

It was recently described by Powers and his associates (McRae et al., Biochemistry, 20, 7196, 1981) that peptide-thiolesters are fairly good substrates for thrombin. According to your data on chymotrypsin and subtilisin, O-esters are much more favourable than S-esters. Is this finding characteristic of proteinases? If not, how would you interpret the contradiction between your and Power's data?

POLGÁR:

Thiolesters are better substrates than alkylesters since they have better leaving groups. They are actually intermediates between alkyl and nitrophenyl esters.

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SUBSTRATE AND SOLVENT ISOTOPE EFFECTS AND "PROTON INVENTORIES" FOR THE ELUCIDATION OF CATALYTIC STRATEGIES OF SERINE PROTEASES*

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Our investigations of serine protease enzymes have been conducted in the perspective of revealing underlying catalytic strategies, that is an assembly of interactions between substrate and dynamically vital functional groups on the enzyme, used in their innate biological functions. Through careful selection of a series of molecular structures, the chemical constraints imposed on catalytic efficiency of each particular enzyme can be discerned (Fersht, 1977). To comply with biological and chemical constraints enzymes have to evolve to an optimal distribution of transition state (TS) stabilization between V/K terms and V terms (Stein et al., 1983). An economical use of the free energy released upon binding of the transition state for the uncatalyzed reaction to the enzyme may be a major, albeit not the only determinant in catalytic acceleration (Gandour and Schowen, 1978 Ch. 2; Jencks, 1975). Other factors are reduction of stable and intermediate state stabilization.

Serine protease enzymes have achieved, in the course of evolution, a remarkable level of catalytic acceleration ($\sim 10^9$ fold) (Zerner et al., 1964) for the release of the acyl fragment of the substrate. The catalytic entity operating with such efficiency includes in addition to the serine a chain of general acid-base residues (ABCE); His, Asp, and perhaps

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others. The dynamics of this machinery is the subject of our inquiry. We obtain information on the nature of the transition state through our kinetic studies, which is often different from conclusions that might be drawn from measurements on the resting state of the enzyme (Polgar and Halász, 1982).

A dual approach has been taken to the mechanistic details of protease catalysis. The acid-base catalytic chain contains exchangeable hydrogenic sites, which, if active in catalysis, should manifest this in solvent isotope effects (SIE). The number of protonic sites contributing to the overall solvent isotope effect can also be estimated from studies of reactions in mixtures of isotopic water, a method dubbed "proton inventory" (PI) (Gandour and Schowen, 1978, Ch.6; Schowen and Schowen, 1983). In a mixture of H_2O and D_2O, populations of isotopically substituted subspecies exist (the substrate has either exchangeable sites or a mixture of isotopically different solvates). The concept of fractionation factors, a ratio of deuterium enrichment relative to protium in a particular site of a molecule in a mixture of $H_2O:D_2O=1:1$ (Eq.(1)), has been introduced to quantitate isotopic content of a site.

$$SH + LOD \overset{\phi}{\rightleftharpoons} SD + LOH$$

$$\phi = \frac{[SD]\,[LOH]}{[SH]\,[LOD]} \quad \text{(inverse of SIE*)} \qquad (1)$$

The populations of various sub-species can be related, with appropriate assumptions, to fractionation factors of TSs (ϕ_T), to those of the reactants (ϕ_R), and to the mole fraction (n) of D_2O in H_2O according to the Gross Buttler equation (2)

$$\frac{k_n}{k_o} = \frac{\prod^{V_T}(1-n+n\phi_{Ti})}{\prod^{V_R}(1-n+n\phi_R)} \qquad (2)$$

(k_o = rate constant in pure H_2O)

*The SIE = 1.64±0.04 at pH = 6.00 is more precise than the value obtained from the pH independent k_{cat} values (1.40±0.14). This discrepancy, if real, may arise from a further shift of the rate limiting step from the chemical steps towards physical rearrangements.

Quite frequently, $\phi_R \approx 1$, consequently Eq. (2) then reduces to the numerator, which can take linear, quadratic or other polynomial forms. An exponential dependence can be derived and observed if generalized solvation or subtle enzyme conformational changes give rise to isotope effects. These are generated from a large number of very small effects.

A complementary investigation of the TS status at the carbonyl and surrounding atoms (core) by the use of α and β deuterium secondary isotope effects has further expanded our insight into the mechanism of protease action. A structural plasticity of the core region can be inferred from such substrate isotope effects. Direct deductions of characteristics of the force field in the transition state, near the isotopic center, can be made from substrate isotope effects (Gandour and Schowen, 1978, Ch. 5). The force fields, on the other hand, can be related to the positions of the atoms (B = Pauling Bond order) in these TSs by using vibrational analysis methods (Hogg et al., 1980; Kovach et al., 1980).

In acyl transfer reactions through the addition elimination mechanism, D-substitution on the carbon adjacent to carbonyl is expected to increase the rate. The reason for this is a certain degree of loss, at the quasi-tetrahedral TS preceding and succeeding the tetrahedral intermediate, of the hyperconjugation from the β-CH(D) bond into the pi orbital of the carbonyl that stabilizes the trigonal ground state. This increases electron density in the bonds at the TS and strengthens them leading to an inverse isotope effect. The limiting effect calculated from comparable equilibria , $K_H/K_D = 0.955$ per D, for complete conversion of an sp^2 carbon to sp^3 (at carbonyl) (Kovach et al., 1980a, b; Kovach, 1982). A single measure of tetrahedrality is expressed by Eq. (3),

$$k_H/k_D = (K_H/K_D)^{\hat{I}} \qquad (3)$$

where $\hat{I}$ is the fractional progress towards tetrahedrality.

A variety of enzyme and substrate combinations have been studied by these tools to assess the extent to which the TS status of proton translocation is correlated with the progress of heavy atom reorganization at the core TS.

Utilization of the proton relay system

In a systematic approach to the resolution evolutionary of constraints to catalysis by serine proteases, initial studies included minimal substrates that lack most of the structural features of the natural substrate. In this circumstance, the enzyme is deprived of subsite interactions with substrate derived structural units in the catalytic TS. These systems exhibit one proton catalysis. The enzymes, presumably, rely on the proximal hydrogen bridge of the ABCE, in the manner of nonenzymic protolytic catalysts. Proton inventories of the deacylation of acetyl-chymotrypsin, acetyl-elastase and acetyl trypsin are good examples with SIE-s 2.4, 2.45, and 1.4 respectively (Elrod et al., 1980). The first two are similar to SIEs for general catalysis in organic reactions. The value of 1.4 is too low even for a single proton solvation bridge and may indicate a partial nucleophilic attack by histidine followed by an N-O transacylation and hydrolysis. Hubbard and Kirsch (1972) found their data for acylation of α-chymotrypsin by reactive esters to be consistent with such a model.

A non-linear proton inventory for the acylation of α-lytic protease by p-nitrophenyl acetate (PNPA) has also been observed (Quinn et al., 1980). The simplest interpretation of this profile is that both reactant and TSs contribute subunit fractionation factors, i.e. have loosely bound protons. The best fit to this model gives $\phi_T = 0.39 \pm 0.01$ and $\phi_R = 0.81 \pm 0.01$. The value 0.8 calculated from this model can be compared with $\phi_R = 0.6$, which is suggested by the pKa difference in H_2O and D_2O taken ϕ_R to be ~ 1 for the protonated enzyme. It might be conjured then that the ionized enzyme has a single protonic site with an unusually "loose" potential, which becomes involved in catalysis and then the bond gets weaker at the TS. Alternatively $(0.8)^2 = 0.6$ so that two protonic sites, which

are generated on conversion of EH to E, may be available one of which participates in catalysis.

A transition toward more natural substrates such as oligopeptide analogs can be shown through a series of examples. The first two of these are with truncated substrates that, however, contain a Cα-chain (Elrod et al., 1980). One of these reactions is the acylation of elastase by N-CbzAla-p-nitrophenyl ester (N-CbzAlaONP). A linear proton inventory with a single protonic site generating a SIE of 1.75 emerges from this study. The other case is linear proton inventories with substantial SIE-s of ∿3.0 for deacylation reactions of N-benzoyl-Arg-thrombin and trypsin.

One of our more recent endevours is the investigation of the acylation by N-CbzGly-ONP and the ensuing deacylation of the bacterial serine protease, subtilisin BPN. Fig. 1 portrays the pL (L=H,D) profile of acylation where the SIE stays close to unity throughout the pH range studied. The pH-s of the free

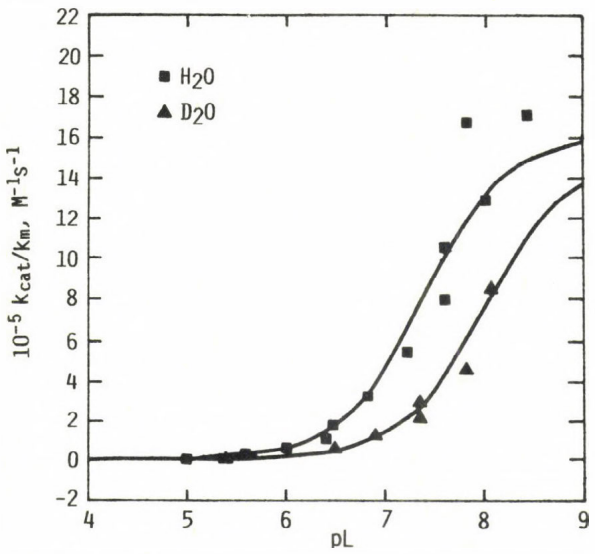


Fig. 1: Bi-molecular rate constants (10^{-5} k_{cat}/K_m, $M^{-1}s^{-1}$) for acylation of subtilisin BPN by N-CbzGlyONP as a function of pL (L=H,D) at 25.0±0.1°C, and μ=0.27.

enzyme are 7.4±0.1 in H_2O and 8.00±0.15 in D_2O and the limiting velocities for the bimolecular reaction between enzyme and substrate are $(1.62\pm0.15)\times10^6$ $M^{-1}s^{-1}$ in H_2O and $(1.51\pm0.20)\times10^6$ $M^{-1}s^{-1}$ D_2O calculated from regressional analysis fit of the data to Eq. (4).

$$k = k^{lim}\, Ka/(Ka + aH) \qquad (4)$$

$$k = k_{cat} \text{ or } k_{cat}/K_m$$

The values obtained in H_2O are in good agreement with those reported by Philipp et al. (1979).

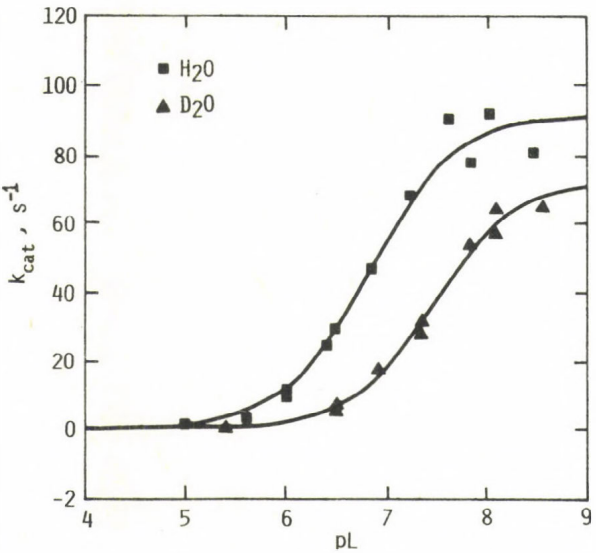


Fig. 2: Unimolecular rate constants (k_{cat}, s^{-1}) for deacylation of N-CbzGly-subtilisin BPN, as a function of pL (L=H,D) at 25.0±0.1 °C and μ=0.27.

In Fig. 2 the pL profile of the deacylation step is presented with SIEs ∿1.5 at points of identical fraction of the catalytically active unprotonated subtilisin. The pKs are 6.85±0.03 and 7.45±0.02 in H_2O and D_2O respectively. The pH independent values of k_{cat} are 105±9 s^{-1} in H_2O and 73.5±6 s^{-1} in D_2O. Thus, the apparent pK of the ES complex is ∿0.4 unit

lower than that of the free enzyme, while the ΔpK of 0.6 between H_2O and D_2O is preserved as close as can be inferred from the data. Such substrate dependent change in the pK of an enzyme upon binding of a substrate may be attributed to conformational changes. Cleland (1982) pointed out the possibility of a partially rate limiting product release from the enzyme in cases when the pK shift occurs outward on the pH-k_{cat} profile. Nonproductive binding may also result in artificial pK values (Fastrez and Ferst, 1973).

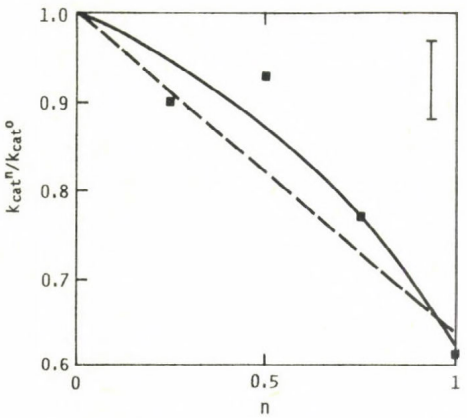


Fig. 3:

PI. Rates of deacylation of N-CbzGly-subtilisin BPN as a function of n, the mol fraction of D_2O in H_2O at 25.0±0.1 °C, pH=6.00 (and equivalent phosphate buffer), and μ=0.27.

A PI has also been carried out and is given in Fig.3 for the deacylation of N-CbzGlyONP at pH=6.00*. This may be another example of a "bulging up" curve, although the quality of data and the small size of the SIE do not permit a clear distinction between a linear one site model with ϕ_T = 0.64±0.03 as the dashed line indicates and various two site models. We find the most consistent with the aforementioned pK shift of the k_{cat} term, the curved proton inventory, and our βDIE results, vide infra, a consecutive two step model in which partially rate limiting product release follows a partially rate limiting breakdown of the tetrahedral adduct of water and the acyl enzyme. Such a scheme is consistent with the kinetic expression of Eq. (5)

$$k_{cat}^n = \frac{k_1^n \, k_2^n}{k_{-1}^n + k_2^n} \qquad (5)$$

with two sequential TSs (ϕ_{T1} and ϕ_{T2}). The proton inventory data fit to such scheme is accounted for in Eq. (6), which simplifies to Eq. (7) in the case of $\phi_{T2} = 1$ as for an isotopically insensitive or physical step (w_1 and w_2 are weighting

$$\frac{k^n}{k^o} = \frac{(1-n+n\phi_{T1})(1-n+n\phi_{T2})}{w_1(1-n+n\phi_{T1}) + w_2(1-n+n\phi_{T2})} \qquad (6)$$

$$\frac{k^n}{k^o} = \frac{(1-n+n\phi_{T1})}{w(1-n+n\phi_{T1}) + (1-w)} \qquad (7)$$

factors $K_1/(k_{-1} + k_2)$ and $k_2/(k_{-1} + k_2)$ respectively and their sum = 1). The curve on Fig. 3 has been generated with $\phi_{T1} = 0.33\pm0.02$, and $w_1 = 0.7\pm0.06$ through nonlinear least squares fit of our data. Consequently, the contribution of the acid base catalytic TS is $\sim$0.3 followed by a TS with a weight of 0.7 and a SIE $\sim$1. SIEs of 3.0 have been observed in the protolytically assisted deacylation of subtilisin Carlsberg by Polgar and Bender (1979) and Matta et al. (1975) with a variety of acyl moieties. It is an unanticipated finding that the minor structural differences between the two species of subtilisin can result in such a shift towards a rate limiting step other than the bond breaking and making steps.

A much stronger response is elicited from α-chymotrypsin to the presence of even a single feature of the natural substrate such as the first peptide unit in N-CbzGlyONP (Stein et al., 1983). Fig. 4 shows comparative PIs for the deacylation of acetyl α-chymotrypsin and porcine elastase and their CbzGly analogs. Both acetyl enzymes demonstrate one proton catalysis with SIEs $\sim$2.4. N-CbzGly-elastase retains this character, whereas N-CbzGly-chymotrypsin shows curvature of the PI plot, which is best described by two proton catalysis with two sites of identical fractionation factors (1/1.8) contributing.

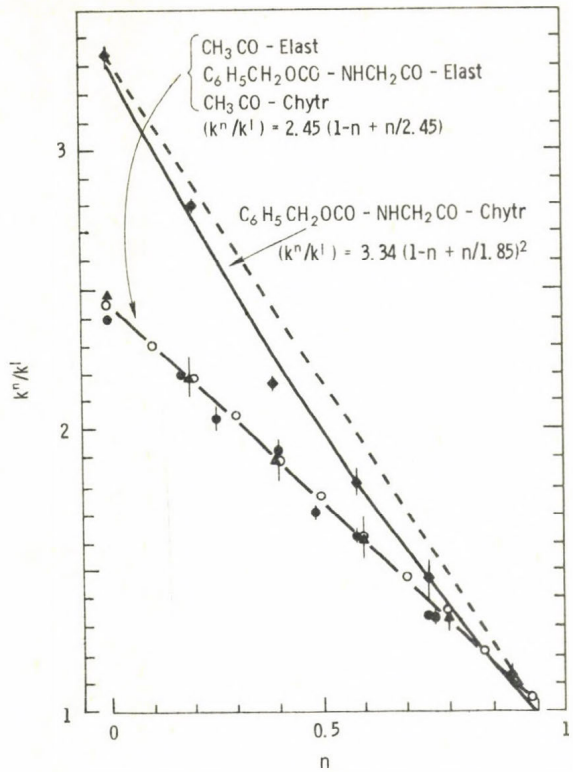


Fig. 4:

PI. The straight line represents combined data for reactions of acetyl-chymotrypsin, -elastase, and N-CbzGly-elastase, the curved line represents deacylation rates of N-CbzGly-chymotrypsin as a function of n, the mol fraction of D_2O in H_2O at pH=4.7, and for 25.0±0.1 °C. The dashed line connects the point n=0 with that for n=1. (Reproduced with permission from Stein et al., 1983.)

It might be noted that the manifestation of the catalytic proton relay network is considerably different in the interaction of chymotrypsin, elastase, and subtilisin with the deacylation TSs of their N-CbzGly-derivatives.

Two or multiproton catalysis has been observed in other cases of protease reactions when some element of the evolutionarily anticipated structure was provided. Addition of the Cα-side chain as in $C_6H_5CH_2CH_2COO$-NP resulted in a concave proton inventory for the deacylation of chymotrypsin with ϕ_{T1} = 1/1.9 and ϕ_{T2} = 1/1.5 as best fit (Elrod et al., 1980). A paradigm of multiproton catalysis is the reaction of trypsin with the oligopeptide BzPheValArg-p-nitroanilide (ONA). Fig. 5 presents a comparison of PIs of trypsin reactions with PNPA, BzArg-ethyl ester, and BzPheValArg-ONA. The progression toward larger SIEs (1.4, 3.0 and 4.3 respectively) as well as to two proton catalysis is obvious. The contributing sites to the

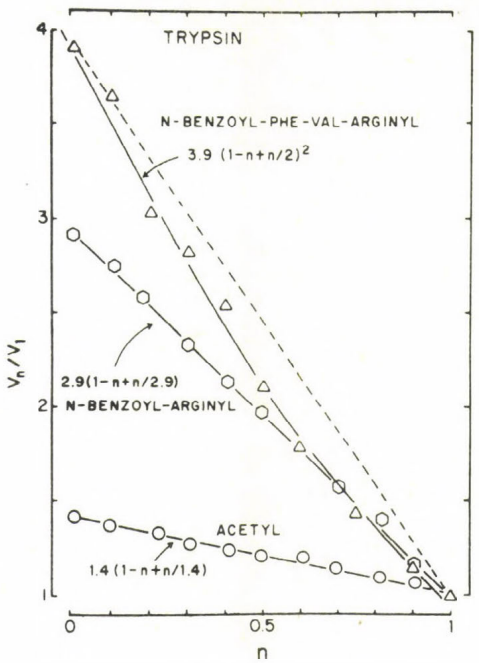


Fig. 5:

PI. Rates of acylation of trypsin by N-BzPheValArg-PNA and of deacylation of N-BzArg- and acetyl trypsin as a function of mol fraction of D_2O in H_2O at pH=8.00 and 25.0±0.1 °C. The dashed line connects points n=0 and n=1 for the acylation reaction.

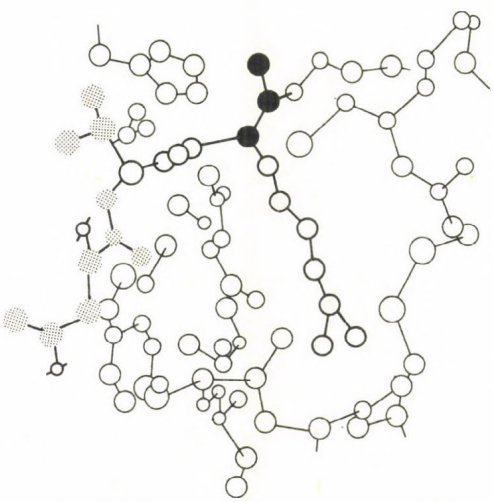


Fig. 6:

A schematic structure of the ES complex of trypsin, which occurs after C-N bond breakage. The dark circles symbolize the acetyl group attached to the serine arm. Addition of the heavy circles depicts the position of a truncated substrate. Inclusion of all these and the shaded circles describe orientation to an oligopeptide substrate. The acid-base catalytic chain begins at the histidine in the upper left corner and extends leftwards. (Reproduced with permission from Schowen and Schowen, 1981.)

BzPheValArg-ONA reaction have SIEs of 2.1 each actually approaching the values obtained in model reactions. A speculative structure of the trypsin complex with peptide analogs is presented in Fig. 6. The core, i.e. the three atom fragment is shown in dark circles and is attached to the serine arm. Inclusion of the heavy circles aids in envisioning the orientation of a truncated substrate. Further addition of elements indicated as shaded circles to the rest of the structure completes the portrayal of probable interactions between trypsin and oligopeptides (Schowen and Schowen, 1981).

Structural variations at the transition state of the core

Secondary α and β-deuterium isotope effects (α or βDIE) have found great utility in the assessment of structural plasticity of the TS in nucleophilic reactions (do Amaral et al., 1979; Kovach et al., 1980a, b; Kovach, 1982; Kovach and Quinn, 1983). Table 1 features βDIEs of two fundamental reactions of phenyl acetate and formate; nucleophilic attack of hydroxide

Table 1

Secondary isotope effects in nonenzymic acyl transfer

System in H_2O, 25°C	k_H/k_D	$\hat{I}^a$
nucleophilic catalysis		
HO^- + PNPA	0.98(β-D_3)	0.15
proteolytic catalysis		
four examples of	0.813-0.833(α-D)	0.58-0.66
hydrolysis of aryl		
formates and acetates	0.914(β-D_3)	0.65

[a]For α-D effects, $\hat{I} = \log(k_H/k_D)/\log 0.73$ (do Amaral et al., 1979); for β-D_3 effects, $\hat{I} = \log(k_H/k_D)\log 0.87$ (Kovach et al., 1980)

ion with a small progress toward tetrahedrality and the general base catalyzed reaction of acetate ion with a quasi-tetrahedral TS more than half way developed. This latter process is remini-

scent of the enzymic deacylation. The $\hat{I}$ values indicate the significant quantitative difference in progress along the reaction coordinate of the two reactions. Yet, the free energy difference between the two TS-s, calculated from the rate constants is only 3.6 kcal/mol. This bespeaks of a great flexibility of the TS structure on the expense of just a small energy. The implication of this phenomenon to biological evolution is nontrivial, since an acyl transfer enzyme may take advantage of a chemical opportunity with a modest expenditure.

Table 2

Secondary isotope effects in the deacylation of serine protease acyl enzymes

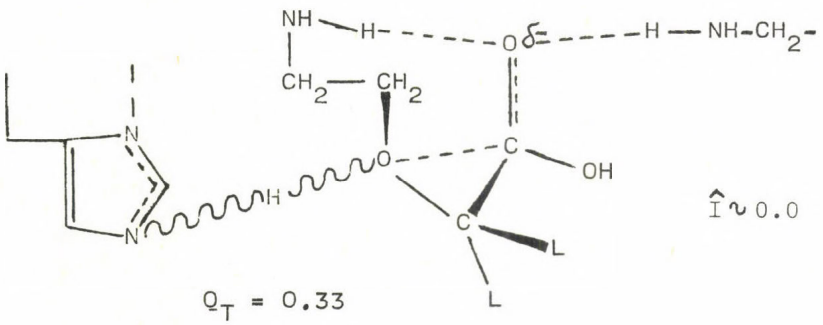
System in H_2O, 25°C	k_H/k_D	$\hat{I}$[a]
LCO-Chymotrypsin	0.870 ± 0.023[b]	0.44
CL_3CO-Chymotrypsin	0.940 ± 0.010	0.44
$CbzNHCL_2CO$-Chymotrypsin	0.925 ± 0.009	0.84
$PhCH_2CL_2CO$-Chymotrypsin	0.950 ± 0.010[c]	0.54
CL_3CO-Elastase	0.975 ± 0.007	0.27
$CbzNHCL_2CO$-Elastase	0.961 ± 0.010	0.43
$CbzNHCL_2COOHp$ + Subtilisin	0.963 ± 0.013	0.40
$CbzNHCL_2CO$-Subtilisin	1.01 ± 0.03	0.0

[a]For β-D_2 effects, $\hat{I} = \log(k_H/k_D)/\log 0.91$.
[b]Lehrman et al., 1980. [c]Fujihara et al., unpublished.

Table 2 lists results of α and βDIEs of reactions of protease enzymes (Stein et al., 1983; Fujihara et al., unpublished; Lehrmann et al., 1980). With minimal substrates, the deacylation of both chymotrypsin and elastase have TS-s less tetrahedral in character than the nonenzymic analog ($\hat{I}$=0.44 and 0.27). The difference between the two enzymes is also marked. Both acetyl-enzymes exert an "explosive" effect, lengthening the bond between the carbonyl cargon and the atom, which interacts with the protonic bridging of the ABCE. βDIEs for the $C_6H_5CH_2CL_2CO$ and N-CbzGly-enzymes are more inverse with chymotrypsin and elastase. Compression of these structures

toward sp^3 hybridization with respect to the acetyl analogs is expressed in the changes of the values of $\hat{I}$; 0.44 → 0.54-0.84 for chymotrypsin and 0.27 → 0.43 for elastase. The latter is still slightly exploded relative to the nonenzymic TS, whereas N-CbzGly-chymotrypsin has a more compressed TS. The main difference between the structure of these two enzymes lies in the specificity pocket "south" of the serine residue (Fig. 6). Chymotrypsin has a large open hydrophobic cavity, while elastase has only a small depression where not more than a C_α-methyl side chain is anticipated. In other words, a small substrate offers much less in terms of interactions than expected by chymotrypsin but not that much less than what elastase can accomodate anyway.

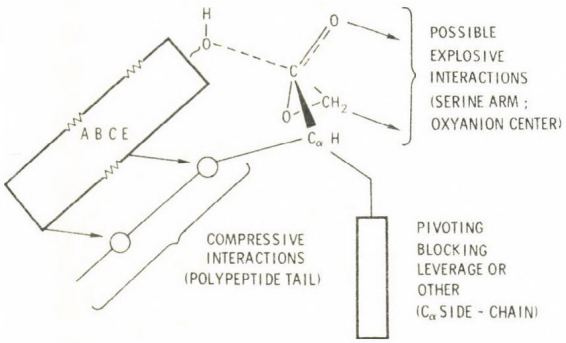
The acylation TS of subtilisin is mostly like the base line TS ($\hat{I} = 0.5$), which fact together with a SIE of unity seems to be compliant with the proposition of a protolytically unassisted nucleophilic attack by the histidine of subtilisin, much like the case, that Hubbard and Kirsh (1972) reported. A negligible alteration of the sp^2 hybridized structure at the TS of CbzGly-subtilisin deacylation can be surmised from the absence of βDIE. This is consistent with the picture that emerged, vide supra, from the SIE studies. If the TS for protonic reorganization and (coupled) heavy atom rearrangement is only 0.3 fraction of the highest energy barrier along the reaction path, the most inverse βDIE to be realized is 1x0.7 + 0.91x0.3 = 0.975. Moreover, such a TS might well be quite late preceding a high energy product state as suggested in structure I and therefore be quasi-trigonal or product-like.



(I)

Correspondence between proton transfer and heavy atom rearrangement in proteases

An attractive interpretation of the combined results of the two diagnostic probes of protease mechanism is that serine proteases use a mechanical leverage on the serine arm and on the carbonyl oxygen of the substrate to explode the carbonyl structure (Scheme I; Schowen et al., 1981). The polypeptide tail via hydrogen bonding interactions can exert a compressive



Scheme I: Proposed interactions of various elements of a substrate with catalytically vital subunits of serine proteases. (Reproduced with permission from Schowen, et al., 1982.)

force across the proton relay chain. A balancing role of the C_α-side chain to proportionate the two effects is evident for chymotrypsin, where the lack of it results in "overshooting"

compression at the carbonyl structure as well as at the proton relay apparatus as it is the case when only N-acyl interactions are present. The compressive effect is less in elastase where the active site is more rigid since a substantial C_α-side chain pivot cannot be accomodated.

A comparison with amidohydrolases

Amidohydrolases, a group of enzymes very similar in their catalytic makeup to serine proteases, have also been studied by the two-fold approach described (Quinn et al., 1980). Highlights of PI results in this connection are the observation of two or multiproton catalysis by asparaginases from E.coli, Proteus vulgaris and Erwinia coratovora in the rate determining expulsion of NH_4^+ from the ES complex, both from the reaction with the natural substrate, Asn, and with Erwinia coratovora, the reaction with succinamate ion (Eq. (8)).

$$\begin{aligned} RCH_2CONH_2 + E\text{-}OH &\rightleftharpoons RCH_2COO\text{-}E + NH_4^+ \\ RCH_2COO\text{-}E + H_2O &\longrightarrow RCH_2COO^- + EOH \end{aligned} \qquad (8)$$

SIE-s in all four cases are 2.6-3.3 with contribution of the two sites each 1.6-1.8. When, however, TS complementarity to the active site is removed, recruitment of the full acid-base catalytic capacity of the enzyme falls short. This was the case with glutamine as a substrate for two asparaginases that showed single site general acid participation. Reactions of Asn and succinamate ion with Erwinia asparaginase are being studied in our laboratory currently (Paborji et al., unpublished). A βDIE = 0.95 and a SIE = 1.5 for the succinamate ion suggests a rate determining nucleophilic attack by the enzymic hydroxyl assisted by a single proton catalysis. Asn also shows a single proton catalysis with SIE = 1.4 akin to the one site of the two proton processes. Rates and SIE-s are the same for Asn and succinamate ion for the expulsion of NH_4^+ from the tetrahedral adduct (ES). βDIEs for this step with several substrates and all three asparaginase enzymes are greater than

one, indicative of a $sp^3 \rightarrow sp^2$ rehybridization as in rate determining breakdown of a tetrahedral intermediate. Such intermediate is expected to accumulate under saturating conditions. These results are supportive of a weak specificity requirement in the catalysis by some asparaginases.

CONCLUSIONS

Serine proteases generally have a greatly variable structure at the active site. In response to the specific substrate structure, reaction conditions such as temperature, pH and salt concentration, any one enzyme can select from an arsenal of pathways and rate determining steps the one with the lowest energy cost. The accessible pathways can converge into one as the physiological substrate structure is approached, i.e. the complementarity between substrate and enzyme is fulfilled.

A speculative suggestion is that serine proteases can achieve mechanical deformations through sufficient leverage (Schowen et al., 1981). Such deformations can serve in intermediate stabilization an extreme case of which is generation of a covalent acyl intermediate. It may be that such deformation is necessary to permit the formation of the optimal TS structure involving the proton relay. The coupling of this hydrogen bond chain to the heavy atom rearrangement can provide the ideal length between the carbonyl carbon and the atom partially bonded to it to give the energetically correct acid-base property at the TS.

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DISCUSSION

POLGÁR:

Did you measure the deacylation rate in the pH independent region?

KOVACH:

Proton inventories have been measured in the pH independent region of the respective parameter in every case except for the deacylation reaction of Cbz-Gly-subtilisin. In the latter case the solvent-isotope effect had been shown to remain 1-5 throughout the pH range studied, thus the proton inventory was measured at pH 6.00 for convenience, i.e. greater precision.

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PROTEINASES AS TOOLS IN PEPTIDE SYNTHESIS. SYNTHESIS OF SUBSTRATES FOR PROTEINASE ASSAY

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Proteolytic enzymes including those produced by microorganisms have found application in various fields. One of the most recent trends consists in their use to catalyse the synthesis of peptide bonds. This line of research might be easily traced until late thirties, but only recently revival of old ideas on the basis of modern enzymology have lead to development of preparatively valuable synthetic procedures.

Among numerous advantages of enzymatic peptide synthesis the most obvious are:

- synthesis at very mild conditions;
- stereoselectivity and the absence of racemization danger;
- structural selectivity that allows to avoid in many cases the protection of functional groups, etc.;
- tolerance towards many admixtures including those found in commercially available starting materials;
- essential absence of by products.

Of course, one should not overlook rather strong limitations imposed by very nature of this approach. Without going into detailed discussion of this matter, one might point out the following ones:

- the methods used to shift the equilibrium toward synthesis are rather limited. Precipitation of the product, its selective transfer to organic phase, the use of the solvents with low water content, the excess of one of the reactants, as well as the application of specifically activated derivatives are predominantly used to this end. Obviously, new ways are

to be searched, e.g., selective sorption of the product or the coupling of the synthesis with other reactions;

- the specificities of the enzymes used do not provide free choise of the peptide structures to be synthesised. To overcome this problem the use of enzymes side specificities might be tried, but substantial broadening of the enzyme collection used for the synthesis appears to be more promising approach.

It appears that in the future the proper choice of the stategy and tactics coined to satisfy the specific demands of enzymatic synthesis would be of crucial importance for successful development of the method. In particular, specially designed protecting groups should open additional possibilities for application of proteinases in peptide synthesis.

In this paper we discuss the experience in the enzymatic peptide synthesis gained recently in our laboratory by L.A. Lyublinskaya, E.N. Lysogorskaya, T.L. Voyushina, E.S. Oksenoit, I.Yu. Filippova and S.E. Boitsova. Our efforts were mainly concentrated on the synthesis of the peptides that might be used as chromogenic substrates for proteinase assay.

In the first series of our experiments we applied thermolysin or related metalloproteinase from B.subtilis to catalyse the synthesis of N-protected tripeptide p-nitroanilides (Lyublinskaya et al., 1982) (Table 1). The choice of these enzymes was dictated by their specificity that demands the P_1 place in the resulting peptide to be occupied by a hydrophobic amino acid. The favourable equilibrium shift was provided by the precipitation of a rather hydrophobic product from the reaction mixture that contained substantial concentration of dimethylformamide.

As a rule, the yields approached the quantitative ones, few deviations are to be explained by inevitable losses during the isolation of the peptides. It appears that in all cases the equilibrium was attained. Rapid formation of the precipitate indicates very high velocity of peptide synthesis. Duration of the synthesis dictated by technical considerations substantially overcomes the time necessary for reaction completion.

Table 1

The synthesis of p-nitroanilides of tripeptides catalysed with thermolysin (TL) and B.subtilis metalloproteinase (BS)

Components		Conditions	Yield
Z-Ala-Ala-OH	+ H-Leu-NH-$C_6H_4NO_2$	TL 3 min, 25°	94 %
Z-Ala-Ala-OH	+ H-Leu-NH-$C_6H_4NO_2$ D,L	TL, 20 min, 37°	93 %
Boc-Ala-Ala-OH	+ H-Leu-NH-$C_6H_4NO_2$	TL, 2 h, 37°	76 %
Boc-Ala-Ala-OCH_3	+ H-Leu-NH-$C_6H_4NO_2$	TL, 4 h, 37°	67 %
Z-Gly-Gly-OH	+ H-Leu-NH-$C_6H_4NO_2$	TL, 17 h, 37°	84 %
Z-Ala-Ala-OH	+ H-Phe-NH-$C_6H_4NO_2$	TL, 30 min, 25°	88 %
Z-Ala-Ala-OH	+ H-Leu-NH-$C_6H_4NO_2$	BS, 5 min, 25°	85 %
Boc-Ala-Ala-OH	+ H-Leu-NH-$C_6H_4NO_2$ D,L	BS, 15 min, 25°	84 %

It is important, that D,L- H-Leu-NH-$C_6H_4NO_2$ might be used instead of L-isomer without noticeable drop in the yield, commercial crude preparations of p-nitroanilides also give satisfactory results. Even more interesting appears the successful synthesis of the tripeptide from leucine p-nitroanilide and benzylocycarbonyl-alanyl-alanine methyl ester. This reaction cannot be considered as a simple reversal of hydrolysis, we did not find any example of peptide bond alcoholysis catalysed by thermolysin. So far as no noticeable saponification of the peptide methyl ester was observed, it appears that the ester participates in the enzymatic reaction. This result might be useful for elucidation of the catalytic mechanism of metalloproteinases. Voyushina and Lyublinskaya have shown that the combined use of thermolysin for peptide bond formation and of subtilisin for subsequent removal of p-nitroanilide moiety from the C-terminus allowed step-wise elongation of the peptide chain. This approach is illustrated by the following examples:

1. Z-Gly-Ala-OH + H-Leu-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 86-93 \%}]{\text{thermolysin}}$

Z-Gly-Ala-Leu-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 87 \%}]{\text{subtilisin}}$

Z-Gly-Ala-Leu-OH + H-Phe-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 71 \%}]{\text{thermolysin}}$

Z-Gly-Ala-Leu-Phe-NH-$C_6H_4NO_2$

2. Z-Gly-Ala-OH + H-Phe-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 91-98 \%}]{\text{thermolysin}}$

Z-Gly-Ala-Phe-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 88 \%}]{\text{subtilisin}}$

Z-Gly-Ala-Phe-OH + H-Leu-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 72-92 \%}]{\text{thermolysin}}$

3. Z-Gly-Gly-OH + H-Phe-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 60 \%}]{\text{thermolysin}}$

Z-Gly-Gly-Phe-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 92 \%}]{\text{subtilisin}}$

Z-Gly-Gly-Phe-OH + H-Leu-NH-$C_6H_4NO_2$ $\xrightarrow[\text{yield 66-77 \%}]{\text{thermolysin}}$

Z-Gly-Gly-Phe-Leu-NH-$C_6H_4NO_2$

Obviously, in these syntheses p-nitroaniline acted as a rather convenient C-protecting group, although its application was handicapped by cumbersome preparation of amino acid p-nitroanilides.

Peptide p-nitroanilides synthesised by this route are regularly used in our laboratory for assay of various bacterial serine proteinases - subtilisins, thiol-dependent serine proteinases, intracellular serine proteinases of bacteria etc. All these enzymes were assayed by the measurement of p-nitroaniline amount set free after hydrolysis. Comparison of their efficiency as the substrates have revealed rather strong influence of the residue P_1 and the participation of at least two preceding residues - P_2 and P_3 in the binding of the substrates by the enzyme.

These substrates are poorly soluble in water that makes it necessary to add an organic solvent - dimethylformamide or dimethylsulfoxide to the assay mixture. Organic solvents, especially, dimethylformamide may act as concurrent inhibitors that induced us to look for less hydrophobic peptides. It turned out that the introduction of pyroglutamic acid residue as an amino protecting group allowed to improve the solubility of p-nitroanilides in water. Thus, Pyr-Ala-Ala-Leu-NH-$C_6H_4NO_2$ has been found to be an excellent substrate for subtilisins and especially for serine enzymes from halophilic bacterium Halobacterium halobium and from Streptomyces rutgersensis. Both these enzymes show no activity against Z-Ala-Ala-Leu-p-nitroanilide apparently being heavily inhibited by dimethylformamide.

The same principle of substrate hydrophilisation via introduction of pyroglutamic acid residue was successfully applied for the synthesis of the substrates suitable for papain and other cysteine proteinases. To this end E.N. Lysogorskaya and her colleagues in our laboratory at Moscow State University pursued the following scheme. Pyr-Phe-OCH_3 was prepared by H-Phe-OCH_3 acylation with pyroglutamic acid pentafluorphenyl ester. The product separation from the residual pyroglutamic acid might be rather cumbersome, but for the enzymatic synthesis this procedure is quite superfluous so far as pyroglutamic acid would not participate in the further condensation with leucine p-nitroanilide catalysed by chymotrypsin:

$$\text{Pyr-Phe-OCH}_3 + \text{H-Leu-NH-C}_6\text{H}_4\text{NO}_2 \xrightarrow[\text{yield 79 \%}]{\text{chymotrypsin}}$$

$$\text{Pyr-Phe-Leu-NH-C}_6\text{H}_4\text{NO}_2$$

This p-nitroanilide was found to be convenient chromogenic substrate for papain that is to be explained by the presence of hydrophobic residue - that of phenylalanine in P_2 position. The hydrolysis of leucine - p-nitroaniline bond proceeds optimally at pH 6 in the presence of 3×10^{-3} M dithiothreitol and 10 % of dimethylsulfoxide, K_M was found equal to 0.34 mM, $k_{cat}=0.13\ s^{-1}$. Comparable data were obtained for the substrate hydrolysis by ficin and bromelain. From the practical point of view it is

important that the specific activities measured against this substrate were found to be equal to 330 mol/A_{280}·min for papain, 330 - for ficin, 43 - for bromelain, whereas the specific activity of the same papain preparation measured against commonly used p-nitroanilide of benzoyl-D,L-arginine corresponded to only 4 units.

Following the same approach, Filippova, Lysogorskaya and others synthesized the substrate for assay of pepsin and other aspartyl proteinases. The synthesis was accomplished as follows. Benzyloxycarbonyl-alanyl-alanine was condensated with phenylalanine methyl ester in the presence of dicyclohexylcarbodiimide (or, eventually, by thermolysin-catalysed reaction). After deprotection with HBr/CH_3COOH the tripeptide methyl ester was acylated by pyroglutamyl acid pentafluorphenyl ester, the crude product was coupled with leucine p-nitroanilide using chymotrypsin as the catalyst, that gave Pyr-Ala-Ala-Phe-Leu-NH-$C_6H_4NO_2$ with the yield of 71 %. On this stage the selectivity of the enzymatic reaction was once more used to make unnecessary rather complicated purification of Pyr-Ala-Ala-Phe-OCH_3.

Swine pepsin was found to split phenylalanyl-leucine bond in this substrate. This reaction might be followed by amino group quantitation with trinitrobenzenesulfonate or by p-nitroaniline liberation from leucine p-nitroanilide with leucine-aminopeptidase.

The substrate is optimally hydrolysed by pepsin at pH 3, by aspergillopepsin - at pH 4.0. K_M was found to be equal to 0.55 mM and k_{cat} = 18.5 s^{-1} for its hydrolysis by pepsin, K_M = 0.66 mM and k_{cat} = 0.33 s^{-1} - by aspergillopepsin. Shorter peptides, e.g., Pyr-Phe-Leu-NH-$C_6H_4NO_2$, were optimally hydrolysed by swine pepsin at pH 4.0. It is to be noted that the increase in the substrate length by addition of Ala-Ala sequence before Phe-Leu moiety left K_M values practically unchanged, whereas k_{cat} was improved by the factor of 1300.

Pyroglutamic acid residue has been successfully used as a hydrophilic N-protecting group in design of specific substrates for other proteinases. Thus, Pyr-Phe-OH was suggested by Tarasova as a convenient substrate for carboxypeptidase A, Pyr-Phe-NH-$C_6H_4NO_2$ was used as a chromogenic substrate for chymotrypsin.

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DISCUSSION

VITALE:

Since many components for peptide synthesis are not readily soluble in water, what sort of solvents and what concentration do you use?

STEPANOV:

Indeed, the reaction mixtures practically always contained a rather high concentration of dimethylformamide - up to 50%. We may accept it as a rule, that the solvent would not hamper the enzyme stability to the extent that would diminish its synthetic activity.

GRÁF:

In your stepwise synthesis of tetrapeptide Gly-Gly-Leu-Phe you were consequently using thermolysin. My question is: could subtilisin also catalyze the final step of the synthesis?

STEPANOV:

Yes, we have observed that subtilisin may catalize the synthesis of the peptide bond specifically in the case of tetrapeptide p-nitroanilides containing rather bulky residues /Leu or Phe/ at P_2 position. But these are only qualitative observations that should be further examined.

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REGULATION OF FIBRINOLYSIS

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Plasmin (EC 3.4.21.7) is the serine proteinase that converts fibrin into soluble products in the last step of physiological fibrinolysis. Antifibrinolytic amino acids, various compounds analogous to lysine, are well known effectors of the fibrinolytic system (Muramatu et al., 1965) and are known to bind the so-called lysine binding sites (LBS) confined to the heavy chain, that is the non-proteinase part of plasmin(ogen) and thus influence various interactions of the components of the fibrinolytic system (Ambrus et al., 1968; Thorsen and Astrup, 1969; Landmann, 1973; Thorsen and Müllertz, 1974; Richli and Otawsky, 1975; Christensen and Clemmensen, 1977, 1978; Christensen, 1978). The presence of two to three classes of LBS of the plasminogens has been demonstrated (Markus et al., 1978a,b), and much focus has been placed on one of these, the high affinity or strong LBS (Wiman and Collen, 1978), whereas the possible importance of the other classes of LBS's have received little attention. Realizing that the ligand specificity of the weak(er) LBS's is probably different from, but shows some resemblance to that of the strong LBS, as is indeed indicated by their different affinities for antifibrinolytic amino acids, 1-carboxyl-5-amino compounds (Markus et al., 1978a,b), prompted us to study the interaction of Glu-, Lys-, and miniplasminogen with AH-Sepharose a potential affinity adsorbent for these proteins, because of its content of 6-amino-hexyl side chains.

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MATERIALS and METHODS

The following materials were obtained from the indicated commercial sources: α-N-acetyl-L-lysine methyl ester (ALME; Sigma), α-N-benzoyl-arginine ethyl ester (BAEE; Sigma), 6-amino-hexanoic acid (6-AHA; Fluka), D-Val-Leu-Lys-pNA (S-2251 Kabi), human serum albumin (HSA; Behringwerke), urokinase (UK; Leo).

Human Glu- and Lys-plasminogen were purified using affinity chromatography on lysine-substituted Sepharose 4B (Deutsch and Mertz,1970) as described by Castellino and Powell (1981). Miniplasminogen was prepared as described by Sottrup-Jensen et al. (1977).

Interactions of the plasminogens with 6-amino-hexyl ligands

The frontal, quantitative affinity chromatography (FQAC) method described by Kasai and Ishii (1978) for the determination of dissociation constants of protein-ligand complexes was used. Affinity chromatography of Glu-, Lys- and miniplasminogen were performed using AH-Sepharose 4B as affinity adsorbent and 6-AHA and ALME as competing ligands. A solution of the plasminogen in question (approx. 100 ml 0.5 μM), where appropriate also containing competing ligand, was continually added to a column (0.9x10 cm, flow rate 5 ml/h) of AH-Sepharose suitably diluted with Sepharose 4B to obtain an elution volume of the plasminogen in the absence of competing ligand, not exceeding approx. 10 times the elution volume of non-adsorbed proteins (HSA). Experimental conditions: 0.1 M phosphate buffer, also containing 0.2 M NaCl to avoid ion exchange effects, pH 7.6, 25°C. The elution pattern was determined by measuring the amount of plasminogen present in each 0.25 ml-fraction collected using an S-2251 assay after addition of UK and 6-AHA to a final concentration of 25 mM. None of the plasminogens were adsorbed to pure Sepharose 4B, here they all showed an elution volume equal to the bed volume.

Conversion of Glu- and Lys-plasminogen to plasmin

Kinetic experiments on UK catalysed conversion of the plasminogens were performed and analysed as described earlier (Christensen and Müllertz, 1977; Christensen, 1977), but in some series of experiments a lysine substrate (S-2251) and not an arginine substrate (BAEE) for plasmin was used.

The reactions were also investigated using a two step assay method where plasminogen is incubated with UK in the absence of a plasmin substrate, and then an aliquot is added to a substrate solution and the plasmin formed is determined from the measured initial rate.

RESULTS

Interactions of the plasminogens with 6-amino-hexyl-ligands

Glu-plasminogen is a one chain protein, Glu-1-Asn-790, plasmin cleaves the Lys-76-Lys-77 bond rather easily so that Lys-plasminogen (Lys-77-Asn-790) is formed. Cleavage catalysed by elastase results in various fragments, one of which is mini-plasminogen (Val-442-Asn-790). The serine proteinase part of plasmin is Val-561-Asn-790 (Sottrup-Jensen et al., 1977).

Glu-, Lys- and miniplasminogen all strongly interact with AH-Sepharose. The dissociation constants of the AH-ligand-plasminogen complexes obtained from FQAC experiments are shown in Table 1. ALME was found to strongly effect these interactions in a simple competitive manner. The K_i-values obtained are shown in Table 1. Also 6-AHA effects the plasminogen-AH-Sepharose interactions, but less strongly than ALME. With 6-AHA two effects could be distinguished on the Glu-plasminogen-AH-ligand interaction, whereas those of Lys- and miniplasminogen both were inhibited in a simple competitive manner (Table 1).

Lys- and miniplasminogen interact with AH-Sepharose equally well and their interactions are inhibited in the same way by ALME as well as by 6-AHA. As seen from Table 1 Lys- and miniplasminogen both interact with the column by weak LBS that binds 6-AHA with K_i approx. 0.3 mM, not found (free) in Glu-plasminogen (Markus et al., 1978a,b). It is further seen that

Table 1

Dissociation constants of various plasminogen-ligand complexes determined by FQAC on AH-Sepharose

	K_d (μM) AH-Sheparose	K_i (μM) ALME	K_i (μM) 6-AHA
Glu-plasminogen	95	240[1]	10[2] 5600[1]
Lys-plasminogen	10	25[1]	295[1]
Miniplasminogen	10	26[1]	290[1]

1. Competitive inhibition of the plasminogen-AH-Sepharose interaction.
2. Noncompetitive inhibition of the Glu-plasminogen-AH-Sepharose interaction.

lysyl-compounds are bound more than 10 times more strongly. That site apparently, in contrast to the strong LBS, prefer lysine compounds not carrying a free carboxylate function, and therefore may interact with lysine side chains of proteins.

Native fibrin does not possess any C-terminal lysine-residues, which fulfil the structural requirements of the strong LBS and in our opinion are the only likely candidates present physiologically for ligands to bind the strong LBS, but fibrin does carry many lysyl-side chains. Miniplasminogen, but not various fragments of plasminogen from the N-terminal residues 77-441 that possess strong LBS, binds rather well to fibrin (Thorsen et al., 1981; Suenson and Thorsen, 1981). Interactions between native fibrin and the plasminogens by a weak LBS, which is actually a lysyl-site located in the miniplasminogen part of the molecules, is strongly indicated.

Blocking of the strong LBS of Lys-plasminogen by 6-AHA (K_i=35 μM; Markus et al., 1978a) has no effect on its AH-Sepharose interaction. This is not so with Glu-plasminogen, as seen from the difference between the effects of 6-AHA and ALME.

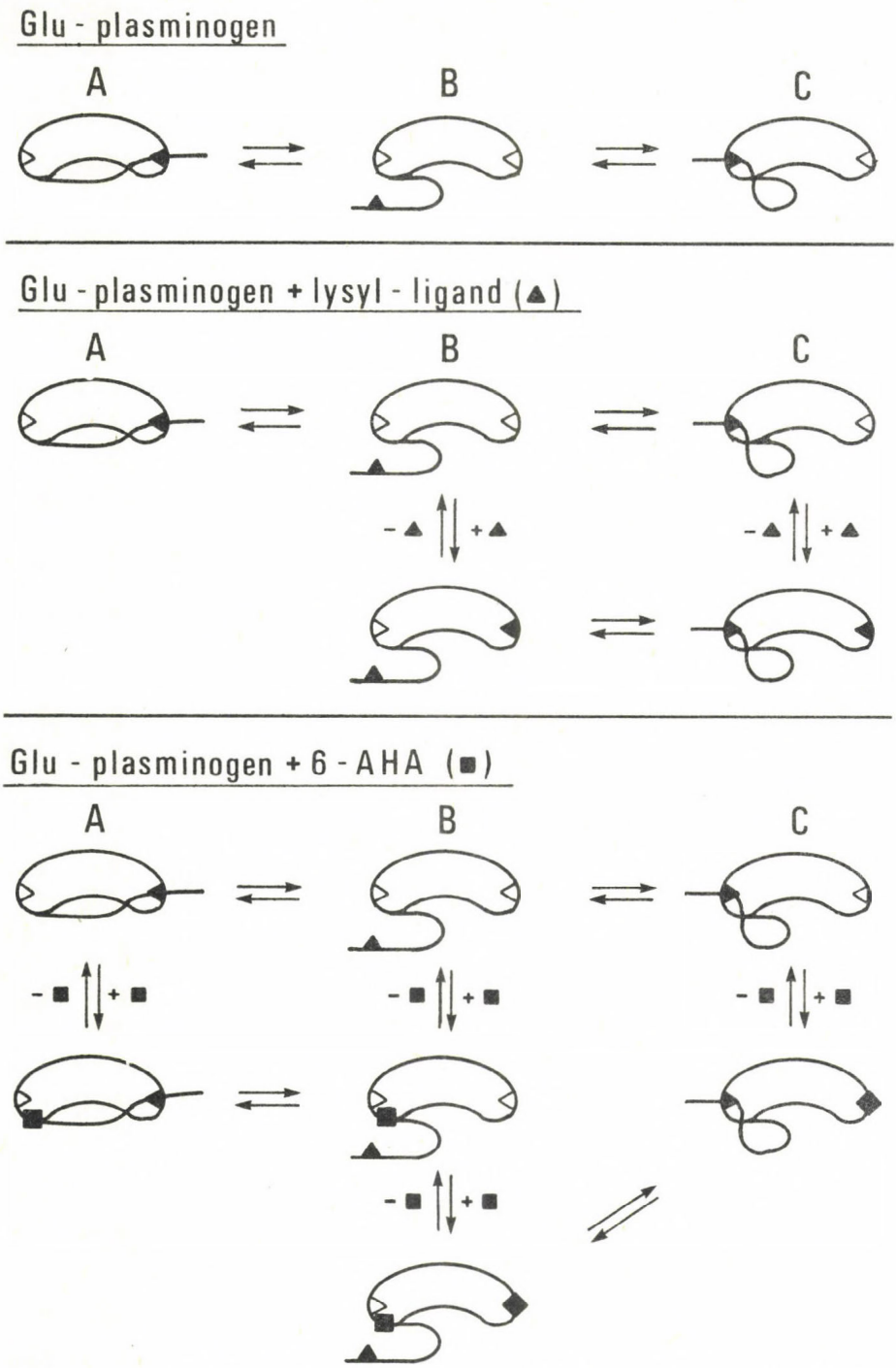

Fig. 1: The various forms of Glu-plasminogen. Dissociation constants are given in Table 1.

The ratios of the Glu- and Lys-plasminogen apparent binding constants (Table 1) for ALME and AH-Sepharose are exactly the same (9.5), but for 6-AHA it is 20.

A model that fits these results and is compatible with other known features of the plasminogens is presented in Fig. 1. We propose that Glu-plasminogen exists in three forms. In the absence of external ligands the equilibrium state between these forms results in the presence of 89.5 % A-form, 5 % B-form and 5.5 % C-form, where the A-form has an internally occupied lysyl-site and the internal bond is between a lysyl-side chain located in the Glu-1-Lys-76 part and the lysyl-site in the miniplasminogen part of the molecule. The B-form has no such internal bond and the C-form has another (weak) internal bond affecting the interaction of 6-AHA with the strong LBS, most probably indirectly by binding of the Glu-1-Lys-76 part of the molecule to a neighbouring weak LBS probably lysyl-site.

In the presence of a lysyl-ligand and equilibrium state between 5 forms: the A-form, two B- and C-forms (with and without bound lysyl-ligand) is established. In the presence of 6-AHA a different 5-form-equilibrium state between the C-form and two A- and B-forms (with and without occupied strong LBS) results at low concentrations of 6-AHA, but at high concentrations of 6-AHA a 7-form-equilibrium state adding the B- and C-forms with occupied lysyl-site is obtained, thus giving rise to the two effects observed with 6-AHA.

Apparent K_d-values of Glu-plasminogen-fibrin and Lys-plasminogen-fibrin complexes (Suenson and Thorsen, 1981) are of the same order of magnitude as those found here of the AH-Sepharose complexes and also approx. 10 times greater for Glu-plasminogen than for Lys-plasminogen. Supporting the suggestion that the (native) fibrin-plasminogen interaction mainly is mediated by a lysyl-site. The result reported by Markus et al. (1978a,b) that Glu-plasminogen binds the second 6-AHA molecule approx. 20 times more weakly than Lys-plasminogen K_d=0.26 mM does, is also compatible with our model.

The presence or absence of the Glu-1-Lys-76 fragment makes the molecular differences between Glu-and Lys-plasminogen. Lerch et al. (1980) studied the isolated CNBr-fragment Glu-

1-Met-57 and found that it interacted with the isolated so-called kringle 1 part (K1) residues 77-approx. 160 of plasminogen, but not by the strong LBS, which is located there (Wiman and Wallen, 1977). It should be noted that the fragment Glu-1-Lys-76 that results from plasmin cleavage does contain a C-terminal lysine residue and therefore may interact with the strong LBS, whereas the Glu-1-Lys-76 part of Glu-plasminogen and the CNBr-fragment do not. An important feature of the C-form of Glu-plasminogen is its inhibition of the interaction of 6-AHA with the strong LBS, no such inhibition was observed by Lerch et al. (1980) using the CNBr-fragment, but that fragment is not a part of the polypeptide chain and has a loose C-terminal end.

The dissociation constants of the internal bonds of the A- and C-forms of Glu-plasminogen found are A=B, K=0.66 (dimensionless) and C=B, K=0.9. Thus the A-form is 15 times stronger bound than the C-form.

Conversion of Glu- and Lys-plasminogen to plasmin

The results on the kinetics of UK catalysed conversion of the plasminogens to plasmin obtained by Wohl et al. (1978, 1980) and those obtained by us previously (Christensen and Müllertz, 1977; Christensen, 1977) are markedly different. The only major experimental difference between these studies is the kind of substrate used to detect the plasmin formed in the course of the reactions. Wohl et al. uses a lysyl- and we use an arginyl-substrate.

The realization, that the presence or absence of a lysyl-ligand possibly interacting with a weak LBS of the plasminogens may be of great importance for the activation process and may lead to completely different kinetic parameters, led us to re-investigate the processes.

The results are presented in Table 2. It is seen that the presence of a lysyl-substrate leads to K_m (Michaelis constants) for both Glu- and Lys-plasminogen activation remarkably low compared to the K_m-values obtained using an arginyl-substrate. The values obtained here are similar to those reported by Wohl

Table 2

Kinetic parameters of UK-catalysed conversion of Glu- and Lys-plasminogen to plasmin

	One step assay[1]			Two step assay[2]		
	K_m (μM)	$k_{c,app}$ (s^{-1})	k_c[3] (s^{-1})	K_m (μM)	$k_{c,app}$ (s^{-1})	k_c[3] (s^{-1})
Glu-plasminogen						
BAEE	≧40	0.3	3	≧40	0.3	3
S-2251	6	3	3	≧40	3	3
Lys-plasminogen						
BAEE	32	3	-	30	3	-
S-2251	5	3	-	31	3	-

1. Activation and plasmin assay in the presence of BAEE or S-2251.
2. No plasmin substrate present during activation.
3. k_c corrected for Glu-plasmin/Lys-plasmin activity differences.

et al. (1980). The apparent catalytic constant of the processes, k_c, is unchanged with Lys-plasminogen, but increases approx. 10 times with Glu-plasminogen as the UK-substrate when determined in the presence of lysyl-ligands.

Binding of a lysyl-ligand to Glu-plasminogen changes the equilibrium state from one where mainly A-form to one where mainly B- and C-forms are present, and that explains nicely the different K_m-values obtained in the presence and absence of lysyl-ligands, if it is assumed that UK is able to bind only the B- and C-forms.

The k_c-values have to do only with the rate of the steps of the catalytic process following the binding of the substrate to the enzyme and should not obviously change because more of the substrate is in a form that binds the enzyme. This was further investigated using a two step assay method where

plasminogen and UK were first incubated in the absence of lysyl-ligands and then the plasmin formed was determined by two initial rate assays one using BAEE and the other using S-2251 as substrates.

With Lys-plasminogen as the UK-substrate the kinetic parameters obtained were those expected in the absence of a lysyl-ligand (Table 2), and no difference was seen between the results of the BAEE- and S-2251-plasmin-assays. In contrast to this the Glu-plasminogen data were markedly different.

According to the BAEE (arginyl-substrate) assay the samples contained much less plasmin than according to the S-2251 (lysyl-substrate) assay; that is when the amount was calculated using the well-known kinetic parameters of Lys-plasmin for these substrates, a fact that can be explained only by assuming a difference in the kinetic properties of Glu-and Lys-plasmin. The different apparent catalytic constants of UK-catalysed conversion of Glu-plasminogen in the presence and absence of lysyl-ligands, obtained in the one step assay, disappears when a change of the activity of Glu-plasmin is taken into account. And that change is explained, if it is assumed that only approx. 10 % of Glu-plasmin is in an active form in the absence of lysyl-ligands. So that Glu-plasmin similar to Glu-plasminogen exists in several forms one of which is blocked and has no catalytic activity (A-form).

No difference in catalytic activity was found between Glu- and Lys-plasmin by Wohl et al. (1977) and that is in accordance with our suggestion, since they used a lysyl-substrate. We have previously found a positive effect of antifibrinolytic amino acids on the k_c-value of plasmin catalysed hydrolysis of BAEE (Christensen, 1978) that was attributed to LBS-interaction of plasmin in general, in fact those results may be better explained by assuming the presence of some Glu-plasmin and the conversion of that to active form.

Not only Glu-plasminogen, but also Lys-plasminogen becomes a better UK-substrate in the presence of lysyl-ligands according to our results (Table 2). We therefore assume that binding of a lysyl-ligand to the weak LBS, in general is important to obtain an efficient activation of the plasminogens, so that the

B- and C-forms of Glu-plasminogen with bound external ligands are even better UK-substrates than the B- and C-forms themselves.

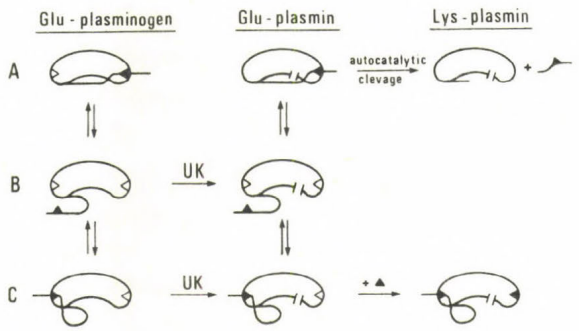

Fig. 2: Model of UK-catalysed conversion of Glu-plasminogen

A model that illustrates the conversion of Glu-plasminogen to plasmin is presented in Fig. 2. Only the B- and C-forms of Glu-plasminogen interact with UK and in the first place, only the B- and C-forms of Glu-plasmin are formed. In the presence of an external lysyl-ligand the ligand is bound to the lysyl-site and the enzyme is kept active and in its absence the enzyme converts to the inactive A-form. Active Glu-plasmin (at least in the C-form) is protected from α-2-antiplasmin, whereas autocatalytic cleavage to form Lys-plasmin apparently facilitates that interaction (Christensen, unpubl.).

The main physiological inhibitor of plasmin, α-2-antiplasmin interacts specifically with the K1+2+3 part of plasminogen (Wiman, 1980). The strong LBS is unoccupied in final plasmin-α-2-antiplasmin complexes and α-2-antiplasmin contains no C-terminal lysine residue and that agrees with our result that a weak LBS is involved (Christensen and Clemmensen, 1977, 1978), but according to Wiman and Collen (1978) α-2-antiplasmin interacts at a strong LBS.

DISCUSSION

The serine proteinases constitute an important group of enzymes that acts as catalysts in a variety of physiological processes. There are many indications that these enzymes each possesses a catalytic site similar to that of other serine proteinases and that they all probably employ a common mechanism to catalyse the hydrolysis of their substrates. It seems reasonable therefore if sites other than the catalytic site are responsible for individual enzyme specificities.

Plasmin has a specific and selective physiological function, i.e. the degradation of fibrin deposits in the blood vessels. It shows catalytic characteristics similar to those of trypsin to low-molecular weight substrates, but the two enzymes are distinctly different in their reaction with fibrin and the inhibitors of the fibrinolytic system. The physiological function of plasmin presumably is controlled by sites other than the active site, the so-called LBS's, and according to the results presented here, a weak LBS, which is actually a lysyl-site that interacts with (specific) lysine side chains of proteins, is of crucial importance in the regulation of fibrinolysis.

Below we present a hypothesis on the regulation of fibrinolysis that explains the efficient and specific hydrolysis of fibrin catalysed by plasmin.

1. In normal plasma only Glu-plasminogen and no Lys- or miniplasminogen is present. Glu-plasminogen exists mainly in the A-form which is not converted to plasmin by activator-enzymes.

2. In the presence of fibrin (native), where the concentration of specific lysyl-ligands is high, Glu-plasminogen binds and fibrin-bound Glu-plasminogen exists only in an activatable form.

3. Activator catalysed conversion of bound Glu-plasminogen results in Glu-plasmin bound to fibrin at the lysyl-site. Internal Glu-plasmin-Kl (weak LBS) interaction protects it from α-2-antiplasmin.

4. The lysyl-site interaction brings a (number of) particular fibrin peptide bonds in close contact with the catalytic site of plasmin, so that cleavages occur only at specific points of the fibrin molecule. During fibrinolysis a number of C-terminal lysine residues are formed, since plasmin preferentially cleaves Lys-X bonds of fibrin and other proteins. Plasmin uses these in getting from one cleavage point to another by alternating LBS[+] and lysyl-site interactions and is not released until the fibrin network is dissolved.

5. Termination of fibrinolysis involves several mechanisms that protect the organism from damaging cleavages catalysed by plasmin. Binding of internal lysyl-ligand to a weak LBS in the miniplasmin part of the molecule converts most of the free Glu-plasmin to the inactive A-form, the interaction with α-2-antiplasmin is no longer inhibited by internal Glu-plasmin-K1 interaction, and autocatalytic cleavage of the primary Glu-plasmin-α-2-antiplasmin complex to a similar Lys-plasmin complex further facilitates the final formation of α-2-antiplasmin-plasmin complex, that irreversibly inhibit plasmin. Further, since lysyl-site interactions apparently are stronger than active site interactions of plasmin, the binding of proteins may preferentially occur at the lysyl-site and reduce plasmin catalysed protein cleavage greatly, because only a limited part of the protein, perhaps containing no Lys-X or Arg-X bonds, is accessible to the active site. The main features of this hypothesis are shown in Fig. 3.

[+]Interactions at a strong LBS are indicated by the classical effect of low concentrations of 6-AHA on fibrinolysis. The fragments of plasminogen have two strong LBS's (K1 and K4 sites). Only that of K1 is open in free Glu- and Lys-plasminogen (Vali and Patthy, 1982). The conformational change involved when external lysyl-ligands bind Glu-plasmin(ogen) may,however, open the K4 site and ligands binding at that site may stabilize the active conformation of Glu-plasmin, as well as lysyl-site interactions.

1. Free Glu-plasminogen :

2. Fibrin-bound Glu-plasminogen :

UK

3. Fibrin-bound Glu-plasmin :

4. Fibrinolysis :

5. Termination :

AP

AP

AP

Fig. 3: Model of fibrinolysis

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DISCUSSION

PATTHY:

From your data presented here it appears that there is little difference between the kinetic parameters of the activation of Glu-plasminogen and Lys-plasminogen by urokinase. How do you reconcile your results with those of Peltz et al. (1982) who found about 10-fold difference in the K_m values for the activation of Glu- and Lys-plasminogen?

CHRISTENSEN:

The kinetic data, you refer to, were obtained in the absence of lysine and Lysyl-compounds. Plasmin was assayed by titration using an arginine-analogous compound in a two step-assay. The results are not very different from ours obtained under similar conditions.

STEPANOV:

Do you have any direct evidence concerning the flexibility of kringle structures in plasminogen and its activation products?

CHRISTENSEN:

There are well-known conformational differences between Glu- and Lys-plasminogen, and in the presence of high concentrations at 6-amino-hexanoic acid some changes of conformation occur in both proteins, more markedly in Glu- than in Lys-plasminogen.

GRÁF:

Do I understand well your suggestion that C-terminal lysines are important in the structure of fibrin for interaction with plasminogen? Do these lysines provide a sort of binding site for the first interaction?

CHRISTENSEN:

C-terminal lysine residues are initially not present. We think, that such residues formed during fibrinolysis may play a role, but not in the initiation of fibrinolysis. We propose initial plasminogen-fibrin interaction at a lysyl-site by binding a lysyl-residue.

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KRINGLE STRUCTURES OF HUMAN PLASMINOGEN: THEIR ROLE IN THE REGULATION OF FIBRINOLYSIS

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INTRODUCTION

Plasmin, a trypsin-like serine protease, is primarily responsible for the removal of fibrin deposits from the walls of blood vessels. Although the enzyme has a broad sequence-specificity, intravascularly it shows a remarkable selectivity for fibrin. The fibrin-specificity of plasmin is due to regulatory mechanisms which ensure that plasmin formation takes place predominantly when and where fibrin formation occurs (Wiman and Collen, 1978). Recent studies have revealed that fibrin greatly facilitates the conversion of plasminogen to plasmin by tissue-type plasminogen activator since both plasminogen and plasminogen activator are adsorbed to fibrin to form a cyclic ternary complex and activation of plasminogen occurs within this complex (Hoylaerts et al., 1982). The plasmin formed remains bound to fibrin polymer, degrades fibrin into soluble fragments thus removing the fibrin deposit. Plasmin released from the clot is rapidly inactivated by the fast-acting plasmin inhibitor, α_2-antiplasmin, therefore other plasma proteins are normally not attacked by plasmin.

The specific interaction of plasminogen and plasmin with fibrin, the rapid binding of plasmin by α_2-antiplasmin thus play important roles in the regulation of fibrinolysis. The structures essential for these interactions are located in the amino-terminal non-protease part of plasminogen (Wiman et al., 1979; Thorsen et al., 1981). The multiplicity of binding functions associated with this segment is reflected in its structure since in this part of the molecule five closely homologous three-disulphide-bridge structures, kringles, are present which carry the binding sites for fibrin, α_2-antiplasmin and low molecular weight compounds (Sottrup-Jensen et al., 1978).

GENERAL CHARACTERISTICS OF KRINGLE STRUCTURES

Kringles are present in several trypsin-type proteases of blood plasma. Thus the non-protease part of human plasminogen contains five kringles (Sottrup-Jensen et al., 1978), human and bovine prothrombin have two kringles (Magnusson et al., 1975; Walz et al., 1977), human high molecular weight urokinase contains one kringle (Günzler et al., 1982) and human tissue-type plasminogen activator possesses two kringles (Pennica et al., 1983). The kringles of the different proteases show a high degree of sequence homology, they appear to be more closely related than the protease parts (Young et.al., 1978).

Several studies have demonstrated that the kringles are independent structural domains. Limited proteolysis of prothrombin and plasminogen is known to yield fragments corresponding to kringles, indicating that

kringles are independent structural units (Magnusson et al., 1975; Sottrup-Jensen et al., 1978). Comparison of the circular dichroism spectra of prothrombin and its constituent fragments obtained by limited proteolysis showed that dissection of the molecule causes little alteration in the structure of the kringles, suggesting that these regions exist as independent structural domains (Bloom and Mann, 1979). Differential scanning calorimetry studies on plasminogen, prothrombin and proteolytic fragments thereof also showed that the kringles are independent structural domains (Ploplis et al., 1981; Castellino et al., 1981). Immunochemical studies using antibodies raised against plasminogen have shown that antigenic structures are conserved in isolated kringles thus providing further evidence for the view that kringles exist as independent domains in the native molecule (Ploplis et al., 1982).

The kringles appear to correspond to functional domains since kringles isolated by limited proteolysis of plasminogen and prothrombin retain their original binding functions. Isolated kringle 1 and kringle 4 fragments of plasminogen bind ω-aminocarboxylic acids (Sottrup-Jensen et al., 1978; Lerch et al., 1980), kringle 5 fragment of plasminogen binds to benzamidine-Sepharose (Váradi and Patthy, 1981). Kringle 1 fragment of plasminogen also preserves its ability to bind to fibrin (Lerch et al., 1980). Fragment 2 of prothrombin, which corresponds to one of the prothrombin kringles, has an intact binding site for factor V_a (Esmon and Jackson, 1974).

In view of these findings it is likely that kringles are structurally and functionally independent domains specialized to bind different proteins or ligands. In the case of prothrombin and plasminogen the kringles determine binding specificities which endow these proteases with some of the properties required for their highly specialized function. Although nothing is known at present about the exact function of the kringles of urokinase and tissue-type plasminogen activator, the homology with the prothrombin- and plasminogen-kringles suggests that they may be essential for binding functions important for the regulation of plasminogen activation.

KRINGLE 4 DOMAIN OF HUMAN PLASMINOGEN

Considering the importance of kringles in the function of several highly specialized proteases it is of interest to define the structural and functional properties of these "miniproteins". In our studies we chose kringle 4 of plasminogen as a model since this kringle fragment can be prepared in high yield, it carries an intact ω-aminocarboxylic acid binding site whose functional integrity can be readily tested by affinity chromatography on lysine-Sepharose.

The ω-aminocarboxylic acid binding site of kringle 4

Kringle 4 is known to bind various ω-aminocarboxylic acids with high affinity (Lerch et al., 1980), the optimum distance between the amino and carboxyl groups of the ligand is 0.68 nm (Violand et al., 1978; Winn et al., 1980). Since both the positive and negative charges of the ligand are essential for ligand binding (Winn et al., 1980) it is probable that complementary charges present in the binding site are involved in electrostatic binding of the ligand. Variation of substituents of ω-aminocarboxylic acids also revealed that a polar group on the carbon α to the carboxyl retards binding, substitution of this polar group with an apolar substituent increases affinity (Winn et al., 1980). Therefore it may be assumed that a hydrophobic interaction between the ligand and the binding site is also important for binding of ω-aminocarboxylic acids.

Chemical modification studies have been carried out in order to identify residues essential for ligand binding by kringle 4. Modification of kringle 4 with 1,2-cyclohexanedione was found to lead to loss of lysine-Sepharose affinity and the abolition of ligand binding was shown to be due to modification of Arg-7o (Trexler et al., 1982). Arg-7o is essential for ligand binding since it is responsible for the electrostatic binding of the carboxyl group of ω-aminocarboxylic acids. The possibility that modification of Arg-7o caused loss of lysine-Sepharose affinity indirectly, through a modification-induced conformational change, is unlikely since nmr studies showed that reaction of Arg-7o did not cause a detectable conformational change of the kringle (Trexler et al., 1983). The direct involvement of Arg-7o in ligand binding is further supported by our observation that ligand protects Arg-7o against reaction with 1,2-cyclohexanedione since it renders the residue inaccessible to the reagent.

Modification of kringle 4 with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide also led to abolition of affinity for lysine-Sepharose, reaction of Asp-56 of kringle 4 being responsible for loss of affinity (Trexler et al., 1982). The importance of Asp-56 for ligand binding is probably due to its involvement in electrostatic binding of the amino group of the ligand.

Reaction of Trp-71 of kringle 4 with dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide (Hochschwender and Laursen, 1981 b) or with hydrogen peroxide-dioxane (Trexler et al., 1983) destroys lysine-Sepharose affinity. The Trp-71 residue is essential for ligand binding probably because it interacts with the hydrocarbon chain moiety of ω-aminocarboxylic acids. Polar substitution of the α-carbon of ligands possibly weakens binding since it interferes with the interaction between the hydrocarbon moiety of the ligand and the indole side-chain of Trp-71.

The presently available informations thus suggest that Arg-7o, Asp-56 and Trp-71 of kringle 4 are directly involved in ligand binding. It is noteworthy that these residues are clustered in the inner loop of kringle 4.

The solution structure of kringle 4

The solution structure of kringle 4 is studied by chemical modification and by nmr spectroscopy of native and chemically modified kringle 4 species in collaboration with the Inorganic Chemistry Laboratory of Oxford. Comparison of the nmr spectra of native and chemically modified kringle 4 species permitted the assignment of resonances to specific residues in the kringle 4 sequence. Nuclear Overhauser experiments defined several residue-residue interactions in the kringle 4 structure, permitting the definition of an outline structure of kringle 4 (Trexler et al., 1983). The most noteworthy feature of the kringle-fold is that Leu-45 interacts with the sequentially distant Trp-25 and Trp-61 residues. Since these residues are conserved in all kringles sequenced so far it seems probable that this structural feature is common to all kringles.

Folding autonomy of kringle 4

The structural and functional independence of kringles raised the possibility that the kringles may be autonomous folding domains. To test this assumption we have carried out experiments with kringle 4 fragment to decide whether this isolated kringle is capable of refolding to its native structure following reductive cleavage of its disulphide bridges (Trexler and Patthy, 1983). Treatment of kringle 4 with reducing agents in the presence of 6 M guanidinium chloride led to loss of lysine-Sepharose

affinity, but when the reduced-denatured kringle was incubated aerobically in the absence of reducing and denaturing agents, the lysine-Sepharose affinity was regained concomitant with the restoration of its disulphide bonds. Refolding and recovery of lysine-Sepharose affinity was significantly faster when refolding was performed in buffers containing oxidized and reduced glutathione.

During refolding aliquots of the refolding mixture were withdrawn for alkylation with iodoacetic acid and the S-carboxymethylated folding intermediates were analyzed by isoelectric focusing. Chromatography of alkylated refolding mixtures on lysine-Sepharose revealed that not only the fully refolded kringle 4 was capable of binding to lysine-Sepharose; a folding intermediate possessing only two disulphide bridges was also bound to the affinant. Structural analysis of this functionally active two-disulphide-bridge folding intermediate showed that it has two of the three native disulphide bonds but lacks the disulphide bond connecting Cys-1 and Cys-79. The ability of this two-disulphide-bridge folding intermediate to bind to lysine-Sepharose indicates that the third disulphide bridge is not essential for the maintenance of the functionally active architecture of kringle 4.

In summary, kringle 4 of human plasminogen is capable of assuming the native, biologically active conformation after denaturation and reductive cleavage of its disulphide bonds, indicating that this fragment harbours an intact folding unit. The kringle 4 domain thus corresponds to an independent structural, functional and folding domain.

Although the different kringles of prothrombin, plasminogen, urokinase and tissue-type plasminogen activator diverged to bind different proteins or low molecular weight ligands they retained the same triple-loop architecture. If the amino acid sequences of kringles with different binding functions are compared, the residues essential for the autonomous folding should be found unchanged in all or most of the kringles, whereas the residues involved in the specialized binding functions may vary from kringle to kringle. Comparison of the sequences of the kringles of human plasminogen, human prothrombin, human high molecular weight urokinase and human tissue-type plasminogen activator reveals that in addition to the six cysteines conserved in all kringles, the conserved residues are clustered around Cys-5o, Cys-62 and Cys-74 (Trexler and Patthy, 1983). Of special interest is that Leu-45, Trp-25 and Trp-61, which were shown by nmr studies to interact in the kringle-fold, are conserved in all kringles. This observation provides further support to the view that the residues conserved in all kringles are essential for the folding autonomy of these domains.

The residues involved in the varied binding functions of kringles are probably found in sequence areas where the different kringles show variability. Such regions are present in the right outer loop (residues 26-44) and in the left and right arms of the inner loop (Trexler and Patthy, 1983). It is noteworthy that Asp-56, Arg-7o and Trp-71, residues essential for ligand binding by kringle 4, are located in these variable regions.

The function of the kringle 1 and kringle 4 domains of plasminogen

Kringle 1 and kringle 4 of plasminogen are similar to each other in that both kringles bind ω-aminocarboxylic acids, the ligand-specificity of the two kringles is apparently identical (Winn et al., 198o). Despite this similarity the binding sites of kringle 1 and kringle 4 probably have different function.

In the case of kringle 1 the binding site appears to be essential for fibrin affinity as the interaction of this kringle with fibrin is abolished by ω-aminocarboxylic acids (Lerch et al., 198o). The fibrin

molecule possesses two types of plasminogen binding sites (Váradi and Patthy, 1983) and the kringle 1 domain appears to be essential for binding of plasminogen to both sites since the plasminogen-fibrin complex is disrupted by ω-aminocarboxylic acids at concentrations that saturate only the site present on kringle 1 (Thorsen et al., 1981). In contrast with kringle 1, kringle 4 has practically no affinity for fibrin (Thorsen et al., 1981) suggesting that it has a function different from that of the first kringle.

Several lines of evidence indicate that the kringle 4 domain and its ω-aminocarboxylic acid binding site are buried in Glu-plasminogen. We have shown previously that the kringle 4 domain of Glu-plasminogen is unable to mediate binding to lysine-Sepharose since intramolecular interactions render it inaccessible to external ligands (Váli and Patthy, 1982). Studies with antibodies raised against isolated kringle 4 also revealed that the antibodies bind twenty times more weakly to Glu-plasminogen than to kringle 4 fragment, confirming that the kringle 4 domain is practically inaccessible in Glu-plasminogen (Hochschwender and Laursen, 1981 a). In Glu-plasminogen the ω-aminocarboxylic acid binding site of kringle 4 is involved in interactions that shield it even from small ligands, therefore in Glu-plasminogen it has only very weak affinity for ligands (Markus et al., 1979). Abolition of these interactions with ω-aminocarboxylic acids causes the transition of Glu-plasminogen from a closed to a loose conformational state (Markus et al., 1979) suggesting that the ω-aminocarboxylic acid binding site of the kringle 4 domain is essential for maintaining the closed conformation of Glu-plasminogen. Since the two conformational states of plasminogen differ markedly in their activability by plasminogen activators (Markus et al., 1979; Peltz et al. 1982) the ω-aminocarboxylic acid binding site controlling the conformation of plasminogen has an important role in the regulation of fibrinolysis.

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DISCUSSION

GRÁF:

The autonomous folding of kringle 4 could be further confirmed by an experiment in which the oxidation of reduced kringle 4 would be studied in the presence of other entities of plasminogen like the large fragment containing kringles 1-3. I wonder whether the oxidation rate /folding/ of kringle 4 was affected by other portions of the plasminogen molecule.

PATTHY:

We have not yet performed such experiments.

CHRISTENSEN:

Did you use the X-ray crystallographic results reported recently on one of the prothrombin-kringles, when your model of kringle 4 was constructed?

PATTHY:

From the low resolution model of prothrombin fragment only the gross dimensions of the kringle-fold are known, but details cannot yet be deduced from these data. For this reason we did not use the X-ray crystallographic data in the construction of our kringle 4 model.

Symposia Biologica Hungarica 25 (1984)

SUBSTRATE DESIGN AND PROTEOLYTIC ACTION

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The fundamental problem in the kinetic investigation of the enzymes which can hydrolyze several bonds of high-molecular-weight substrates is that the size and the structure of the substrate and, thus, the availability of the bond to be split next are changing continuously as a result of each attack by the hydrolytic enzyme. Therefore, a basic demand of kinetic analysis, that every catalytic step has to take place exactly under the same conditions, is poorly satisfied in these cases. For example, in the pioneering studies on proteolytic enzymes /Northrop et al., 1948/ various proteins, denatured hemoglobin, casein, etc. were evidently assayed as substrates. The heterogeneity of the substrate in an assay mixture, however, prevents the exact determination of the kinetic parameters and the time-velocity function does usually not result in a straight line. Therefore, only apparent kinetic constants can be calculated from the data obtained on high-molecular-weight substrates. To avoid this, low-molecular-weight substrates, esters, amides, etc. of amino acid derivatives were introduced for the kinetic studies of proteolytic enzymes /Schwert et al., 1948/. These compounds

Abbreviations: Boc: -t-butoxycarbonyl; Bz: -benzoyl; pNA: -p-nitroanilide; Suc: -succinyl; Tos: -tosyl; Z: -benzyloxycarbonyl.

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contain only one scissile bond and the product of enzyme action does not get involved again with the enzyme after hydrolysis. Nevertheless, the low-molecular-weight synthetic substrates meet certain requirements of kinetic investigations, however, their affinity to enzymes is usually rather poor, it is somewhere in the milimolar range.

A satisfactory compromise was found in the last decade by the introduction of chromogenic peptide substrates of various lengths /Blombäck et al., 1967; Svedsen et al., 1972/ containing a chromogenic or fluorogenic group at the C-terminus of the peptide. The labeling group is usually bound to the peptide by an amide or ester bond. The properties of the synthetic substrates are similar to those of natural substrates in many respects and surpass the characteristics of amino acid derivatives in the following:

1. They contain only one bond to be split.
2. Their affinity is superior to substrates that contain a single amino acid and their K_m value may approach the micromolar range. Thus, their affinity to enzymes is greater by orders of magnitude than that of the single amino acid derivatives.
3. By applying peptide substrates more information can be obtained than with compounds containing a single amino acid. To the primary-specificity site we can add residues differing in size, polarity, charge and other properties at P_2, P_3 positions /notation of Schechter and Berger, 1967; Table 1/. The investigation of these substrates may reflect the properties of the S_2, S_3, etc. substrate-binding sites of proteinases. The data obtained with substrates of various amino acid sequences may permit us to draw conclusions about the size, polarity or hydrophobicity of the binding site, or about the presence or absence of charged side chains in the binding area.
4. The convenience of measurements with chromogenic or fluorogenic substrates, the spectrophotometric recording of the changes in absorption, allowed the widespread application of chromogenic and fluorogenic peptide substrates for practical purposes in clinical and other laboratories /see for a review Huseby and Smith, 1980/.

It seems to be evident that both the affinity of the enzyme to the substrate and the velocity of the cleavage of a sensitive bond may strongly depend on the size and composition of the rest of the peptide part joined to the primary-specificity residues of the substrate. If so, what should the composition of a substrate expected to work satisfactorily with a proteinase be like?

The are at least three different ways for finding an answer to this question: 1. imitation of the structure of the native substrate around the scissile bond, 2. synthesis of peptides of different composition by trial and error, and 3. application of a systematic method, if any, which enables us to predict the properties of good substrates. The first reasoning, which looks very logical at first sight, is based on the assumption that the structure of a good substrate should be similar to that of the sequence around the scissile bond of the natural substrate. This suggestion can be easily accepted as far as the structural requirements of enzymes in the blood-clotting system are concerned. However, in the case of less selective proteinases it may bring about difficulties.

The members of the blood-clotting cascade are fastidious enzymes. It was demonstrated, for example, that the last enzyme of the cascade system, thrombin, which was investigated most extensively, can split only very few bonds. The substrates available commercially for the determination of thrombin activity /Blombäck et al., 1977; for reviews see Jackson and Nemerson, 1981; Lottenberg et al., 1981; Elődi, 1983/ have slightly different sequences in the vicinity of the primary-specificity side chain, arginin /Table 2/, compared to those found in the natural substrates. In addition, they may contain non-natural residues, e.g. pipecolate, at P_2 position, or D-derivatives of amino acids at P_3 position appear to be at least as favourable as the side chain of natural amino acids.

The second method of finding good sequences is trial and error /Claeson et al., 1977; Claeson and Aurell, 1981; Ondetti and Cushman, 1981/. It is easy to admit that the selection of proper substrates by trial and error may be a time-consuming task, since, in principle, each substrate

Table 1

Amino acid sequences adjacent to bonds cleaved by thrombin
/cf. Blombäck et al., 1977/

Substrate	P_3	P_2	P_1	P_1'
HUMAN				
Fibrinogen /Aα/	.Gly.	Val.	Arg.	Gly.
	.Gly.	Pro.	Arg.	Val.
/Bβ/	.Ser.	Ala.	Arg.	Gly.
Factor XIII	.Val.	Pro.	Arg.	Gly.
Factor VII	.Gly.	Val.	Arg.	Ile.
Prothrombin	.Thr.	Pro.	Arg.	Ser.
	.Thr.	Ala.	Arg.	Thr.
	.Asn.	Pro.	Arg.	Thr.
Antithrombin III	.Ala.	Gly.	Arg.	Ser.
Actin	.Ala.	Pro.	Arg.	Ala.
	.Arg.	Pro.	Arg.	His.
	.Asn.	Pro.	Lys.	Ala.

subsite may be occupied by one of the twenty natural amino acids. For good many reasons, only a limited number of amino acid residues was applied for the synthesis of peptide substrates and inhibitors containing peptidyl parts of various lengths /Bajusz et al., 1975; Kettner and Shaw, 1981/.

When we started our work on substrate design we were looking for some kind of a solid basis that may ensure a simpler and safer way for finding out the best composition of a substrate built up from a given set of amino acids.

Table 2

Kinetic parameters of the hydrolysis of peptidyl-p-nitroanilide substrates by human thrombin
/cf. Lottenberg et al., 1981/

Substrate	K_m /μM/	k_{cat} /s^{-1}/	k_{cat}/K_m /$\mu M^{-1}s^{-1}$/
N-protected peptides			
Tos-Gly-Pro-Arg- /Chromozym-TH/	4.2	130	31.0
Z-DPhe-Pro-Arg-	20.0	32	1.6
Z- Arg-Pro-Arg-	32.0	85	2.7
Unprotected peptides			
H-DPhe-Pro-Arg-	91.0	82	9.0
H-DVal-Pip-Arg-	120.0	68	5.9
H-DPhe-Pip-Arg- /S-2238/	1.5	90	60.0
H-DIle-Pro-Arg- /S-2288/	3.0	120	40.0
H-DVal-Pro-Arg- /S-2234/	14.0	63	4.3

There are some simple and common mathematical methods in use in pharmacological research that are successfully applied for designing drugs. It seemed to be promising to test these methods, since we were aware of the fact that the data obtained by enzyme-kinetic measurements are based on more precise and reliable techniques than those applied in pharmacological investigations, therefore, it was hopeful to expect better quantitative correlations, as well. These methods are based on the linear free-energy relationship of the macro-

molecule-ligand interaction /for a review see Martin, 1981/. Our original assumption was that a similar interaction may occur in the formation of the enzyme-substrate complex, too /Grieco et al., 1977/. Thus, we selected a simple method describing the quantitative structure-activity relationship /QSAR/ suggested by Free and Wilson /1967/.

At the beginning of our work on substrate design we expected that the mathematical treatment of the data by regression analysis would provide reasonable answer to the following:

1. Can a quantitative correlation between the substrate sequence and the kinetic parameters be expected?
2. Can we find a general standard, if there is any, for this correlation?
3. Is it possible to demonstrate an interaction or connection between the role of P_1 primary-specificity site and the residues of the secondary P_2, P_3, etc. sites? Are the primary and secondary sites equivalent in importance in the enzyme-substrate interaction?
4. Is it worthwile to substitute the trial and error method by an exact one based on calculations?

To answer these questions we measured the kinetic parameters of eight proteolytic enzymes with substrate series composed of free and N-protected tripeptidyl-p-nitroanilides and K_m, the Michaelis constant, k_{cat}, the catalytic constant, and k_{cat}/K_m were derived from the measured values /Pozsgay et al., 1977; 1979; 1981; 1981a; Cs-Szabó et al., 1980; 1980a; Marossy et al., 1980/. Then, these data were treated by regression analysis and the contribution of each side chain to the kinetic parameters was deduced. Since these contributions calculated by the QSAR method are additive, the values found by calculation permit us to predict the kinetic parameters of any kind of a substrate sequence composed from the investigated set of amino acids. Moreover, those residues that have the greatest contribution will form the best substrate, and the composition of an optimal substrate can be predicted.

Table 3

Contribution of tripeptidyl-p-nitroanilide substrates to the kinetic parameters of the human granulocyte elastase /15 substrates/
/Marossy et al., 1980/

Subsite	Substituent	Contribution $1/K_m$ mM^{-1}	Contribution k_{cat} s^{-1}	Contribution k_{cat}/K_m $M^{-1}\ s^{-1}$
P_4	Boc	57.02	0.388	72 137.3
	Bz	7.98	0.146	4 337.4
	Z	4.49	0.178	6 226.5
	Suc	- 26.37	-0.534	- 33 128.3
P_3	DPhe	33.40	0,218	31 419.6
	Arg	- 10.56	-0.686	4 599.8
	Gly	- 15.03	-0.059	- 13 818.1
	Asp	- 21.96	-0.673	- 27 949.0
	Ala	- 24.39	0.624	- 34 629.3
P_2	Ala	16.16	-0.093	44 033.0
	Pro	11.66	0.129	38 738.3
	Val	- 6.30	0.183	5 986.5
	Nle	-109.37	-1.135	-432 507.7
P_1	Nle	110.09	1.315	401 645.9
	Val	37.34	-0.0001	13 883.1
	Ala	- 2.72	0.091	- 14 831.0
	Leu	- 6.73	-0.035	- 37 172.0
	Arg	- 70.77	-0.866	- 95 869.4
Overall /μ/		24.32	0.387	20 517.6
Correlation coefficient /r/		0.8996	0.9365	0.9163

The mathematical treatment of the kinetic data provided us with three kinds of results. First, the predicted best susbstrate was a member of the assayed series, as was found with thrombin /Pozsgay et al., 1981/. Secondly, the sequence obtained by calculation was characteristic of a compound insoluble in aqueous media /Pozsgay, 1979/. Thirdly, when a new sequence was obtained, that seemed easy to handle, it was synthesized and assayed kinetically. The predicted and experimentally measured kinetic parameters showed good agreement /Gáspár et al., 1981/. In most of the cases the correlation coefficients of these values were over or near 0.9.

Let us take some examples that may illustrate the versatility of the QSAR-treatment of kinetic data. As the first example, we shall take elastases isolated from porcine pancreas and human granulocytes. They are similar in their substrate requirements, both can cleave peptide bonds at the aliphatic side chains. A slight difference between the two elastases is that the pancreatic enzyme prefers smaller side chains, like Ala, whereas, the granulocyte enzyme works faster when a bulkier side chain, like Leu, Val or Nle, forms the scissile bond.

It is worth to compare the values of contribution calculated from kinetic measurements. It can be seen in Table 3 that the best residues are not equally good in every respect. For example, DPhe at P_3 position has the greatest contribution to the K_m value for both elastases. In contrast, it attenuates the substrate when its contribution to k_{cat} is taken into account. Both enzymes prefer Pro or Ala at P_2 for a good K_m.

From the above data it was concluded that the aldehyde derivatives /Bajusz et al., 1975/ of compounds of the general formula

...DPhe.Pro/Ala/..X.H

may be reasonably good competitive inhibitors for elastases. A small set of such aldehydes was synthesized and assayed with Boc-Ala-pNP as substrate, and the K_i inhibitory constants were calculated. At P_1 the good inhibitor contained valine

Table 4

K_i inhibitory constants of peptide aldehydes measured with elastases

/Substrate Boc-Ala-pNP; in 50 mM Tris HCl, pH 8.0; at 37°C/

Compound	K_i inhibitory constant /μM/ human granulocyte	bovine pancreatic
Suc-DPhe-Pro-Ala.H	620	900
Nle.H	380	210
Val.H	40	1 300
Suc-Pro-DPhe-Pro-Nle.H	65	850
Val.H	38	520
Val.H	3[x]	-

[x]substrate: human lung elastin

for the granulocyte enzyme, whereas, alanine seemed to be more effective for pancreatic elastase /Table 4/. The K_i inhibitory constants of these compounds were in the 10^{-4} to 10^{-5} molar range. When, however, human elastin was used as substrate, the effect of Suc-Pro-DPhe-Pro-Val.H inhibitor was improved, and the K_i value decreased near to micromole.

The lengthening of Suc-DPhe-Pro-Val.H by a Pro residue at the N-terminus did not increase the inhibitory capacity of the compound when assayed with low-molecular-weight synthetic substrates. In contrast to the findings of Atlas /1974/ and MacRae et al. /1980/, the extension of peptide substrates and inhibitors for elastase definitely improves their catalytic productivity.

Another enzyme that became a point of interest recently is plasmin. A new line of research aims at some other aspects then the classical studies of enzyme-substrate or enzyme-inhibitor interactions, which encouraged us to reinvestigate our earlier data on plasmin /Cs-Szabó et al., 1980/.

It was suggested by Chakravarty et al. /1983; 1983a/ that peptides containing Lys residues at the C-terminus could be successfully employed in cancer therapy. They had bound drugs to be directed to the site of cancerous poliferation to the carboxylic group of the C-terminal lysine of a peptide, and thus a prodrug was formed. This, due to the relatively high plasmin activity around the proliferating cells, is expected to decompose and, consequently, the drug will exert its effect on the very site of malignant proliferation and hit practically the cancerous cells only. It is reasonable to suppose that the composition of the peptide part of the prodrug will not be indifferent in respect to its efficacy.

A few years ago /Cs-Szabó et al., 1980/ we carried out investigations to find "optimal" substrates for plasmin. These studies resulted a DIle-Phe-Lys-pNA sequence as a promising compound. Substrates having Z-, Bz-, Boc- or other protective groups appeared to be less favourable than their unprotected derivatives. The blemish of these studies was that all investigated compounds contained D-amino acids at P_3 position.

Recently we repeated these experiments with a different and larger series of substrates containing both L and D derivatives of the same amino acids at P_3 position DIle-Phe-Lys-pNA, the best member of the previous series was also included in the new substrate set, and the kinetic parameters were determined /Table 5/. Recent investigations revealed further remarkable features concerning both the reliability of regression analysis in predicting the kinetic parameters of peptide substrates and the plasmin-substrate interaction. First, again DIle-Phe-Lys appeared to be the best in respect of $1/K_m$ and k_{cat}/K_m /Table 6/, although, at P_3 the contribution of DPro to $1/K_m$ and that of DVal to k_{cat}/K_m seemed to be greater than the contribution of DIle to the same kinetic parameters. Secondly, the calculated contribution of pGlu to k_{cat} at P_3 was much greater than that of other ten side chains, whereas, its contribution to K_m seemed to be rather poor. In spite of this, we may list pGlu-Phe-Lys-pNA in the group of reasonably good substrates. Substrates No 16-18 in

Table 5

Kinetic parameters of plasmin determined with unprotected tripeptidyl-p-nitroanilides

Number	Substrate subsites P_3	P_2	P_1	$1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}\ s^{-1}$
1	DIle	Phe	Lys	33.8	3.13	106 000
2	DPro	Phe	Lys	28.0	2.16	60 370
3	DVal	Phe	Lys	20.0	4.93	98 520
4	DGln	Phe	Lys	19.2	1.72	33 100
5	DAla	Phe	Lys	16.0	2.05	32 870
6	DVal	Leu	Lys	9.7	7.95	77 180
7	DGlu	Phe	Lys	8.0	3.18	25 500
8	Ile	Phe	Lys	7.5	2.89	21 770
9	DIle	Leu	Lys	6.5	2.65	17 230
10	Pro	Phe	Lys	5.5	1.87	10 290
11	pGlu	Phe	Lys	5.0	15.92	79 620
12	Ala	Phe	Lys	5.0	4.90	24 500
13	pGlu	Leu	Lys	3.0	7.96	23 910
14	Val	Leu	Lys	2.5	1.38	3 450
15	Ile	Leu	Lys	1.0	3.43	3 430
16	DLeu	Met	Lys	10.0	2.76	27 600
17	DSer	Tyr	Lys	5.5	3.10	17 260
18	Gly	Tyr	Lys	5.0	2.85	14 230

Table 5 did not meet the requirements of regression analysis, therefore they were omitted from the calculation of contributions listed in Table 6.

Without exception, the D derivatives in the series of substrates appeared to be superior to L ones /cf. Bajusz, 1979/. For example, the K_m values of the D derivatives turned out to be three to six times higher /Table 7/.

According to our experience, plasmin seems to be somewhat

Table 6

Contribution of subsites to the kinetic parameters
/plasmin, calculated from a 15-member substrate series/

Subsite	Residue	Contribution to $1/K_m$ mM^{-1}	k_{cat} s^{-1}	k_{cat}/K_m $M^{-1}\ s^{-1}$
P_3	DPro	12.78	-2.65	3 840
	DIle	10.69	-1.31	28 100
	DVal	5.39	2.23	54 340
	DGln	3.98	-3.09	-23 430
	DAla	0.78	-2.76	-23 660
	Val	-1.20	-2.21	- 7 040
	Ile	-5.21	-1.04	-20 900
	pGlu	-5.46	7.73	18 260
	DGlu	-7.22	-1.63	-31 030
	Pro	-9.72	-2.94	-46 240
	Ala	-10.22	0.08	-32 030
P_2	Phe	3.84	4.06	15 350
	Leu	-7.68	-0.81	-30 690
Overall /μ/		11.38	4.41	41 180

exceptional among the proteolytic enzymes assayed with chromogenic peptide substrates, since the k_{cat} values found with these substrates appeared to be fairly insensitive to the composition of the substrates, in particular to the P_3 residue. It can be supposed that the P_3-S_3 interaction in the enzyme-substrate complex-formation is of limited importance in the case of plasmin.

In conclusion, we believe that regression analysis is a powerful tool for the study of protein-ligand interactions /Grieco et al., 1977; Martin, 1981; Gáspár et al., 1981/ and

for the prediction of the sequence of peptides that will function well with their protein counterparts. This assumption is valid probably not only for enzyme-substrate and enzyme-inhibitor interactions, but in other cases, as well, where a protein-peptide interaction may occur.

Table 7

The effect of D and L configuration on the $1/K_m$ values of plasmin

Subsites				$1/K_m$	Ratio
P_3	P_2	P_1		mM^{-1}	$1/K_m^D$ $1/K_m^L$
DIle	Phe	Lys	/1/	33.8	4.5
LIle			/8/	7.5	
DPro	Phe	Lys	/2/	28.0	5.1
LPro			/10/	5.5	
DAla	Phe	Lys	/5/	16.0	2.9
LAla			/12/	5.0	
DVal	Leu	Lys	/6/	9.7	3.9
LVal			/14/	2.5	
DIle	Leu	Lys	/9/	6.5	6.5
LIle			/15/	1.0	

Definite quantitative correlations could be demonstrated between the properties of amino acid side chains and their contribution to the kinetic parameters with all proteinases investigated in our laboratory /Gáspár et al., 1981/. Our prime hypothesis was convincingly supported by correlation coefficients calculated by comparing the predicted and measured kinetic values, by synthesizing and assaying the predicted

sequences as well as by preparing and testing inhibitors the structure of which was selected according to predicted values. These results may permit us the assumption that the analysis of quantitative structure-activity relationship can be successfully extended not only to the investigations of proteolytic enzymes but also to various other biological phenomena where a protein-peptide ligand may play a role.

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DISCUSSION

KOVACH:

How good is the correlation of the structure-reactivity relationship for k_{cat}/K_m, which is the true kinetic parameter and is reflective on transition state stabilization? Is the correlation better, as might be expected, for K_m, which may bespeak of intermediate stabilization?

ELŐDI:

The correlation coefficients of predicted and measured data obtained by the comparison of calculated and experimentally determined kinetic values were satisfactory. In 22 out of 28 investigated series the values were over 0.95 and were less than 0.9 only in two cases.

As a matter of fact, I do not know what kind of correlation could be expected for K_m. Anyway, in the majority of cases the best fit for expected and measured values was found for K_m.

STEPANOV:

I am very much impressed by your success in the prediction of kinetic constants. The question that immediately appears concerns the principles underlying such an approach. Is it based on the assumption that the interaction S_1-P_1, S_2-P_2 etc. are independent ones? If so, how do you evaluate the limits of this assumption?

ELŐDI:

Very probably, the interaction between the subsites of the binding sites and those of the substrate are not a sum of independent phenomena. They are rather influenced by some kind of cooperative phenomena hard to express by mathematical terms. Nevertheless, the additive character of regression analysis provided values which can be applied for practical purposes to design "optimal" structures. We believe that this kind of analysis may supply useful information in investigating substrate specificity of other proteolytic enzymes, too.

PROTEINASES AND REGULATION

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PRINCIPLES OF THE REGULATION OF INTRACELLULAR PROTEOLYSIS

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SUMMARY

A survey is given on the principles of the control of intracellular proteolysis in rat liver. Compartmentation, specificity of proteinases and inhibitors, and the activity of these enzymes are described as possible factors of control.

In particular, this survey deals with changes in the molecular properties of substrate proteins as an important principle of the regulation of intracellular proteolysis.

INTRODUCTION

It is important that a cell, when faced with a change in the environment, should be able to alter its enzyme composition in order to adapt oneself efficiently. Such adaptation occurs by the synthesis of enzymes appropriate to the new environment as well as by the removal of those proteins that are no longer needed. This degradation of proteins should be selective to be optimal. Thus, controls during adaptation are directed towards the activation or inhibition of the breakdown of individual proteins rather than towards the process of degradation as a whole (Bohley, 1968, 1974, 1979; Ballard, 1977).

The effect of this adaptation is that one group of proteins (enzymes) is replaced by another more appropriate group without any change in the total amount of proteins.

There is an enormous range of turnover rates of different proteins, i.e., half-lives from minutes to weeks in rat liver, which is the best investigated organ in this field. Goldberg and St. John (1976) showed that rat liver enzymes with very high turnover rates are sited at important metabolic control points, and they suggested that proteins with particularly long half-lives are relatively unimportant in metabolic control.

Enzymes which do not catalyze rate-limiting processes do not need to be changed in activity, and therefore have very slow turnover rates.

We propose to distinguish three main populations of proteins with respect to turnover rates in rat liver:

1./ very-fast-turnover proteins with half-lives of less than one hour, i.e., mainly proteinogen processings and abnormal proteins.

2./ fast-turnover proteins with half-lives of 1-24 hours, i.e., mainly enzymes which are important for metabolic control points, and

3./ slow-turnover proteins with half-lives of more than 24 hours without important changes in mass or activity.

Which molecular mechanisms are responsible for such extremely different turnover rates? How is the selectivity of intracellular proteolysis regulated? What are the primary reactions of intracellular protein catabolism? We will attempt to answer these questions, in the following paragraphs describing the principles of the regulation of intracellular proteolysis.

Compartmentation

It was shown by labeling experiments that probably all proteins of the liver turn over, but at variable rates. Many proteins of the outer mitochondrial membrane turn over more rapidly than the proteins of the matrix and particularly those of the inner membrane, a relatively stable organelle. A comparison of the turnover rates of short- and long-lived proteins in vivo and all proteins in vitro is shown in Table 1.

Table 1

Degradation of protiens in subcellular fractions of rat liver in vivo and in vitro

	In vivo		In vitro
	6-24 h	24-120 h	
Homogenate	2,3 %	0,9 %	1,6 %
Nuclei	8,7 %	0,6 %	1,3 %
Mitochondria	2,4 %	0,4 %	2,1 %
Lysosomes	17,5 %	0,4 %	5,9 %
Microsomes	3,8 %	1,0 %	1,2 %
Plasma membranes	2,6 %	1,8 %	1,9 %
Cytosol proteins	5,6 %	0,6 %	0,5 %

(in vivo: decay of ^{14}C between 6-24 h (Miehe, 1974), decay of ^{14}C between 24-120 h (Arias et al., 1969), in vitro: autoproteolysis at pH 6.8 in 0.2 % Triton X-100. All values in K=% of protein degraded per hour (Bohley, 1966, 1968), see also Waterlow et al., 1978; Schön and Bohley, 1977)

The half-life of short-lived proteins is shortest in lysosomes, nuclei and cytosol. The very short half-life or proteins found in lysosomes, between 6 and 24 hours, represents mainly the degradation of autophagocytosed, short-lived substrate proteins from other organelles, particularly from cytosol. The values of autoproteolysis in vitro show that the proteolytic capacity of cytosol is very low to explain the high turnover rate observed in this compartment in vivo. Therefore, we have to postulate an intracellular cooperation in vivo in which cytosol proteins are substrates of protein-degrading systems in other organelles, especially in lysosomes (Bohley et al., 1969, 1971, 1974, 1979).

On the other hand, all true lysosomal enzymes hitherto investigated have been found to be long-lived. It can therefore be supposed that lysosomes contain short-lived proteins (their substrates) and long-lived proteins (their enzymes). Resident membrane proteins seem to be sterically protected against proteolysis. On the other hand, the cytoplasmic sur-

face of lysosomes is susceptible to hydrolytic attack by Kupffer cell lysosomes. Henell et al. (1983) demonstrated that lysosomal membranes are degraded if their outer surface is exposed to lysosomal enzymes and that proteins are degraded faster than lipids.

In any case, the compartmentation results in a high concentration of substrates and enzymes but also of effectors as protons and thiols in the lysosomes which constitute more than 10 % of the total cell volume in Kupffer cells but less than 1 % of the total cell volume in parenchymal cells of rat liver. More than 60 % of total proteolysis occurs in lysosomes, and the other degradative compartments, especially for the very short-lived proteins, are mainly unknown as yet.

Specificity of proteinases

A few proteinases with extremely high substrate specificity are known to be able to select substrates for degradation without any additional control mechanisms (renin, angiotensinase, etc., see Holzer and Heinrich, 1980).

This, however, cannot be the only type of regulation of proteolysis. Namely, for each protein individually degraded, an individual proteinase is required. Furthermore, a regulatory mechanism is needed for all these proteinases.

In general, the specificity of proteolytic enzymes alone is insufficient to explain the selectivity of intracellular proteolysis and its control.

Nearly all intracellular proteinases preferentially split peptide bonds between hydrophobic amino acids (Ala-Leu, Leu-Tyr, Tyr-Leu, Leu-Val, Phe-Phe,...). On the other hand, a variety of proteolytic enzymes with very different specificities degrade preferentially short-lived proteins. This means that the short-lived proteins are more susceptible to attacks by any of these proteinases.

Specificity of proteinase inhibitors

Hirado et al. (1981) isolated a proteinase inhibitor from the cytosol of rat liver cells (molecular mass 12 400, IP 5.04) which is very effective against cathepsin B and cathepsin H, it is less effective against cathepsin L and is ineffective against cathepsin A and cathepsin D. Such an inhibitor seems to function mainly as a safeguard against proteolytic damage which might arise from leaky or broken vacuoles. There are, however, some hints that in yeast the inhibitors, besides the safeguard function, may also serve as a control element regulating proteinase activity (Holzer and Heinrich, 1980). Speiser and Etlinger (1983) found an inhibitor in rabbit reticulocytes that can be repressed by ATP in the presence of ubiquitin. During reticulocyte maturation, the inhibitor and protease remain, but the ubiquitin-containing fraction is less effective in repressing the inhibitor. Etlinger's results (Speiser and Etlinger, 1983) do argue against an obligatory role for ubiquitin-conjugates in proteolysis.

In rat liver there exists a relatively high amount of calpastatin, an inhibitor against the Ca^{2+}-dependent cysteine proteinases calpain I and calpain II (Murachi, 1983). In general, the role of a cytosol proteinase (Hershko and Ciechanover, 1982) is not important in the overall degradation of rat liver proteins, as will be shown in the next paragraph.

Activity of proteinases and inhibitors

The most active proteinase in rat liver parenchymal cells is cathepsin L, which degrades proteins as myosin, actin, calmodulin, hemoglobin, histones, collagen, insulin, glucagon, azocasein and especially cytosol proteins at least 10 times more rapidly than cathepsin B and cathepsin H do even at pH 6 (Bohley et al., 1979). The role of cathepsin T (Gohda and Pitot, 1980) in comparison with these enzymes, however, was unknown as yet. The molecular weight of cathepsin T is 35 000 and therefore, we compared the activities of lysosomal supernatants (60-fold enriched) after fractionation on Sephadex G-75 under different conditions to estimate the share of cathepsin T in

overall proteolytic activity. As is shown in Figure 1, the activity of proteinases of a molecular weight of 35 000 is less than 5% in comparison with the activity of proteinases of a molecular weight between 20 and 30 000 (mainly cathepsin L).

It is shown in Table 1 that even under conditions specially selected for cathepsin T according to Gohda and Pitot (pH 6.5; glycerol; dithiothreitol and azocasein) the activity of cathepsin T is very low (less than 8 %) in comparison with the activity of cathepsin L.

Summarizing, the share of different proteinases in the degradation of cytosol proteins at pH 6 and pH 5 is compiled in Table 2, which shows that cathepsin L has indeed the maximum share and that cathepsin T and all cytosolic proteinases together have only a very small share in the overall proteolysis. They may, however, have an important function in degrading special proteins (as cathepsin T for tyrosine aminotransferase).

Table 2

Share of different proteinases in vitro in the degradation of cytosol proteins

	pH 6	pH 5
Cathepsin B	< 20 %	< 10 %
Cathepsin D	< 10 %	< 20 %
Cathepsin H	< 10 %	< 5 %
Cathepsin L	> 40 %	> 50 %
Cathepsin T	< 10 %	< 5 %
Cytosol proteinases (Calpains ATP-ubiquitin-dependent proteinases)	< 10 %	< 5 %

It is necessary to obtain further information about other, as yet unknown, proteinases and their contribution in cytosolic proteolysis.

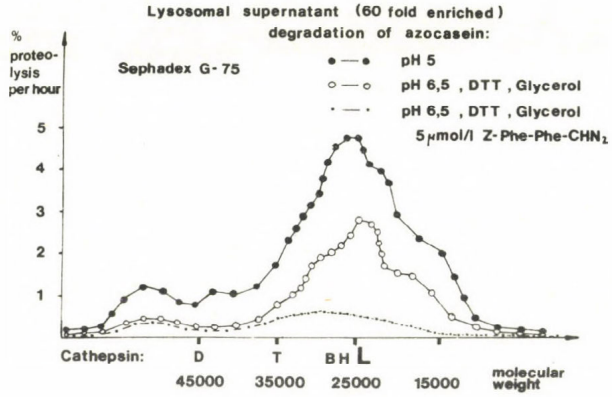

Fig. 1: Sephadex G-75 chromatography of 60-fold concentrated lysosomal supernatant at various pH values. Fractions were characterized according to their proteolytic activity with azocasein as substrate. The elution of catepsins is indicated by capital letters on the abscissa. T: tyrosine amino transferase.

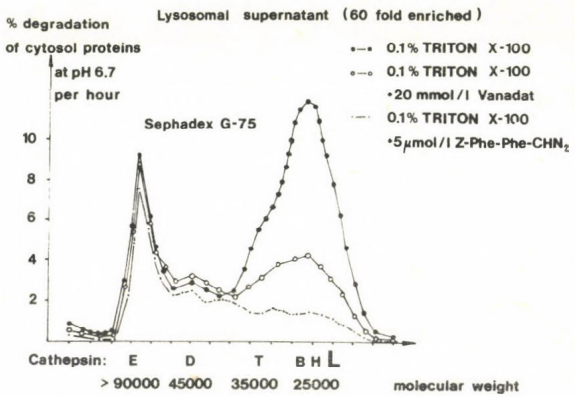

Fig. 2: Fractionation of 60-fold enriched lysosomal supernatant on Sephadex G-75 at pH 6.7 in the presence of 0.1 % Triton X-100. The degradation of the cathepsins and T (tyrosine amino transferase) is expressed as percentage.

It is unclear as yet, whether the very small overall proteolytic activity in cytosol is due to the high inhibitor activities or to low proteinase activities. In any case, many cytosolic proteins need another degradative compartment in vivo. This is evident from a comparison of the turnover rates of cytosol proteins in vivo (0.6-5.6 % per hour) and in vitro (<0.5 % per hour), as was shown in Table 1.

Saus et al. (1982) demonstrated the limited scope of the ATP-ubiquitin-proteinase system and assumed therefore a highly specialized function. Mortimore and Ward (1981) found that even at pH 7 less than 5 % of total proteolysis of rat liver occurs in the cytosol and more than 95 % in the particulate fractions, especially in lysosomes.

On the other hand, the proteinase activity in rat liver lysosomes (even the cathepsin-L-activity alone!) at pH 6 is much higher than it is needed for the in vivo turnover rates of cytosol proteins.

We conclude that except for some special proteins, the main part of cytosolic proteins require another environment for their overall degradation.

Neither the specificity of lysosomal enzymes is high enough nor their activity is limited enough to explain the selectivity of intracellular proteolysis and its regulation.

Changes in molecular properties of substrate proteins

Presumably, the primary reactions of intracellular proteolysis are the most important ones for its regulation.

Although in some special cases a first enzymatic attack by proteinases or thiol-proteindisulphide-oxidoreductases may occur (followed by conformational changes of the substrate proteins), we think that the main part of cytosol proteins first undergoes some conformational changes, afterwards is transferred to the degradative environment and finally is degraded by the enzymes.

It was first presumed that the size and charge of substrate proteins are important for their degradation rates. However, in 1981 it became evident from data published by Susan Russell

et al. and Ann St. John et al. that the size and charge of substrate proteins are not correlated well enough with turnover rates to explain the selectivity of proteolysis by these properties.

More important are some other properties of the substrate proteins, such as wrongly placed amino acids (Goldberg, 1976; Ballard, 1977), glycosylation (Goldstone and Koenig, 1974; Kalish et al., 1979), phosphorylation (Ekman et al., 1978) or, finally, the quantity of hydrophobic areas on the surface of the substrate proteins (Bohley et al., 1968, 1977, 1979, 1981).

Since the most degradative enzymes of the cells are separated from the substrate proteins by membranes, it seemed reasonable to think that the rate of proteolysis is controlled by the rate of transfer of substrate proteins across the membranes. Clearly, proteins with hydrophobic surface areas may be expected to associate with membranes, and thus have the best chance of being transferred across them.

Experimental evidence supports this idea. It has been found that short-lived proteins have a special affinity for liver fat, organic phases, hydrophobic surfaces such as siliconized glass beads, phenyl sepharose and octyl sepharose (Bohley et al., 1977; Bohley and Riemann, 1977) and for lysosomal and other membranes (Dean, 1975).

If, indeed, hydrophobicity of a protein tends to cause it to be turned over rapidly, this property should be correlated with other physical properties that are also conducive to a rapid turnover. A positive correlation has indeed been found between hydrophobicity and glycosylation (Hieke, 1983), but not between hydrophobicity and low isoelectric point for the substrate proteins from rat liver cytosol. Further investigations on this topic are in progress.

It can be seen that control of protein turnover mediated by surface hydrophobicity could account for many of the observed characteristics of the process. As a rule, in native proteins the majority of hydrophobic amino acid sidechains are buried in a hydrophilic environment.

Reversible changes which increase the rate of turnover of a protein, and can be expected to increase the hydrophobicity

of its surface, include the following:

a.) dissociation of the protein from an organelle,

b.) dissociation of stabilizing ligands - substrates, cofactors, etc.,

c.) dissociation of monomers from an oligomeric protein.

Further irreversible processes likely to have the same effect include:

a.) the incorporation of amino acid analogs (e.g., canavanine for arginine),

b.) sequence errors in biosynthesis,

c.) denaturation at interfaces (membranes, other proteins, aggregates) and

d.) limited proteolysis.

In extreme cases, the modified proteins may aggregate before being degraded, as has been shown for both bacterial and rat liver cytosol proteins (Bohley et al., 1974; Goldberg, 1974).

In summary of this view, we see that the increase in surface hydrophobic areas could be a common factor in the accelerated degradation of many kinds of proteins, not only because it facilitates entry into the degradative compartment, but also because the cellular proteinases tend to act most rapidly on peptide bonds between hydrophobic residues.

We have concentrated our work on surface hydrophobicity, mainly because this property of substrate proteins can be changed and therefore allows regulation of protein degradation.

For instance, in the absence of stabilizing ligands (such as substrates or, for example, cofactors), superfluous substrate proteins (for example, enzymes without substrates) might be degraded preferentially. There are some hundred different proteins in rat liver cytosol, and in the strictest sense we would need a hundred-pathway-model to describe their degradation. We can hope, however, that many of these pathways are similar.

In order to understand the molecular properties which may be important for the control of proteolysis in many of these pathways, we have investigated surface hydrophobicity of cytosol proteins from rat after a 4-day period of a protein-free

diet or after a 20-day period of a protein-rich diet.

Such nutritional changes involve firstly changes in the microenvironment of substrate proteins (changes in the concentration of substrates and other stabilizing ligands) and secondly, may produce changes in the composition of cytosol proteins. If surface hydrophobicity is really associated with an increased rate of degradation, the preferential degradation of short-lived proteins in the livers of protein-deprived rats with deficient resynthesis should be followed by a decrease of hydrophobic proteins in rat liver cytosol.

Indeed, the affinity of cytosol proteins from protein-deprived rats to hydrophobic surfaces is significantly decreased in comparison with the two other groups (Bohley et al., 1981).

To test the influence of small molecules as possible stabilizing ligands (substrates, etc.) we investigated the effect of ultrafiltration and washing on the affinity to hydrophobic surfaces. After ultrafiltration and washing (which results in the removal of stabilizing ligands) we found that cytosol proteins bound more strongly to both hydrophobic surfaces. Furthermore, addition of the ultrafiltrates diminished the binding of cytosol proteins. We have not so far identified the molecules responsible for this protecting effect (protecting here means: a decrease in the binding to hydrophobic surfaces).

In rats kept on a protein-rich diet these low molecular weight protecting substances most probably are the substrates, cofactors and free amino acids.

Furthermore, cytosol proteins of young rats bind better to phenylsepharose than those of old rats, as was shown by B. Wiederanders (1978).

The degradative compartments can be: intracellular membranes especially for short-lived proteins, and lysosomes mainly for long-lived proteins (leupeptin-treatment in vivo results in a prolonged apparent half-life of the slowly turning over proteins in rat liver, as we found by a triple labeling technique using ^{14}C- and ^{3}H-arginine and ^{15}N-lysine).

Surprisingly, we have not any detailed knowledge of the intracellular place for the degradation of short-lived proteins.

Most probably, microsomal membranes may be responsible for this process (Ballard, 1977).

Quite clearly, the investigation of the crude mixture of perhaps a hundred cytosol proteins can only give a faint insight into the mechanisms which must next be studied with isolated, well defined substrate proteins.

On the other hand, the use of fresh cytosol is the best way to preserve most of the substrate proteins in their natural environment in the presence of other proteins and stabilizing ligands in as native a state as possible.

Transfer to other organelles (mainly microsomal membranes and lysosomes) is not the only process to be facilitated by increased surface hydrophobicity. There is, furthermore, an increased susceptibility to proteinases which attack mainly in the neighbourhood of hydrophobic amino acids as for instance cathepsin L.

We still do not know whether such enzymes are also located on the cytosolic face of the lysosomal membrane and in such a manner can initiate proteolysis by splitting only one or a few peptide bonds. This might be followed by an increase in surface hydrophobicity of the nicked substrate protein molecule, which afterwards facilitates its transfer to an organelle more active in degradation.

Summarizing, we wanted to show you that changes in the metabolic state really are followed by changes in surface hydrophobicity of substrate proteins and therefore can be important for the control of intracellular proteolysis.

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DISCUSSION

GLAUMANN:

The cell adapts itself to environmental changes; many regulatory enzymes increase. This is not the case for lysosomal enzymes, is it? If not, why?

BOHLEY:

The activity of lysosomal proteinases is much higher than it is necessary for the turnover rates of proteins found in vivo.

VITALE:

It is known that diabetes is accompanied by some protein glycosylation which is considered to be a cause for some late effects of the disease. How does this fit to the existing hypothesis; are there any enzymatic studies on their degradation?

BOHLEY:

We did not study this question. There is, however, a paper from Fred Dice showing that in diabetic state the behavior of cytosol proteins is completely changed. Glycosylation causes a more rapid turnover and might be in part responsible for such changes.

ELŐDI:

As far as your classification i.e., short-, medium- and long-living group is concerned, what would you suggest for the definition of intracellular stability of proteins?

BOHLEY:

This is only an operational classification. To find a definition - that is what we have to find out in the future.

ELŐDI:

When enzymes are considered, their activity may be regarded as a sign of stability and/or destruction. What method would you suggest for characterizing the intracellular protein stability?

BOHLEY:

Slow-turnover proteins are more stable against proteolytic enzymes hitherto investigated.
Hydrophobicity on the surface of substrate proteins always tends to cause a more rapid turnover in vivo and this means a limited stability.
Therefore, hydrophobic chromatography is a good tool to isolate the less stable proteins from cytosol.

SOHÁR:

How can you explain the higher cathepsin B activity in the homogenate during autolysis?

BOHLEY:

We did not investigate this problem.

TURK:

The increased proteolytic activity of cathepsin B results in the cleavage of cathepsin B and cytosolic protein inhibitor complex.

BOHLEY:

Whether an inhibitor-proteinase complex is split also in vivo remains to be clarified.

GLAUMANN:

Since we know there are several endogenous proteolytic inhibitors present in the cytosol, can we really estimate the role of cytosolic enzymes in overall proteolysis, by simply incubating the cytosol without first removing the inhibitors?

BOHLEY:

Yes, we can. Since the in vivo-conditions in cytosol include proteinase-inhibitor-complexes (safeguard function of inhibitors) it would be wrong, in my opinion, to separate the inhibitors for the estimation of overall proteolysis,and for the comparison with proteolysis in vivo.

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INHIBITION OF PROTEINASES IN ISOLATED CELLS

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INTRODUCTION

Aspartic proteinases presumably act synergistically with cysteine proteinases and exopeptidases in intracellular protein degradation. This essential living process takes place in rat liver mainly in the lysosomes /Bohley, 1968; Dean and Barrett, 1976; Goldberg and St. John, 1976; Holtzmann, 1976; Holzer and Heinrich, 1980/. In the last years especially the lysosomal cysteine proteinase, cathepsin L /EC 3.4.22.15/ and the lysosomal aspartic proteinase, cathepsin D /EC 3.4.23.5/ have been shown to play an important role in intracellular proteolysis in the rat liver /Huisman et al., 1974; Dean, 1975; Wiederanders et al., 1976; Kirschke et al., 1977/.

About 60-70% of the total cell number of the rat liver consists of parenchymal cells /hepatocytes/ and about 30-40% is occupied by nonparenchymal cells, i.e. Kupffer cells, endothelial cells and fat-storing cells /Wisse, 1977; van Berkel, 1979; Knook et al., 1982/. In the last decade numerous authors have determined the activity of cathepsin D with hemoglobin substrate at pH 3-4, and they measured a

Abbreviations: Z-Phe-Phe-CHN_2: benzyloxycarbonyl-phenylalanyl-phenylalanyl-diazomethane; SPA: specific proteolytic activity.

significantly higher activity in nonparenchymal cells than in the parenchymal cells /Arborgh et al., 1974; van Berkel et al., 1975; Munthe-Kaas et al., 1976; Knook, 1977/.

However, nothing is known of the occurrence and distribution of cathepsin L activity in the individual cell types. Thus, we have developed two methods that can be applied to different substrates at different pH values and allow a specific determination of cysteine proteinase activity /mainly cathepsin L/ and of aspartic proteinase activity /mainly cathepsin D/. Since specific substrates for both proteinases are still lacking, we have used specific inhibitors: the synthetic inhibitor Z-Phe-Phe-CHN_2 /Leary and Shaw, 1977; Green and Shaw, 1981/ which inhibits specifically isolated rat liver cathepsin L /Kirschke and Shaw, 1981/, and the microbial inhibitor pepstatin which inhibits cathepsin D /Knight and Barrett, 1976/. Details concerning some essential presuppositions for the development of the tests were published /Riemann et al., 1982/.

Applying a method using urea-denatured azocasein as substrate we investigated several organs of carp, frog, pigeon and rat. The kidneys of carp, frog and rat were found to have a particularly high Z-Phe-CHN_2-inhibited activity, therefore they are very promising sources for the isolation of cathepsin L /Riemann et al., 1982/.

Some authors reported on the involvement of lysosomal proteinases in tumor-genesis /Grabske et al., 1979; Oshima et al., 1979; Geyer et al., 1980; Mort et al., 1980; Recklies et al., 1982/. Thus, we have investigated human tumors of different tissues, Ehrlich-ascites-tumor cells from mice and L-cells with regard to the occurrence of pepstatin- and Z-Phe-Phe-CHN_2-inhibited activities.

MATERIALS AND METHODS

1. Isolation of various rat liver cells

Parenchymal cells, Kupffer cells and endothelial cells were isolated according to van Bezooijen et al. /1974/ and

Knook and Sleyster /1976/. Besides parenchymal cells were isolated according to the method of Seglen /1976/. The preparation of the whole liver homogenate was described by Riemann et al. /1982/.

2. Preparation of tumors, Ehrlich-ascites tumor cells and L-cells

The preparation of human tumors was performed as described by Riemann et al. /1982/. Isolation of Ehrlich-ascites tumor cells was carried out 8 days after inoculation into mice /Agnes-Bluhm/. Under ether narcosis the abdomen was opened, the tumor cells were removed and centrifuged at 5 000 g. The sedimented cells were homogenized with an equal volume of Triton X-100, 0.1% /v/v/. L-cells, transformed fibroblasts were cultivated for 5 days at 37°C in Eagle's MEM containing 10% calf serum. After harvesting, the cells were washed twice in 150 mmol/l NaCl solution, centrifuged and homogenized with the equal volume of Triton X-100, 0.1% /v/v/.

3. Preparation of substrates

Azocasein was prepared according to Langner et al. /1973/. ^{3}H- and ^{14}C-labeled cytosol proteins were obtained by the method of Bohley et al. /1971/.

4. Assay of proteolytic activities

The two methods used were published in detail /Riemann et al., 1982/. The final concentration of Z-Phe-Phe-CHN_2 in the assay mixtures was 5 µmol/l and that of pepstatin was 0.5 µmol/l.

5. Calculation of the results

The results are expressed as specific proteolytic activity /SPA/. For method I, which used urea-denatured azocasein at

pH 5.0 as substrate, SPA is defined as follows:

$$SPA = \frac{\%\ \text{degradation of azocasein}}{\text{mg protein} \times 1\ \text{h}}$$

/The degradation of azocasein in the test tubes never exceeded 10% per hour of incubation./

For method II, which used double-labelled cytosol proteins at pH 3.0 as substrate, SPA is defined as follows:

$$SPA = \frac{\%\ \text{degradation of cytosol proteins}}{\text{mg protein} \times 1\ \text{h}}$$

/The degradation of double-labelled cytosol proteins in the assay tubes was always below 1% during incubation for 1 h./

6. Protein determination

The amount of cellular protein was determined by the modified LOWRY procedure, according to Langner et al. /1971/, using human serum albumin as standard.

RESULTS AND DISCUSSION

Figures 1 and 2 show the results obtained with the isolated rat liver cells. Both methods yielded striking results. In Kupffer cells the specific proteolytic activity /control value/ was about 10 to 30 times higher than in parenchymal cells. Compared to the endothelial cells the proteolytic activity was 2 to 3 times higher in Kupffer cells. This may be due to the fact that Kupffer cells contain more lysosomes than the other liver cells. As reported by Blouin et al. /1977/, lysosomal structures contribute to the cell volume of parenchymal cells with 0.8%, to that of endothelial cells with 7% and to that of Kupffer cells even with 14%.

These results were confirmed by other authors, i.e. Kupffer cells have higher cathepsin D activity than endothelial and parenchymal cells. In the presence of 0.5 µmol/l pepstatin we have found an inhibition of 80 to 100% with

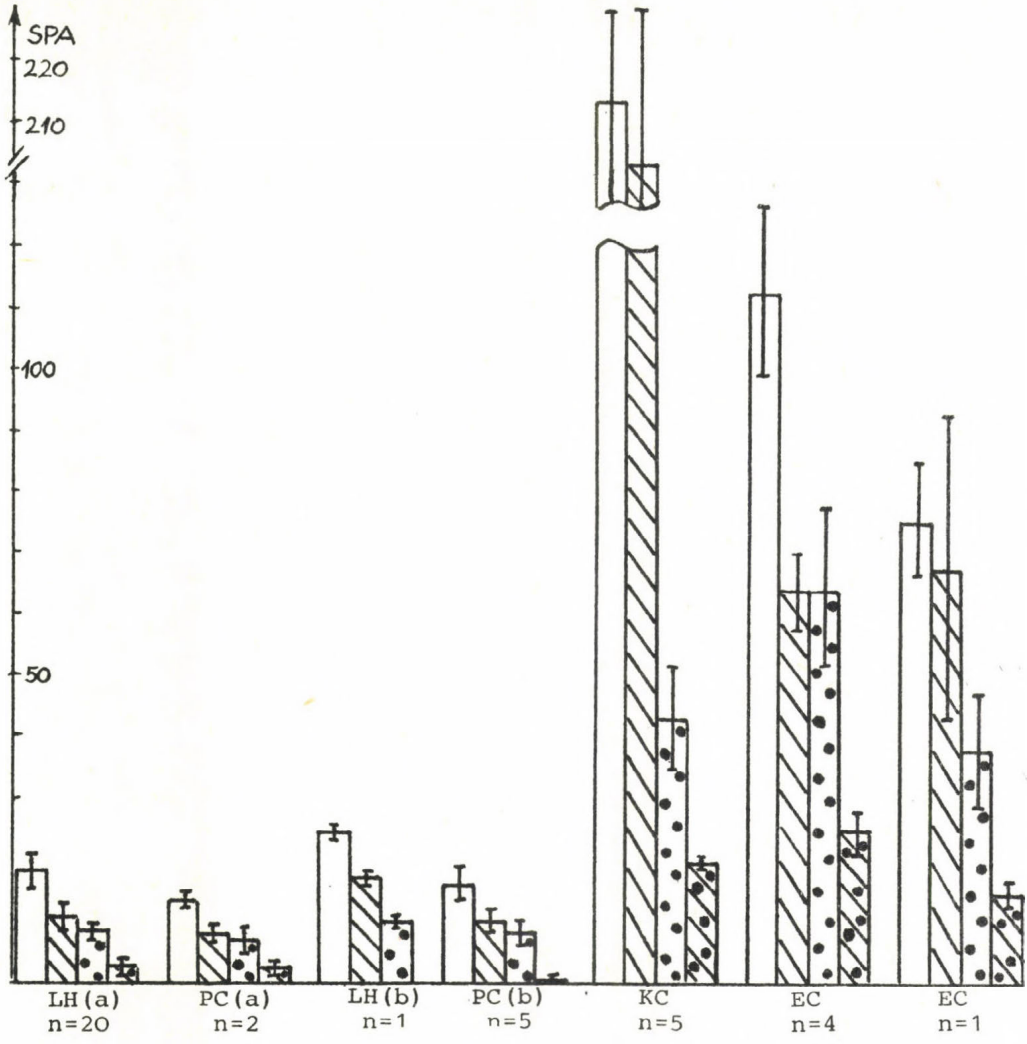

Fig. 1. Specific proteolytic activity of isolated rat liver cells as determined by method I /see methods/.

n = number of cell preparations. Each value is the mean ±SD of at least 8 determinations.
LH = whole liver homogenate /a/ prepared in Halle
/b/ prepared in Rijswijk
PC = parenchymal liver cells /a/ prepared in Halle
/b/ prepared in Rijswijk
KC = Kupffer cells
EC = endothelial liver cells
SPA of the control value /without inhibitor/
SPA after inhibition with 5 µmol/l Z-Phe-Phe-CHN_2
SPA after inhibition with 0.5 µmol/l pepstatin
SPA after inhibition with the mixture of both inhibitors at the given concentrations.

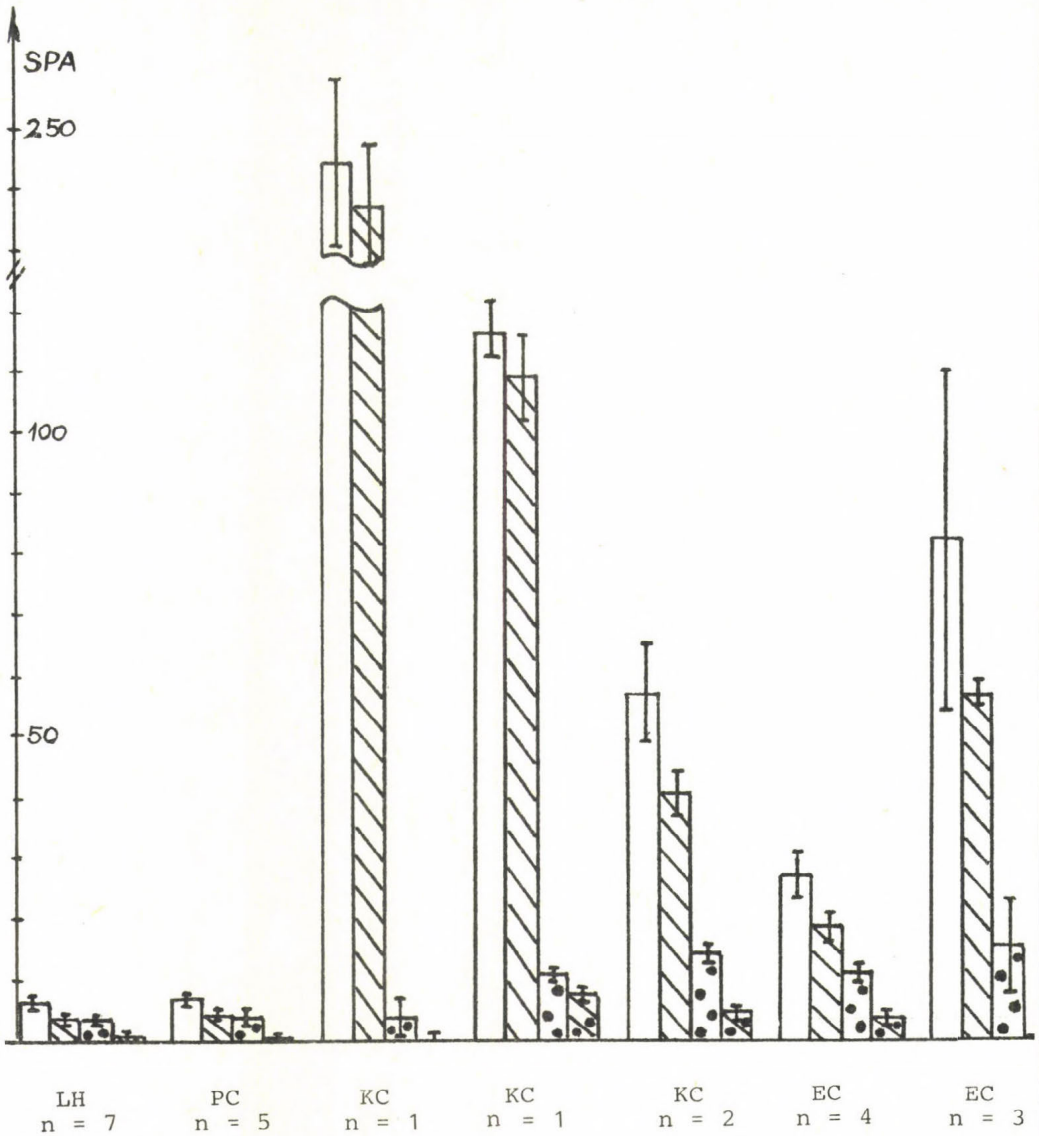

Fig. 2. Specific proteolytic activity of isolated rat liver cells as determined by method II /see Methods/

Each value is the mean ±SD of at least 12 determinations. For symbols see Fig. 1.

Kupffer cells, 60 to 70% with endothelial cells and 50 to 55% with parenchymal cells, respectively.

Ansorge et al. /1979/ have obtained similar results by immunological determination. They found that the amount of cathepsin D in the endothelial cells is three times higher and in the Kupffer cells even 15 times higher than in parenchymal cells. The share of the aspartic proteinase cathepsin E, which is assumed to play a minor role compared to cathepsin D in the pepstatin-inhibited activity, still has to be explained.

We measured 2 to 10% inhibition in Kupffer cells with 5 µmol/l Z-Phe-Phe-CHN_2, 10 to 30% inhibition in endothelial cells, and about 40% inhibition in parenchymal cells. This result is a first hint that cathepsin L is more concentrated in the parenchymal cells.

Recently, it has been shown that Z-Phe-Phe-CHN_2 at concentrations of 0.1 mmol/l and 1 mmol/l inhibits the lysosomal protein degradation in isolated viable rat hepatocytes to 60 and 70%, respectively /Grinde, 1983/. This finding confirms our result and emphasizes the importance of cysteine proteinases probably cathepsin L, in hepatocytic protein degradation.

However, the previously discovered lysosomal cysteine proteinase, cathepsin T, /Gohda and Pitot, 1981/ cannot be excluded as being inhibited also by Z-Phe-Phe-CHN_2. The same may be true for the calcium-dependent cysteine proteinases isolated from the cytosol of rat liver cells /Kishimoto et al., 1981/. A possibly disturbing influence of the cysteine proteinases cathepsin B and cathepsin H is almost completely excluded, as was described recently /Riemann et al., 1982/. In the presence of a mixture combining the two inhibitors at the given concentration a relatively high activity which was not inhibited remained only in the endothelial cells. It seems to be very likely that in these cells not only the cysteine and aspartic proteinases participate in the total cellular protein breakdown under these conditions.

Moreover, endogenous cysteine proteinase inhibitors /Kominami et al., 1981; Hirado et al., 1981; Anastasi et al.,

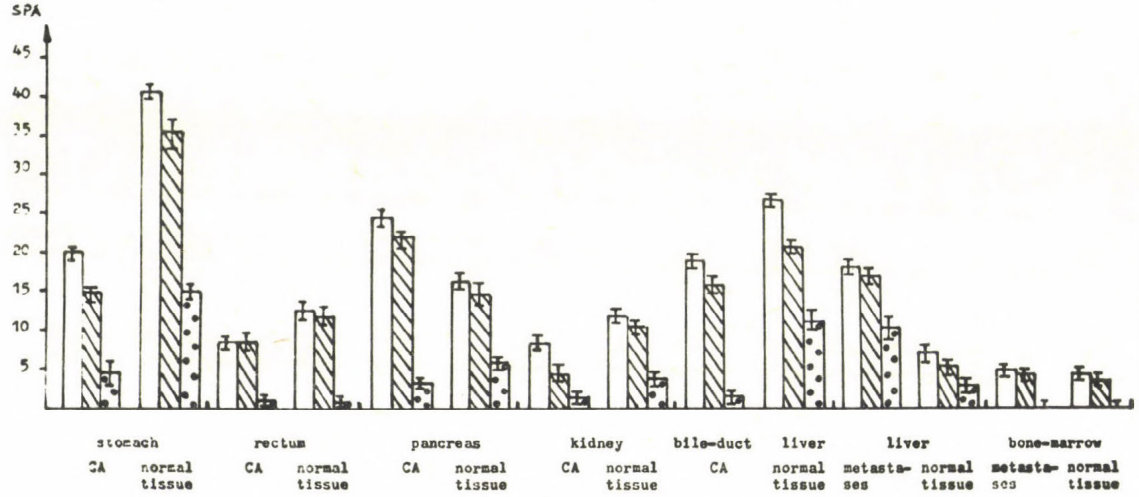

Fig. 3. Specific proteolytic activity of different human carcinomas and normal tissues as determined by method II

n = 1 throughout. CA = carcinoma.

Each value is the mean $\pm$ SD of at least 6 determinations.
For symbols see Fig. 1.

1983/ and aspartic proteinase inhibitor/s/ /Turk et al., 1981/ should be considered to possibly influence the described results.

Several authors /van Berkel, 1979; Brouwer et al., 1981/ suppose a close relation between the high amount of cathepsin D in Kupffer cells and their high activity in heterophagic processes. Whether or not there is any connection between the relatively high cathepsin L content and autophagic processes in parenchymal cells should be investigated.

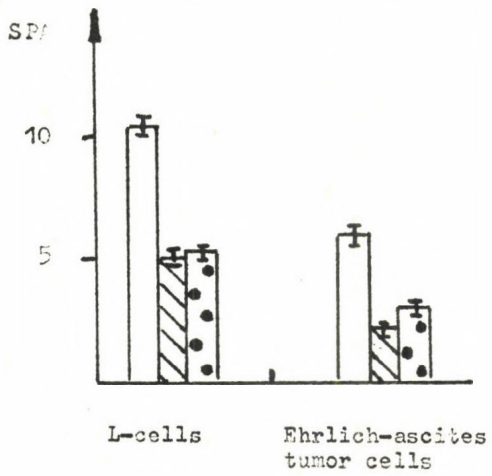

Fig. 4. Specific proteolytic activity of L-cells and of Ehrlich-ascites tumor cells method II
Each value is the mean ± SD of 12 determinations.
For symbols see Fig. 1.

The results concerning the tumors are summarized in Figure 3. In all tumor homogenates Z-Phe-Phe-CHN_2- as well as pepstatin-inhibited activity could be identified. Contrary to some reports /Grabske et al., 1979; Geyer et al., 1980/ we found a higher specific proteolytic activity in comparison to the normal tissues only in two cases /pancreas carcinoma and liver metastases. In normal tissues, except L-cells and Ehrlich-ascites tumor cells /see Fig. 4/, as well as in tumorous

tissues we always measured markedly higher inhibition by pepstatin than by Z-Phe-Phe-CHN_2. Compared to the normal tissues only carcinomas of the bile-duct, the kidney and the pancreas showed stronger inhibition by pepstatin. Whether this more pronounced inhibition was due to contaminating macrophages could not yet be clarified.

The results are encouraging and ask for further experiments whereby isolated tumor cells should be incubated with pepstatin-loaded liposomes to provide more information about the role of cathepsin D in tumor-genesis and its share in tumor proteolysis.

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ENDOPEPTIDASE-24.11, A MEMBRANE ENZYME WITH DIVERSE ROLES IN MANY TISSUES

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BACKGROUND

The purpose of this report is to review some of our recent work on this endopeptidase. The first evidence for the presence of a membrane bound enzyme in kidney that was capable of hydrolysing [^{125}I] insulin B chain was obtained about 15 years ago (Wong-Leung and Kenny, 1968). Subsequent work showed it to be localized in the brush border membrane (George and Kenny, 1973). It was characterized in detail after purification from rabbit kidney (Kerr and Kenny, 1974a,b) and was shown to be a Zn-metallo endopeptidase that cleaved insulin B chain at bonds -X-Y, provided Y was a hydrophobic amino acid residue. In both these respects it resembled the microbial metallo-endopeptidases typified by thermolysin. Phosphoramidon, a potent inhibitor of thermolysin, also inhibited the kidney enzyme (K_i=2 nM, Kenny, 1977). The EC name, kidney brush border neutral proteinase, 3.4.24.11, was appropriate at that date, but subsequent work has shown that the enzyme has a much wider distribution, being present not only in the brush border of the intestine (Danielsen et al., 1980) but also in membranes of other tissues (Kenny and Fulcher, 1983). We have therefore proposed the name "endopeptidase-24.11" in order to embrace the wider role of this enzyme (Matsas et al., 1983a).

In rabbit and pig, it is the only endopeptidase in the renal microvillar membrane. The other peptidases in this membrane are exopeptidases that exhibit a wide range of specific-

ities (Table 1). It seems likely that an initial attack by the endopeptidase may be the essential step in the hydrolysis of some peptides, e.g. those with blocked termini, before the exopeptidases can be effective.

Table 1

Peptidases so far identified in pig kidney microvilli

Enzyme	Active site	Specificity	Specific inhibitors
Endopeptidase-24.11 (EC 3.4.24.11)	Zn^{2+}	-0-0$\overset{\downarrow}{-}$●-0- (hydrophobic)	Phosphoramidon Thiorphan
Aminopeptidase N	Zn^{2+}	●$\overset{\downarrow}{-}$0-0-0- (many)	
Aminopeptidase A	Ca^{2+}	●$\overset{\downarrow}{-}$0-0-0- (Glu/Asp)	
Aminopeptidase P	?	0$\overset{\downarrow}{-}$●-0-0- (Pro)	
Proline carboxypeptidase	?	-0-0-●$\overset{\downarrow}{-}$0 (Pro)	
Dipeptidylpeptidase IV	Serine	$\overset{\downarrow}{}$ 0-●-0-0- (Pro)	Dip-F
Peptidyldipeptidase	Zn^{2+}	-0-0$\overset{\downarrow}{-}$●-● (nonspecific)	Captopril
γ-Glutamyl transferase	?	●$\overset{\downarrow}{-}$0-0-0- (γ-Glu)	

Topology of endopeptidase-24.11 in pig kidney microvilli

The rabbit enzyme had been successfully released from the membrane by toluene-trypsin treatment (Kerr and Kenny, 1974a) and the purified product was a monomer of M_r 93 000 with

hydrophylic properties. In contrast to most other microvillar hydrolases, the endopeptidase cannot be released by treatment of the membrane with papain or trypsin (Kenny, 1977) a feature possibly suggesting a different type of association with the membrane from the well-studied "stalk proteins" (for review see Kenny and Maroux, 1982). We therefore sought to study the detergent-solubilised form of endopeptidase-24.11 purified from pig kidneys (Fulcher and Kenny, 1983). The purification was achieved by an immuno-adsorbent step employing a polyclonal antibody raised in rabbits. This form was dimeric and, being amphipathic, bound a micelle of Triton X-100. It was successfully reconstituted into liposomes and electron micrographs established that it, too, is a stalked protein. Morphometry of the micrographs revealed that it possessed an unusually short stalk (2 nm) (Kenny et al., 1983). Aminopeptidase N (EC 3.4.11.2), an enzyme that is readily released from the membrane by papain, has a 5 nm stalk (Hussain et al., 1981). We have therefore suggested that the inability of papain to release endopeptidase-24.11 relates to the limited space between the mass of the protein and the membrane surface, which is clearly insufficient to accommodate proteinases of the size of papain with a minimum diameter of 3.7 nm.

A monoclonal antibody to endopeptidase-24.11

Although the purified enzyme was not highly antigenic when injected into rabbits, a monoclonal antibody to it was obtained at an early stage in a programme in which Balb/c mice were immunized with pig kidney microvilli. This antibody, GK7C2 (Gee et al., 1983), has been valuable for purifying the enzyme in batches of 1 mg or more, by immunoadsorbent chromatography, the elution step being achieved by bicarbonate buffer at pH 10.6. Immunofluorescence with GK7C2 has also shown that the enzyme is located predominately in the brush borders of the proximal tubule of the kidney and of mucosal cells of the intestine including those lining the crypts. However, weak fluorescence was also demonstrated over the glomeruli and in basolateral membranes of the enterocytes, an observation that

indicates that the antigen is probably not unique to microvilli. Other regions of the nephron - distal tubules and collecting ducts - appeared to lack the antigen.

Endopeptidase-24.11 in pig tissues

The availability of a specific inhibitor, phosphoramidon, provided the opportunity to search for this type of endopeptidase in other tissues.

Table 2

Endopeptidase activity in pig tissues

The fractions were assayed with 6 μM [^{125}I] insulin B chain as substrate in the presence and absence of 1 μM-phosphoramidon.

Tissue	Membrane fraction	Activity (pmol·min^{-1}mg^{-1} of protein)	Inhibition by phosphoramidon (%)
Spleen	microsomal	76	38
Aortic endothelium	microsomal	54	50
Lung	microsomal	16	75
Myocardium	microsomal	31	34
Skeletal muscle	microsomal	20	7
Liver	microsomal	40	0
Brain caudate nucleus	synaptic	30	50
Brain cerebral cortex	synaptic	25	5
Intestine	microvilli	1700	100
Kidney	microvilli	8000	100

Table 2 shows the activities, using [^{125}I] insulin B chain as substrate, in membrane fractions prepared from several sources. For spleen, aortic endothelium, lung, myocardium and skeletal muscle, a crude microsomal pellet was prepared. For brain, the results are for synaptic membranes and for kidney and intestine, microvilli were prepared. It is clear that the

activities of microvilli are about two orders of magnitude higher than those for any other membrane and that the microvillar activity is wholly inhibited by phosphoramidon. In other membranes, the proportion of the activity sensitive to phosphoramidon varied from 7-75 %, the highest being in lung. In brain, the activity in synaptic membranes was non-uniform. In the caudate nucleus, 50 % of the activity was phosphoramidon-sensitive, but in cerebral cortex only 5 % could be attributed to endopeptidase-24.11. When the phosphoramidon-sensitive activity in lung, heart and spleen was titrated with the polyclonal antibody raised to the kidney enzyme, inhibition was observed. This supports the view that endopeptidase-24.11 rather than an unrelated protein was present in these tissues (Kenny and Fulcher, 1983).

Endopeptidase-24.11 and the hydrolysis of neuropeptides

Much attention has been given in the literature to the inactivation of [Leu] and [Met]enkephalins:

Tyr-Gly-Gly-Phe-Leu

Tyr-Gly-Gly-Phe-Met

The main points of hydrolysis by membrane preparations of brain are the Tyr^1-Gly^2 and the Gly^3-Phe^4 bonds. The first is attributable to a membrane aminopeptidase sensitive to inhibition by puromycin (Vogel and Altstein, 1979; Barclay and Phillips, 1980; Hersh, 1981). The second has attracted greater interest and has been variously attributed to peptidyl-dipeptidase A (EC 3.4.15.1) (Swerts et al., 1979a; Benuck and Marks, 1979).However, the lack of inhibition by captopril (Swerts et al., 1979b) made this view untenable. It was then suggested that "enkephalinase" was another peptidyldipeptidase for which a specific inhibitor, thiorphan N-(DL-2-benzyl-3-mercaptopropionyl)glycine, had been designed (Roques et al., 1980). This reagent not only inhibited enkephalin hydrolysis in vitro, but was able to protect [Met]enkephalin released from brain slices (Patey et al., 1981). However, the hydrolysis of the -Gly-Phe- bond is also consistent with an endopeptidase attack as suggested by Blumberg et al. (1981) and is the predictable site of hydrolysis by endopeptidase-24.11.

When we compared thiorphan with phosphoramidon as an inhibitor of the hydrolysis of insulin B chain or [Leu]enkephalin by the purified kidney enzyme, the two inhibitors were indistinguishable, giving IC_{50} values in the range 10-13 nM. Partial inhibition by both was also observed for enkephalin hydrolysis by brain synaptic membranes (Fulcher et al., 1982). A comparison of the structures of the two inhibitors (Fig. 1) shows that both are substituted dipeptides with an anionic group ($-PO^-$ of $-S^-$) adjacent to the P_1' residue which has a hydrophobic side chain.

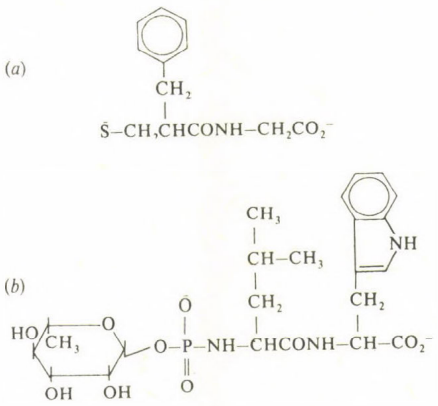

Fig. 1:
Chemical structures of (a) thiorphan and (b) phosphoramidon

We have also compared the hydrolysis of another neuropeptide, substance P, by the purified kidney enzyme and synaptic membranes prepared from caudate nuclei (Matsas et al., 1983a). This peptide has the sequence:

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met NH_2

and thus contains several possible sites for endopeptidase-24.11 attack. The peptides produced by the kidney enzyme and synaptic membranes were found to be identical when resolved by hplc and were all derived from hydrolysis at three bonds:

$-Gln^6-Phe^7-$, $-Phe^7-Phe^8-$ and $-Gly^9-Leu^{10}-$

the last being slightly more favoured than the other two. The

hydrolysis of the substrate was fully inhibited by phosphoramidon in both preparations. Moreover, the hydrolysis of Tyr-DAla-Gly-Phe-Leu (which is attacked by synaptic membranes only at the -Gly-Phe- bond) could be titrated by phosphoramidon and gave an IC_{50} value of 8 nM. The polyclonal antibody also gave identical inhibition curves for the hydrolysis of substance P and the enkephalin analogue by detergent solubilized membranes from kidney and caudate nuclei.

On the basis of these experiments we have argued that endopeptidase-24.11 probably has a general role in the hydrolysis of neuropeptides at the surface of synaptic membranes and that there is no need to postulate the existence of a series of peptide-specific peptidases each recognizing only one type of neuropeptide.

Purification of brain endopeptidase-24.11 by monoclonal antibody GK7C2

The close similarity of the kidney and the brain caudate endopeptidase activities, as revealed by inhibitors, bond specificity and titration by a polyclonal antiserum, has now been confirmed by the isolation of the enzyme from caudate nuclei (Relton et al., 1983). The very low abundance of the enzyme in caudate membranes required a rapid and specific method for its isolation. An immunoadsorbent column containing monoclonal antibody GK7C2 achieved essentially a single step purification. The overall purification factor was 24 000. The product (118 µg from 130 g of striatum obtained from 25 brains) was homogeneous on SDS-PAGE and had an apparent subunit M_r of 87 000. This is lower than that for pig kidney (89 000) and for pig intestine (94 000) (Fulcher et al., 1983) and it appears to be explained by the lower content of carbohydrate. Brain contains 12.9 %, kidney 15.0 % and intestine 18.3 % carbohydrate. All three have similar amino acid compositions and all show identity by Ouchterlony immuno diffusion (Fig. 2).

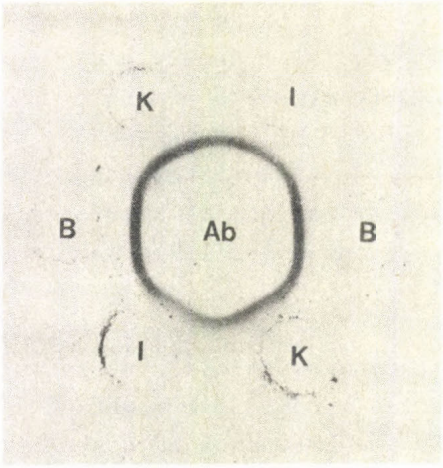

Fig. 2:

Ouchterlony immunodiffusion

Ab, polyclonal antibody in centre well; peripheral wells contain purified endopeptidase-24.11 from: B, brain; K, kidney; I, intestine. Precipitate stained for protein.

Hydrolysis of synthetic and natural peptides by endopeptidase-24.11

Although the primary attack can be predicted from the specificity originally defined with insulin B chain (Kerr and Kenny, 1974), it is becoming increasingly clear that the efficiency of hydrolysis may be greatly influenced by residues in positions somewhat remote from those adjacent to the bond hydrolysed. This was clear from some careful studies by Orlowski and Wilk (1981) on a partially pure preparation of endopeptidase-24.11 from bovine pituitaries in which a marked influence of residues at P_2, P_3 and P_4 on hydrolysis rates was demonstrated. Similar conclusions were obtained with the purified rabbit kidney enzyme (Almenoff and Orlowski, 1983).

In regard to natural peptides, our own work (Matsas et al., 1983b) has shown a very considerable range in K_{cat}/K_m values for their hydrolysis by purified pig kidney endopeptidase-24.11. Some are shown in Table 3. Substance P and the deamidated form were the best substrates. Enkephalins were also hydrolysed well, but the efficiency was lowered when amidated, though not when the peptide was extended by one or two residues. Luteinising hormone releasing hormone (LHRH) was

Table 3

Relative K_{cat}/K_m values for the hydrolysis of some neuropeptides and analogues by kidney endopeptidase-24.11

Peptide	K_{cat}/K_m (relative to Substance P) %
Substance P	100
Substance P (deamidated)	115
[Leu] enkephalin	15
[Leu] enkephalinamide	0.5
[DAla^2,Leu^5] enkephalin	21
[DAla^2,Leu^5] enkephalinamide	0.6
[DAla^2,Met^5] enkephalinamide	5
[Leu^5,Arg^6] enkephalin	14
[Leu^5,Arg^6,Phe^7] enkephalin	54
Neurotensin	5
LHRH	0.3

a very poor substrate. These preliminary studies suggest that the role of endopeptidase-24.11 in inactivating biologically active peptides, at cell surfaces in the central nervous system and elsewhere, may be more selective than had been originally thought.

CONCLUSIONS

We have now purified endopeptidase-24.11 from kidney, intestine and brain. Apart from some differences in post-translational processing, the three preparations have similar properties. One practical gain from this information is that the purified kidney enzyme can be confidently regarded as a valid model for investigating properties of the brain enzyme and, since it is 100-fold more abundant in kidney than caudate nuclei, the advantages of working with the former are obvious.

The various roles of the endopeptidase in its different locations are to some extent a matter for speculation. In kidney microvilli, the enzyme accounts for 4 % of the membrane protein, comparable to dipeptidyl peptidase IV and exceeded in amount only by aminopeptidase N and the principal core protein, actin. Yet its role and that of the other hydrolases in this membrane remain to be clarified experimentally. It seems unlikely that the endopeptidase can initiate the hydrolysis of any native proteins that are filtered by the glomerulus. Its natural substrates are probably peptide hormones exhibiting minimal secondary and tertiary structure. Unlike the isolated chains of insulin and glucagon, insulin itself resisted attack (Kerr and Kenny, 1974a). However, since the potential substrates in the glomerular filtrate are essentially excluded from exerting a biological action on sites other than those bathed by the fluid in the lumen of the nephron, we may assume that the existence of this intensive battery of peptidases, located in the first segment of the nephron, must relate to a need to ensure that one or more peptide does not cause an unphysiological effect lower in the nephron. But the identity of the potentially hazardous peptides is at present unclear. On the other hand, the presence of the endopeptidase and its attendant exopeptidases in the intestinal microvillar membrane can be explained with more confidence. It is clear that many peptides generated by the action of gastric and pancreatic proteinases may well require the attack of the membrane peptidases, including endopeptidase-24.11, before absorption can take place. However, the precise role of endopeptidase-24.11 in this context remains to be experimentally determined. In synaptic membrane of at least some regions of the brain, we may be nearer to understanding the role of this enzyme. If we can assume that its topology in these membranes is like that in microvilli (i.e. at the extracellular surface facing outwards), it is ideally placed to terminate the signals generated by neuropeptides at synapses. Since its specificity is broad, it is likely that a wide spectrum of neuropeptides may be so inactivated, but it is clear that other peptidases also play major roles in respect of different regions of the central

nervous system and in respect of neuropeptides that are vulnerable to attack by other means, e.g. that by aminopeptidase on enkephalins. The precise role of endopeptidase-24.11 in the membranes of other cell types in hydrolysing peptides at cell surfaces is at present an unexplored field.

ACKNOWLEDGEMENTS

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KOPITAR:

I am interested in the pH dependent stability of endopeptidase 24.11. How long does this enzyme retain its (total) enzyme activity at pH 10.6, at 37°C and at room temperature?

KENNY:

When we first used the immunoadsorbent column we neutralised each fraction as it was collected. But this does not seem to be necessary - later we bulked the fractions and then dialysed against pH 7.4 buffer, at 4°C and we know that this process takes several hours before the pH falls significantly. So the stability of the endopeptidase to pH 10-6 is high.

VITALE:

What are K_i values for captopril and other angiotensin - converting enzyme inhibitors for your enzyme?

KENNY:

Captopril does not inhibit endopeptidase - 24.11, even at 1 mM concentration.

GRÁF:

I have not seen β-endorphin among the endopeptidase substrates shown in your last slide. Has β-endorphin been tested? I wonder if β-endorphin is a similarly good substrate of Endopeptidase 24.11 as enkephalin (the N-terminal fragment of β-endorphin) is.

KENNY:

No, it has not been tested, we are beginning to look at it. We don't yet know exactly the rules of the game, we don't quite know how to describe which substrate is going to be good or bad, and I think we need to look at lot more.

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SOME PROPERTIES OF LYSOSOMAL CYSTEINE PROTEINASES AND THEIR COMPLEXES WITH ANTIBODIES

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SUMMARY

Cathepsin L (M_r 29,000) can be cleaved in two chains of M_r 24,000 and about 5,000 as it has already been described for cathepsins B and H.

Labelling of the active center cysteine by Z-Phe-Phe-$^{14}CHN_2$ and by the fluorescent labelled Dans-Ala-Ala-Phe-CH_2Cl shows that the active site thiol of cathepsins L and H is located in the heavy chain (M_r 23 - 24,000). This finding is in contrast to the location of the active site thiol of cathepsin B which has been found in the light chain.

Enzyme activity of cathepsin I was retained in the heavy chain after removing the light chain.

Cathepsins H and L have been found to precipitate with their antibody at a pH as low as 6.0, where the enzymes are enzymatically active. Cathepsin B has only shown precipitation after alkaline denaturation.

Antigen-antibody-complexes of cathepsins H and L exhibited enzyme activity against low molecular weight substrates.

A high molecular weight precursor protein of cathepsin B and H could not be detected in lysosomal extracts, whereas such an active proenzyme of cathepsin D is present in lysosomal extracts.

Abbreviations: Dans: dansyl-
Z : benzyloxycarbonyl-

INTRODUCTION

In search of proenzymes of lysosomal cysteine proteinases we use the following technique: after incorporation of ^{35}S-labelled methionine of the cells in culture the enzymes and their precursors were specifically bound to their respective antibody. The molecular weight of the bound antigens was examined after SDS-gel electrophoresis. The interpretation of the results of these and the related experiments using specific radio-labelled inhibitors (Docherty et al., 1983) needs knowledge of the dissociation behaviour of the cysteine proteinases on SDS-gels both under reducing and non-reducing conditions. The determination of the active site in the separated chains would be of equal importance. On the other hand, we are also lacking detailed knowledge of the reactions of lysosomal cysteine proteinases with their antibodies.

In the present paper we describe properties of cathepsins B, H and L concerning their dissociation behaviour on SDS-gel electrophoresis and location of the active site cysteine in the separated chains. In the second part some results are given of experiments with cathepsins B, H and L and their antibody.

Separation of heavy and light chains of cathepsins B, H and L and location of the active site

Cathepsin B from pig liver (Takahashi et al., 1979, 1980) and beef spleen (Pohl et al., 1982) has been studied in detail with respect to the noncovalently bound chains. According to the authors the cleavage of cathepsin B (M_r 28 - 29,000) after reduction resulted a heavy chain (M_r 22 -25,000) and a light chain (M_r 5 - 6,000). The latter forms the N-terminus of the native molecule (M_r 28 - 29,000). The active site thiol of cathepsin B was located in the light chain.

In contrast to cathepsin B the light chain (M_r ca. 6,000) of cathepsin H from rat liver originated from the C-terminus of the aggregated molecule (M_r ca. 28,000) (Takio et al., 1982). Molecular weight of ca. 22,000 for the heavy chain was deter-

mined by the same authors. The location of the active site thiol of cathepsin H remained undecided.

We could show that cathepsin L also consists of two chains.

The separation on SDS-gel electrophoresis of the chains of cathepsins B, H and L is shown in Fig. 1.

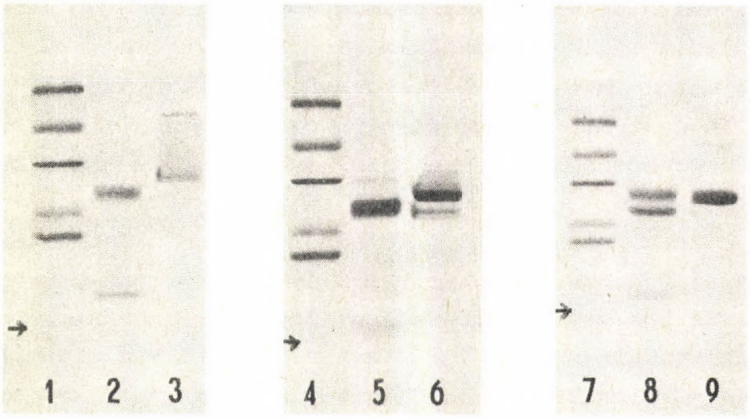

Fig. 1: SDS-polyacrylamide gel electrophoresis of cathepsin L and B and H
The samples were run on 15 % polyacrylamide gel slab, 1, 4, 7; protein standards: bovine serum albumin (68,000), egg white albumin (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), myoglobin (17,800), insulin B chain (3,495), indicated by arrow. 2: cathepsin L after reduction with 2-mercaptoethanol; 3: cathepsin L without reduction; 5: cathepsin B after reduction; 6: cathepsin B without reduction; 8: cathepsin H after reduction; 9: cathepsin H without reduction

Cathepsin L has a molecular weight of ca. 29,000, the heavy chain of 24,000 and the light chain of about 5,000. The chains of cathepsin L can be separated completely by SDS-gel electrophoresis already under mild reducing conditions (0.1 % 2-mercaptoethanol). With cathepsin H, however, under these conditions and even after reduction with 1 % 2-mercaptoethanol a part of the intact molecule remained undissociated on the gel

(Fig. 1). We determined the same molecular weight of about 5,000 for the light chains of cathepsin L and H, whereas the light chain of cathepsin B has a slightly lower molecular weight of about 4,000.

The molecular weights have been determined by SDS-gel electrophoresis using 15 % polyacrylamide gels (2,6 % cross linked). The low molecular weights could be only roughly calculated by this method.

It was of interest to know whether the active site cysteine of cathepsin L was located also in the light chain as has been described for cathepsin B (Takahashi, et al., 1980; Pohl et al., 1982). In contrast to the results with cathepsin B, however, we detected the active site thiol in the heavy chain of cathepsin L by the following methods.

1./ Cathepsin L reacts irreversibly and fast with Z-Phe-Phe-CHN_2 (Kirschke and Shaw, 1981). This reagent can be used to titrate the active site of cathepsin L. The inactivation of cysteine proteinases by diazomethyl ketones was shown to result from the alkylation of the essential SH-group (Leary et al., 1977).

After reaction of cathepsin L with ^{14}C-labelled Z-Phe-Phe-CHN_2 the chains were separated by SDS-gel electrophoresis. The gel was stained for protein and afterwards sliced in sections which were solubilized for measuring radioactivity. Radioactivity was detected only in the gel slices of the heavy chain. It was shown by autoradiography of the dried gel that the labelled inhibitor had reacted only with the heavy chain. A non-reduced sample of enzyme-inhibitor-complex exhibited the radioactivity in the intact molecule with M_r 29,000.

2./ There was no reaction of cathepsin L with ^{14}C-labelled Z-Phe-Phe-CHN_2 after the enzyme had reacted with a different inhibitor as iodoacetic acid or E-64. This results is a piece of evidence that the labelled inhibitor reacted with the active site thiol of the enzyme.

3./ Z-Ala-Ala-Phe-CH_2Cl is an irreversible and fast reacting inhibitor of cathepsins B, H and L (unpublished results). For the experiments described here we used the inhibitor after substitution of the N-terminal protective group (Z-) by dansyl-residue.

After reaction of the enzymes with Dans-Ala-Ala-Phe-CH_2Cl and separation of the chains by SDS-gel electrophoresis, a bright fluorescence was detectable of the heavy chains of cathepsins L and H and of the light chain of cathepsin B. This experiment shows clearly that the inhibitor Dans-Ala-Ala-Phe-CH_2Cl reacts with an amino acid in the active center, otherwise the heavy chain of cathepsin B would have been marked, as well. Studies of the reaction of chloromethyl ketones with papain have shown an alkylation of the active center thiol group (Glazer and Smith, 1971).

4./ The heavy chain (M_r 24,000) of cathepsin L retained the enzyme activity after removing the light chain. The chains of cathepsin L have been separated after reduction by gel electrophoresis at pH 3.7 with cetylpyridinium chloride. A gel strip was stained for protein and a duplicate gel strip was sliced in sections. The enzyme activity was determined in the extracts of the gel slices with Z-Phe-Arg-methyl coumaryl amide as substrate. Only the gel slices of the heavy chain exhibited catalytic activity.

Reaction of cysteine proteinases with their antibody

The formation of insoluble antigen-antibody-complexes of cathepsin B from rat is in a special way pH dependent. Already at pH values between 6 or 7, where the enzyme exhibits enzyme activity, cathepsin B does not precipitate with its antibody. This has been described in detail for cathepsin B from rabbit and human (Graf, 1981). Cathepsins H and L precipitate with their antibody at pH 8-9, as well as at pH 6-6.5. The antigen-antibody-complexes of cathepsin H and L precipitated at pH 6 retained the enzyme activity against low molecular weight substrates.

In the search of proenzymes of lysosomal cysteine proteinases, first we tried to detect such precursors in the lysosomal extract. A lysosomal extract from rat liver was run on Sephadex G-75. The fractions were tested by fused rocket immunoelectrophoresis. We were unable to detect antigens of molecular weight higher than 30,000 either for cathepsin H or

for cathepsin B. On the other hand, we could show that an antibody against cathepsin D (aspartic proteinase) precipitated with an antigen of M_r about 100,000.

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DISCUSSION

TURK:

In your presentation you mentioned the molecular weight of the precursor of cathepsin D to be about 100 000 daltons. In my opinion this value is too high. Reports of e.g. Hasilik, Neufeld, Blotel and our own results show that the Mr of cathepsin D precursor is about 50 000 daltons.

KIRSCHKE:

The molecular weight of the precursor of cathepsin D which has been detected outside the lysosomes is 53 000. We found a compound with a molecular weight of about 100 000 in the lysosomes which reacted with an antibody against cathepsin D. Further experiments will show whether this is an aggregate of cathepsin D with other proteins and if this compound exhibits enzymatic activity.

STEPANOV:

Would you tell me something about the inhibitory effect of diazomethanes?

KIRSCHKE:

Leary and Shaw showed that diazomethanes react specifically with cysteine proteinases by alkylation of the active site SH-group. They do not react with low molecular weight SH-compounds and do not inhibit serine proteinases. Aspartic proteinases react only in the presence of Cu^{2+} ions with diazomethanes.

BARRETT:

Although the largest precursor of cathepsin D detected so far is only 50 000 molecular weight, I think that still larger precursors might exist, as indicated by your results.

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CHARACTERIZATION AND STRUCTURAL STUDIES OF CATHEPSIN B, H AND L AND THEIR PROTEIN INHIBITORS

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INTRODUCTION

Tissue cysteine proteinases are responsible for the degradation of intracellular proteins under both normal and pathological conditions. This fact is supported by several observations which suggest their role in the processes of hormone metabolism generating their active peptides from precursors (Quin and Judah, 1978; Smith and VanFrank, 1975), in the metabolism of opiate peptides (Suhar et al., 1981), the inactivation of some enzymes involved in glycolysis (Otto, 1971; Nakai et al., 1983), collagen degradation (Eeckhout and Vaes, 1977) and degradation of myofibrilar proteins (Matsukara et al., 1981). Furthermore, cysteine proteinases are also involved in the pathology of cancer (Mort et al., 1980; Graf et al., 1981; Giraldi et al., 1982).

Recent studies have shown that the activities of lysosomal cysteine proteinases may be regulated by their endogenous protein inhibitors, which are located in the cytosol (Kopitar et al., 1978; Lenney et al., 1979; Hirado et al., 1981; Järvinen and Rinne, 1982; Brzin et al., 1982; Kominami et al., 1982; Kopitar et al., 1983). The mechanisms by which these inhibitors regulate the activities of the corresponding enzymes are not yet defined.

Although lysosomal cysteine proteinases and their endogenous protein inhibitors isolated from various sources have many catalytic properties in common, there are numerous discrepancies in their other characteristics. In this paper the purification, properties and structural data of cathepsins B, H and L from bovine spleen as well as the primary structure and some characteristics of cytosolic protein inhibitor from human leucocytes stefin and the egg white inhibitor cystatin, are presented.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine spleens were used for the isolation of cysteine proteinases. A cytosolic protein inhibitor, stefin, was isolated from fresh human blood. The egg white inhibitor, cystatin, was isolated from fresh chicken eggs. All chemicals used in the experiments were of reagent grade.

Methods

Proteolytic activities of papain and cathepsin B on benzoyl-DL-Arg-2-naphtylamide (BANA) substrate and cathepsin H on L-leucyl-2-naphthylamide (Leu-NA) substrate were determined according to Barrett (1972). Cathepsin L activity was determined according to the Anson method (1939) using haemoglobin as substrate in the presence of 10^{-7} M of the inhibitor pepstatin. Stefin inhibitory activity was determined under the conditions described for proteinase assay. A known amount of inhibitor was preincubated with the enzyme for 5 min at room temperature before adding the substrate. Cystatin inhibitory activity was determined with papain using BANA as substrate (Barrett, 1972). One inhibitory unit of stefin and cystatin was defined as the amount of the inhibitor which completely inhibits 1 µg of papain. Protein was determined by the method of Lowry (1951).

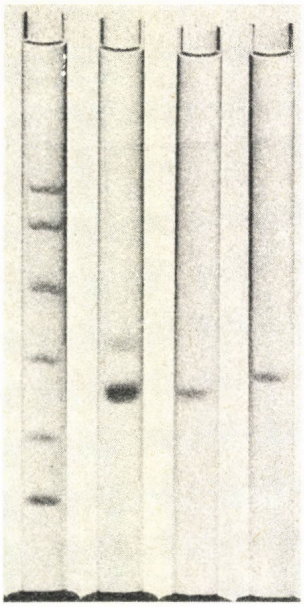
Molecular weight was determined by gel chromatography according to Whitaker (1963) and by SDS polyacrylamide gel electrophoresis (PAGE) according to Laemli (1970). Analytical gel electrofocusing was carried out on 1 mm thick 5% polyacrylamide plates with carrier ampholines (pH 4-8) and gels were stained for protein with Coomassie brilliant blue. Amino acid analysis, and sequence analysis by automated solid-phase Edman degradation were determined (Machleidt et al., 1983, in press).

The purification procedure of cathepsin B, H and L is based on the method for the isolation of bovine lymph node cathepsin B and H (Zvonar et al., 1979) and the modified method for the isolation of cathepsin B, H and S (Ločnikar et al., 1981). The purification steps include acid extraction, acetone fractionation, ammonium sulphate precipitation, gel filtration chromatography on Sephadex G-50, covalent chromatography on thiol-Sepharose 4B, CM-cellulose chromatography and as the last step for cathepsin B fast protein liquid chromatography (FPLC) from Pharmacia, Uppsala. The details of this new purification procedure will be published elsewhere. The human leucocyte inhibitor, stefin, was isolated from leucocyte cytosol using affinity chromatography on carboxymethylated papain-Sepharose 4B and DEAE-Sephacel chromatography (Brzin et al., 1983, in press). The egg white inhibitor, cystatin, was isolated from egg white after alkaline and heat treatment, gel filtration chromatography on Sephadex G-50 and DEAE-Sephacel chromatography (Turk et al., 1983a, in press).

RESULTS AND DISCUSSION

Properties of cathepsins B, H and L

As we reported earlier (Ločnikar et al., 1981) cathepsins B, H and L (former cathepsin S) were resolved by ion exchange chromatography on CM-cellulose. The purity of all three cathepsins (Fig. 1) was analyzed by SDS-PAGE under denaturing (i.e. SDS) and non-denaturing conditions. Whereas cathepsin H and L contain only one protein band with Mr of about 28 000 and 24 000 daltons, respectively, cathepsin B consists of two major polypeptide chains of Mr of about 30 000 daltons and 25 000 daltons (heavy chain) and minor (light chain) of Mr of about 5 000 daltons (only weakly visible). It was suggested recently,



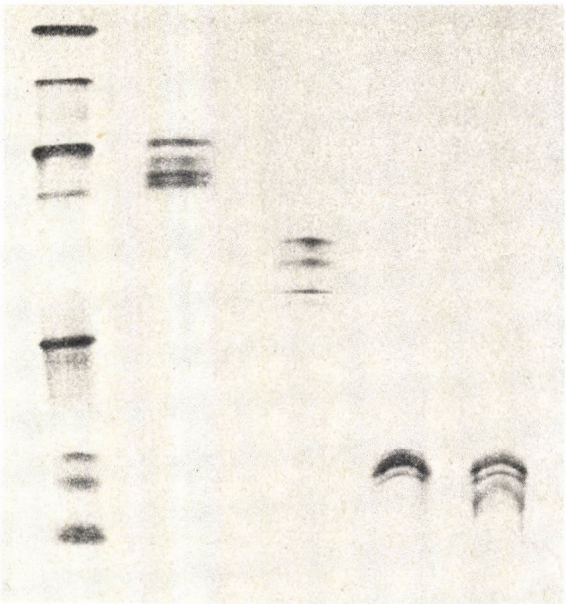


Fig. 1. SDS-PAGE : from left to right: standard proteins, cathepsin B, cathepsin L, cathepsin H.

Fig. 2. Isoelectric focusing: standard, cathepsin H, cathepsin L, two forms of cathepsin B (from left to right).

that limited proteolytic cleavage might occur to form the heavy and light chain of cathepsin B (Katunuma et al., 1981).

Isoelectric focusing of all three purified cathepsins showed that for cathepsin B pI values are between 4.7 and 5.5, for cathepsin H, between 7.0 and 7.3 and for cathepsin L between 6.3 and 6.8 (Fig. 2). The pI values for bovine spleen cathepsin B and H have been found to be similar to rat and human enzymes, whereas the pI values for cathepsin L are slightly higher (Barrett and Kirschke, 1981).

Cathepsin B was also purified by the fast protein liquid chromatography method. The enzyme was eluted in two proteolytically active peaks, without contamination with inactive proteins (Fig. 3). It is possible that these two peaks are multiple forms, which differ slightly in their isoelectric points.

Recently we observed that cathepsin S, isolated in our laboratory (Turnšek et al., 1975; Ločnikar et al., 1981) corresponds to cathepsin L on the basis of the substrate specificity, using hexapeptide Leu-Trp-Met-Arg-Phe-Ala as substrate (Turk et al., 1983b, in press). The cleavage of Met-Arg bond can be compared with similar results obtained for rat liver cathepsin L (Katunuma et al., 1981). Some other properties of isolated bovine spleen cathepsin L are generally in good agreement with published data (reviewed by Barrett and Kirschke, 1981). Small differences may be due to species and tissue variations.

Partial primary structure of cathepsins B and L

Our purification procedure enabled us to prepare enzyme samples in higher amounts to carry out the first structural studies. In Table 1 the amino-terminal sequences of cathepsin B and L are presented and compared with those of papain (Drenth et al., 1971) and rat liver cathepsin B (Katunuma et al., 1981).

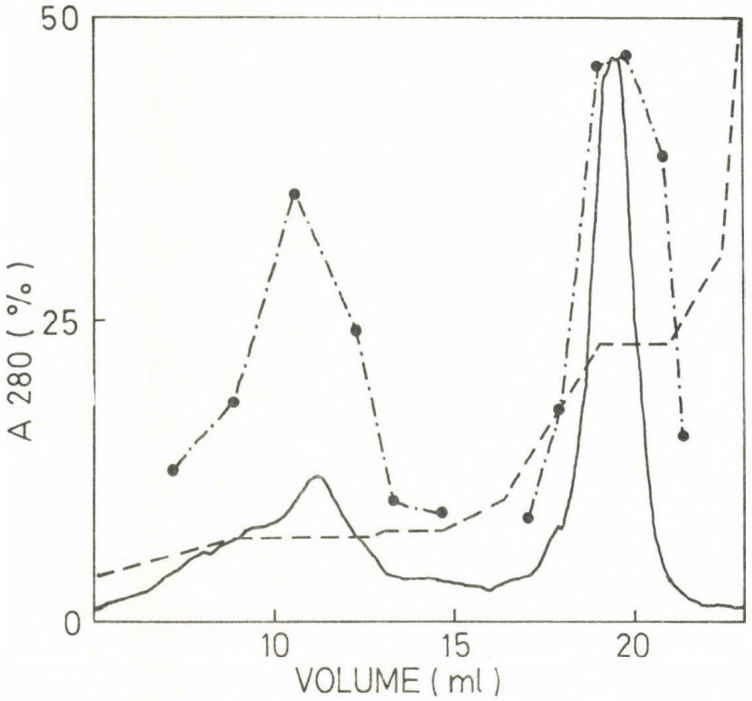


Fig. 3. FPLC of cathepsin B on Mono S column: 2.7 mg of cathepsin B was applied; starting buffer: 20 mM Na-acetate in the presence of 1 mM EDTA, pH 5.0; elution buffer: 0-0.3 M NaCl in the starting buffer; elution volume 25 ml, separation time 25 min; —— protein, -.-. BANA activity, ---- NaCl gradient.

High degree of homology between bovine spleen cathepsin B and rat liver cathepsin B is evident. It is also evident that the amino terminal part of cathepsin L is more homologous to papain than to cathepsin B which can be seen from a short loop (residues 7-10) which is missing in papain and cathepsin L. Cys-29 was found to be in the active site of cathepsin B and L, using active site radiolabelling. The complete homology of amino acid residues 26-32 (cathepsin B numbering) in all four enzymes indicates that the structure of the cysteine part of the active site might be the same. In addition, a highly homologous region in cathepsin B and papain was found around Cys-69 from residues 68-73, apparently as a consequence of the common disulphide bridge Cys-26 - Cys-69. An additional disulphide bond between Cys-14 and Cys-43 was found in cathepsin B, which does not exist in papain and cathepsin L. Furthermore, our preliminary sequence studies of cathepsin H further suggest a common evolutionary origin of the family of cysteine proteinases (in preparation for publication).

```
                    10                  20                  30
a) L P E S F D A R E Q W P N C P T I K E I R D Q G S C G S C+W
b) L P E S F D A R E Q W S N C P T I A Q I R D Q G S C G S C W
c) L P D S V D - - - - W R E K G G V T P V K Y Q G A C G S C+W
d) I P E Y V D - - - - W R Q K G A V T P V K N Q G S C G S C+W

                    40              47  50
a) A F G A V E A I S D R I C I H S - N V N V E V S A E D M L T
b) A F G A V E A M S D R I C I H T - N V N V E V S A E D L L T
c) A F S A V V L A Q -
d) A F S A V V T I E G I I K I R T G N L N - Q Y S E Q E L L D

   60                  70                  80
a) C C G G E C G D G C N G G F P S G A W N F W T V -
b) C C G I Q C G D G C N G G Y P S G A X N F -
c)
d) C D - - R R S Y G C N G G Y P W S A L Q L V A Q -
```

+ indicates active site Cys

Table 1. Amino-terminal sequences of a) bovine spleen cathepsin B, b) rat spleen cathepsin B (Katunuma et al., 1981), c) bovine spleen cathepsin L, d) papain (Drenth et al., 1971).

The amino acid sequence of cathepsin B light chain (residues 1-47) is in agreement with recently published results (Pohl et al., 1982). In conclusion, the elucidation of the complete structure of lysosomal cysteine proteinases will contribute towards further understanding of the mechanism of their action.

Properties and structure of cysteine proteinase inhibitors

Whereas egg white cystatin has been previously isolated (Fossum and Whitaker, 1968; Sen and Whitaker, 1973; Keilova and Tomašek, 1974) and very recently reinvestigated (Anastasi et al., 1983), mammalian cytosolic protein inhibitors of cysteine proteinases were discovered and characterized only recently (see references in Introduction). Due to many similar properties it was assumed that egg white cystatin and cytosolic protein inhibitors are members of a homologous family. Therefore our studies were oriented in this direction. Introducing the new isolation procedure for egg white cystatin and the human leucocyte inhibitor stefin we isolated both inhibitors in the electrophoretically pure forms (Turk et al., 1983a, in press; Brzin et al., 1983, in press). Some of the properties of cystatin and stefin are presented in Table 2. It can be seen that both inhibitors have some common properties, e.g. temperature and pH stability. In addition, egg white cystatin and mammalian cytosolic inhibitors form complexes with inactive papain. This fact was successfully introduced into the purification procedure by the use of the inactive papain as a ligand for the affinity chromatography step (Anastasi et al., 1983; Järvinen and Rinne, 1982; Brzin et al., 1983, in press).

	Cystatin	Stefin
Isolation procedure	standard methods affinity chromatography	affinity chromatography
Usual assay	with papain	with papain
pH stability	pH 3-12	pH 4-12
Temperature stability	20 min at 80 ^{o}C	20 min at 80 ^{o}C
Multiple forms	several, main at pI 5.6 and 6.5	pI 4.7
Molecular weight	form I about 13 100 form II 12 181	11 006
Inhibition	cathepsin B,H,L, papain	cathepsin B,H,L, papain
K_i	about 10^{-11} on papain	10^{-7} - 10^{-11} on different enzymes

Table 2. Properties of egg white cystatin and human leucocyte inhibitor stefin

```
                    10                  20                  30
a) S E D R S R L L G A P V P V D E N D E G L Q R A L Q F A M A
b)                  G A P V P V D E N D E G L Q R A L Q F A M A
c) M I P G G L S E A K P A T P E I Q E I V D K V K P Q L E E K

                    40                  50                  60
b) E Y N R A S N D K Y S S R V V R V I S A K R Q L V S G I K Y
c) T N E T Y G K L E A V Q Y K T Q V V A G T N Y Y I K V R A G

                    70                  80                  90
b) I L Q V E I G R T T C P K S S G D L Q S C E F H D E P E M A
c) D N K Y M H L K V F K S L P G Q N E D L V L T G Y Q V D K N

                   100                 110
b) K Y T T C T F V V Y S I P W L N Q I K L L E S K C Q
c) K D D E L T G F
```

Table 3. Amino-acid sequence of a)cystatin form I (partial), b)cystatin form II, c) stefin.

Therefore our further studies were oriented towards the determination of the amino acid sequence of egg white cystatin and the human leucocyte inhibitor stefin. Amino acid sequences were determined by automated solid-phase Edman degradation and the results are presented in Table 3. (Turk et al., 1983a, in press; Machleidt et al., 1983, in press).

The human leucocyte inhibitor stefin does not contain tryptophane and only one arginine and histidine. An outstanding feature is lack of cysteine which

this inhibitor has in common with thermostable yeast proteinase B inhibitor (Maier et al., 1979) and eglin c (Seemüller et al., 1981).

Two major forms of egg white inhibitor cystatin with pI values 6.5(form I) and 5.6 (form II) were isolated and form II was sequenced completely. Form II consists of 108 amino acid residues and a homogenous amino-acid sequence, starting with Gly-Ala-Pro was obtained. When form I was sequenced two different sequences were obtained and one of them was identical with that of form II. The second sequence turned out to be an elongation of the short form with 8 residues starting with Ser-Glu-Asp. A single N- terminal sequence Ser-Glx-Asx was reported recently for two forms of cystatin with the same pI values compared to our results (Anastasi et al., 1983). Two different isoelectric points, namely pI 6.5 and 5.6 were explained by small differences in the pattern of tryptic digest. It must be assumed that our long form represents the more complete sequence of inhibitor, whereas the short form very likely originated from the cleavage of a peptide bond. As is evident from our results, we sequenced only the short form completely, whereas the longer form was sequenced only partially. Cystatin contains 4 cysteine residues, which form presumably two disulphide bridges, as already proposed (Anastasi et al., 1983). In this respect cystatin differs from stefin which contains no cysteine at all. Also both sequences of cystatin and stefin are different. Therefore we can assume that egg white cystatin and the human leucocyte inhibitor stefin belong to two different families of cysteine proteinase inhibitors.This is additionally supported by the completely different circular dichroism spectra of both inhibitors (Turk et al., 1983,in press).

Both inhibitors are powerful inhibitors of lysosomal cysteine proteinases and papain. Since stefin does not contain cysteine and cystatin does not contain a free thiol group it may be concluded that neither inhibitor is involved in a thiol-disulphide exchange mechanism. Further investigations in this direction in order to understand the mechanism of action of the cysteine proteinases which are regulated by these protein inhibitors are needed.

ACKNOWLEDGEMENT. The investigations were supported by the research grant from the Research Council of Slovenia, Ljubljana, Yugoslavia and by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 0207, München, BRD.

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DISCUSSION

WIEDERANDERS:

With respect to your first slide, I would like to ask you about the differences in the properties of lysosomal and cytosolic insulin-glucagon proteinases.

TURK:

This is described in the literature. It is hard for me to recall it at the moment.

WIEDERANDERS:

Did you look for the presence of cathepsin T in bovine spleen?

TURK:

No, we didn't.

PATTHY:

Have you performed experiments to find out whether the inhibitors form covalent complexes with the proteases?

TURK:

Yes, and the results clearly indicated that there is no covalent bond between the enzymes and protein inhibitors.

PATTHY:

What were the conditions of eluting the inhibitors from the affinity column?

TURK:

For example in the case of purified papain it was nicely eluted from egg-white affinity column with urea at higher pH. In the case of cysteine proteinases, cathepsin B, H and L we had some troubles because when the pH was higher than 7.0 they were unstable. But with papain we have got very nice elution using eluents of higher pH than 7.0 + urea. These are unpublished results.

GRÁF:

How did you determine the K_i constants for your inhibitor?

TURK:

K_i was determined by the method of Green and Work.

GRÁF:

By which method was the sequence work done?

TURK:

Sequence was determined by automatic solid-phase sequanator using Edmann degradation. Details will be published soon in Hoppe Seyler's Z. Physiol. Chem.

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CHARACTERIZATION OF HUMAN ALANINE AMINOPEPTIDASES

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Exopeptidases are peptide hydrolases whose assault upon a peptide bond is limited to one of the two terminus of the peptide chain.

The N-terminal exopeptidases require a free α-amino group and are denominated aminopeptidases, if they split off free amino acids. They can be classified according to their subcellular localization, e.g. membrane bound, lysosomal or cytosolic.

The alanine aminopeptidase /AAP, EC 3.4.11.2/ is the best known example of a particulate N-terminal exopeptidase and has been isolated and characterized from various sources by Pfleiderer and Celliers /1963/, F.J. Behal et al. /1966/,

Abbreviations:

AAP: alanine-aminopeptidase
NANA: N-acetyl-neuraminic acid
DIFP: diisopropyl fluorophosphate
PMSF: phenyl-methylene-sulfonylfluoride
NEM: N-ethylmaleimide
pCMB: p-chloromercuribenzoate
DTNB: dithio-bis-nitrobenzoic acid
NBS: N-bromosuccinimide
DTT: dithiothreitol

H. Hanson et al. /1967/, N. Rehfeld et al. /1967/, H.J. Hütter et al. /1972/, S. Maroux et al. /1973/, Garner and Behal /1974/, Y.J. Kao et al. /1978/.

The enzyme is localized in the brush border region of the proximal convoluted tubules of the kidney, in the brush border region of the small intestinal mucosa and was found in the globular knobs of plasma membrane fractions of the liver cells.

At least, in human liver, kidney, pancreas, small intestine and placenta, different electrophoretic variants have been detected. They are usually found in the microsomal fraction by subcellular fractionation. The AAPs, isolated from human liver, kidney, pancreas and placenta have a molecular mass of 230 000, the enzyme of the jejunum one of 295 000 and that from the ileum 310 000.

Sepharose chromatography in the presence of 8 M urea and 0.1 M mercaptoethanol provided evidence for subunits of 30-35 000 molecular weight. Thus, six subunits would presumably make up the molecular weight of the native enzyme.

The molecular weight of the liver-AAP was calculated by Little and Behal /1976/ using sedimentation and gel filtration data as 234 000. In the presence of 6 M guanidine and 0.1 M mercaptoethanol agarose chromatography provided evidence for subunits of 38 000 molecular weight. In an other communication a subunit molecular weight of 120 000 was reported by Garner and Behal /1974/. According to Feracci and Maroux /1980/ the completely denaturated enzyme after detergent solubilization from the small intestine of the rabbit corresponds to a molecular weight of 130 000 and exists in native form as a monomer.

According to Böhme et al. /1976/ the different electrophoretic mobility of the alanine aminopeptidases, isolated from human liver, kidney and pancreas is due to a different net charge as demonstrated by different isoelectric points. The results of the carbohydrate analysis point to NANA as the main factor causing these differences in electrophoretic mobility. The NANA content varies from 96 mol for the liver AAP to 16 mol for the kidney- and zero for the pancreas-enzyme /Table 1/.

Table 1

Carbohydrate content of alanine aminopeptidases from human liver, kidney and pancreas in percent with regard to protein /Böhme et al., 1976/

Carbohydrate	Liver AAP	Kidney AAP	Pancreas AAP
Glucose	1	18	18
Galactose	2	2	3
Fucose	0	5	3
Amino sugars	9	9	9
N-Acetylneuraminic acid	11	2	0

The isoelectric point of jejunum- and ileum-AAP corresponds partly to that of the pancreas enzyme. This means, the high molecular weight of the small intestinal AAP could be the cause for the lowest electrophoretic mobility found till now /Table 2/.

Table 2

Molecular weight, R_f-value and isoelectric point of human alanine aminopeptidases

AAP isolated from human	Molecular weight	R_f-values on 7.5% polyacrylamide gels	Isoelectric points pH	Mol NANA/mol enzyme
liver	230 000	0.39	3.62	96
kidney	230 000	0.31	4.35/4.70	16
pancreas	230 000	0.25	4.70/4.44	0
jejunum	310 000	0.20	4.45	n.e.
ileum	295 000	0.20	4.45	n.e.
placenta	220 000	0.35	4.25	n.e.

Metal ion chelators are usual inhibitors for AAP. EDTA and o-phenanthroline affect a significant reduction in enzyme activity. Dialyzing the enzymes against 1 mM EDTA, the activities can be restored by the addition Co^{2+} only. Other ions tested, failed to bring about any restauration of activity /Fig. 1/.

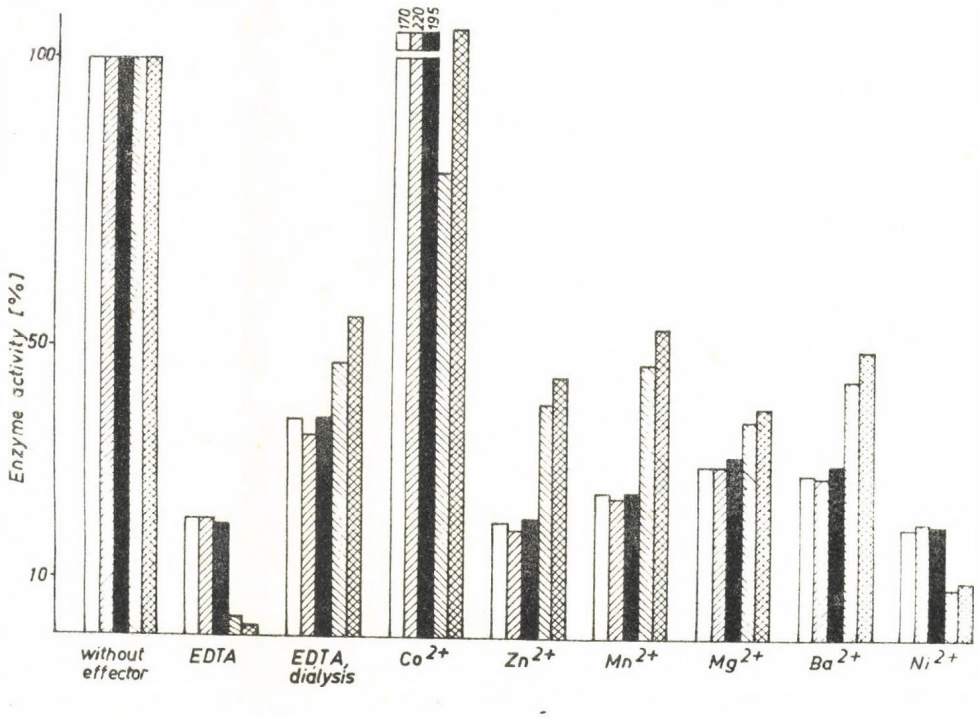

Fig. 1. Effect of metal ions and EDTA on human alanine aminopeptidases. Effector concentrations: 5 mM

liver-AAP, kidney-AAP, pancreas-AAP, jejunum-AAP, ileum-AAP

The high affinity of the enzymes towards metal ions as well as to chelating substances could be demonstrated by estimation of the inhibition constants for Ni^{2+}, Hg^{2+} and EDTA, evaluated in the range of 10^{-5} to 10^{-7} M /Table 3/.

Table 3

Inhibitory and affinity constants of various effectors

AAP from liver, kidney, pancreas	K_i /mol/l/	K_m /metal/ /mol/l/
Co^{2+}		2.5×10^{-4}
Ni^{2+}	1×10^{-5}	
Hg^{2+}	9×10^{-7}	
EDTA	1×10^{-7}	
Dithiothreitol	4×10^{-3}	

Using L-alanine-2-naphtnylamide as substrate, the Michaelis constants of the enzymes investigated were not effected during the activation process.

Co^{2+} activated the cleavage of all other substrates contained L-alanine at N-terminal position, such as L-alanyl-amide, L-alanyl-L-alanine, L-alanyl-glycine, L-alanyl-L-leucine and L-alanyl-glycyl-glycine. On the other side, the turnover of the L-alanyl-p-nitroanilide was nearly completely inhibited by Co^{2+} ions /Table 4/.

The hydrolysis of substrates containing L-leucine in N-terminal position is not influenced by Co^{2+} and is by Mn^{2+} rather inhibited. This could be found using L-leucyl-2-naphthylamide, L-leucyl-p-nitroanilide, L-leucyl-amide, L-leucyl-glycine and L-leucyl-glycyl-glycine. These results suggest that the enzyme molecule might consist of two catalytic units, or the active center may possess two indistinguishable metal binding sites.

Table 4

Influence of cobalt ions on the turnover of different substrates using kidney-AAP enzyme

Substrate	Effector /1 mM/	Activity /U/l/	Relative activity /%/
L-Ala-NA	-	780	100
"	Co^{2+}	1435	184
L-Ala-NH_2	-	645	100
"	Co^{2+}	1185	184
L-Ala-L-Ala	-	560	100
"	Co^{2+}	572	102
L-Ala-Gly	-	378	100
"	Co^{2+}	542	144
L-Ala-L-Leu	-	197	100
"	Co^{2+}	405	200
L-Ala-Gly-Gly	-	2020	100
"	Co^{2+}	2429	118
L-Ala-p-N	-	20.3	100
"	Co^{2+}	1.95	10.4

A broad range of substrate specificity is characteristic of the alanine aminopeptidase.

In the case of the aminoacyl-2-naphthylamide substrates with nonpolar aminoacyl residues are preferred. The enzyme has very low activity with acidic /e.g. aspartyl-/ and basic /e.g. lysyl-/ 2-naphthylamides. In addition, D-aminoacyl-2-naphthylamides are not hydrolyzed. Generally, a free α-amino

group is required for enzymatic activity and substrates having no aryl-group, e.g. dipeptides and tripeptides are split quite well, too.

Using the relation of V/K_m as a constant of effectiveness of the substrate turnover, alanyl-2-naphthylamide is the substrate split most effectively /Table 5/.

Table 5

Michaelis constants of kidney-AAP determined with various arylamides and peptides

Substrate	K_m /mM/	V /U/l/	V/K_m
L-Ala-2-NA	0.25	7 520	30 080
L-Phe-2-NA	0.66	5 500	8 333
L-Leu-2-NA	0.20	855	4 275
Gly-2-NA	0.67	565	843
L-His-2-NA	1.25	48	38.4
L-Leu-p-N	0.72	196	272
L-Leu-NH_2	57.0	5 263	92.3
L-Leu-NH.NH_2	0	0	
L-Leu-Gly-Gly	9.1	6 849	753
Gly-L-Leu	11.0	735	67
Gly-Gly	333.0	8 333	25

The physiological role of the alanine aminopeptidases seems to be still unknown. On the other side, peptide substrates like e.g. oxytocin and vasopressin, are split readily by the enzyme, isolated from human kidney, as demonstrated by Barth et al. /1969/. The enzyme was able to hydrolyze angiotensin-II-amide as found by H.J. Hütter et al. /1970/. In view of these results, besides the aminopeptidase A, which hydrolyzes angiotensin and other peptides containing aspartic or glutamic acid as amino terminal, the particle bound alanine

aminopeptidase may also be considered as possible factor of angiotensin and angiotensinamide regulation in the kidney.

The alanine aminopeptidases were not influenced by reagents specific for the serine residue at the active site. In the same way, we failed to detect histidine in the active centre, as indicated by the absence of photooxydation effect in the presence of methylene blue.

Preincubation of the enzymes with SH-blocking reagents e.g. pCMB, NEM, DTNB, JAM inhibited the activity in nearly identical relations. Preincubation with mercaptoethanol or DTT in 10 mM concentration inhibited the enzyme markedly.

Table 6

Influence of active site specific reagents on AAP

Effector	Concentration /M/	Relative activity /%/		
		liver AAP	kidney AAP	pancreas AAP
DIFP	0.1	100	100	100
PMSF	0.001	100	100	100
NEM	0.001	78	76	72
pCMB	0.005	61	73	63
DTNB	0.005	50	57	51
NBS	0.001	0	0	0
DTT	0.010	24	9	31

As seen in Table 6, NBS inhibited the enzymes completely in 0.001 M concentration.

The titration of bound tryptophane in proteins by NBS can give some clue for the presence of reactive and unreactive tryptophanes. Treating tryptophanes according to Green and Witkop /1964/, different values were found in the untreated proteins as well as in the enzymes denaturated with urea /Table 7/.

Table 7

Oxydation of tryptophane residues of the alanine aminopeptidases by 1 μM NBS

	mol tryptophane/mol enzyme			
AAP from	pH 7.0	pH 4.0	8 M urea	Calculated according to Behal et al. 1974, 1978
kidney	40	40	36	32
liver	18	18	59	62
pancreas	15	17	60	-

According to these results, in the case of liver and pancreas AAP the primarily unreactive tryptophanes are buried by the tertiary structure indicating that both enzymes could be of a more compact structure. These results correspond quite well to the values published by Starnes and Behal /1974/, and Y.J. Kao et al. /1978/.

On the other hand, the results lead to the assumption that the decrease of activity following tryptophane oxydation is caused partly by conformational changes of the protein molecule as by some contribution of some tryptophane residues to the enzyme activity of AAP /Table 7, Fig. 2/.

Membrane bound enzymes are generally defined as enzymes appearing in a membranous fraction, when a homogenate is subjected to a subcellular fractionation.

The enzymes can be displaced from the membranes by the effect of detergents and proteolytic enzymes. But only in the detergent form the native primary structure is preserved. The proteinase form lacks the site of the molecule originally associated with the lipids of the membrane.

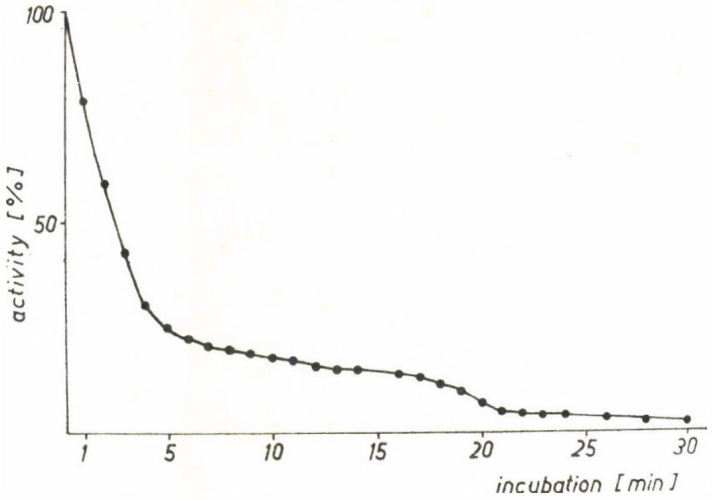

Fig. 2. Effect of tryptophane oxydation by 1 mM NBS on the activity of kidney AAP

Such membrane bound enzymes are not so well characterized as soluble enzymes, due to the difficulties in the isolation procedure. Sometimes, during the last years they were reconstituted with other well defined components in lamellar or vesicular model systems, to reproduce the physiological surrounding for the catalytic activity. This interaction between the enzyme and the remainder of the cell membrane may affect the enzyme activity and may influence some kinetic characteristics /Table 8/.

Table 8

Comparison of the substrate specificity of the protease and detergent-forms of alanine aminopeptidases isolated from human kidney and rabbit small intestine

Substrate	Relative activity, %			
	Human kidney		Rabbit small intestine	
	protease form	detergens form	protease form	detergens form
L-Ala-NA	100.0	100.0	100.0	100.0
Gly-L-Leu	10.7	72.4	39.9	272.9
" + Ca^{2+}/Zn^{2+}	10.5	68.8	55.4	289.7
Gly-Gly	30.4	153.4	57.8	67.9
" + Co^{2+}	31.2	147.4	58.2	60.5
Gly-L-Pro	0	1.2	0.9	49.1
" + Mn^{2+}	0	5.6	9.3	65.0
L-Pro-Gly	2.4	6.2	5.8	68.9
" + Mn^{2+}	2.6	10.1	6.8	54.2
L-Leu-NH_2	43.8	327.2	246.3	450.0
" + Mn^{2+}	32.5	185.9	121.0	450.0
L-Leu-Gly-Gly	-	-	222.0	847.0
" + Mn^{2+}	-	-	122.0	736.0

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DISCUSSION

KENNY:

Alanine aminopeptidase in other species, e.g. rabbit or pig, has a large subunit /130-160 kDa/ but the protease forms clips yielding apparent "subunits". Your data showing an oligomeric structure comprising smaller subunits is very unexpected. Have you determined zinc in your preparations? Other data /e.g. by Wacker/ have shown 2 Zn/dimeric molecule /~300 kDa/ indicating one active site per subunit. In regard to your experiments with metal ions after EDTA treatment, the reactivation with Zn^{2+} may have a sharp optimum concentration with higher concentrations being strongly inhibitory.

HÜTTER:

Results obtained by SDS electrophoresis show M_r 30 000-35 000 for the subunits, but higher M_r of 140 000 was also detected. Up to now, we cannot say that the enzyme exists only as a dimer, as it was reported by Maroux et al. in the case of rabbit and pig enzyme.

STEPANOV:

What was the relationship between the concentration of Co^{2+}-ion or the other ions and your substrates? If you have really high concentration of Co^{2+} compared to the concentration of substrate, practically a part of the substrate could be converted into Co-complexes.

HÜTTER:

The Co^{2+}-concentration was in every case 5 mM in the test tube and the substrate concentration was 0.75 mM. In comparison to the substrate concentration there exists a metal ion excess.

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DIFFERENT EXOGENOUS AND ENDOGENOUS CONDITIONS AFFECTING THE ACTIVITY OF PROTEINASES IN SKELETAL MUSCLES

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Faster protein degradation and enhanced activity of proteinases can be observed in the different kinds of skeletal muscle damaged by changes in exogenous and/or endogenous conditions. Higher proteinase activities can be measured not only in the atrophied muscle but also in hypertrophied muscle. The enhanced proteinase activity may originate a.) from faster proteinase synthesis, or b.) as a result of an activation process. Very often these causes play a role together in the faster process of protein degradation. It is known that a higher proteinase activity is only one of the factors operating in muscle damage. The skeletal muscles are very sensitive tissues compared to others, e.g. liver, kidneys, heart, in spite of their lower proteinase activities. Therefore, muscle tissues proved to be a good media for the study of several influences on proteinase activity. According to Szent-Györgyi (1981) "muscle is the most wonderful material of study for anybody who wants to penetrate into the deeper secrets of life".

Recently some of the factors which play a role in the elevated proteinase activities in skeletal muscles were studied in our laboratory, e.g. mechanical activity, hormone levels, redox state, energy level, proteinase inhibitor content, nutritional changes, energy level, alien proteins.

The results of hormonal changes and reduced redox state were summerized this year (Sohár et al., 1983). It was found that dexamethasone, a synthetic glucocorticoid, caused higher lysosomal and calcium-activated proteinase activities in the

fast-glycolytic type semimembranosus muscle, but there were no changes in the proteolytic enzyme activity of the slow oxidative type soleus muscle (Sohár, 1982). The soleus muscle became hormone-sensitive when the rats were kept under reduced air pressure. Under these conditions the lysosomal proteinase activity in soleus muscle upon dexamethasone-treatment was higher than that measured with the animals kept under reduced air pressure and without hormone administration. This result showed us that redox condition may play a role in the elevated lysosomal proteinase activity in glucocorticoid-treated animals.

The role of testosteron in the protein synthesis of skeletal muscle has been known for long. Its role in protein degradation is not unambiguous. Four weeks after castration higher lysosomal proteinase activity was measured in the androgen-dependent levator ani muscle. The increased activity could be inhibited by testosteron or progesteron. The Levadosin administration could inhibit only the higher azocaseinolytic activity (Levadosin is an ATP-containing substance, produced by Boehringer, Mannheim). The influence of redox state on hormonal effect on proteolytic enzyme activity showed that oxidative capacity may play a role in the higher proteinase activity. Hypoxia of rat skeletal muscles was produced by ligature of the abdominal aorta. The activity of cathepsin D, L and chymotrypsin-like proteinase of soleus and semimembranous muscles was measured after surgery. The cathepsin-L-like activity increased markedly on the fourth day, and the changes in cathepsin D activity were similar, but the activity of chymotrypsin increased only slightly in the soleus muscle. The alterations were more expressed in the slow-oxidative type muscle.

Physical activity. The effect of decreased movement can be examined under immobilization by plaster cast (right hind leg) fixation in full extension. In this position the soleus muscle is in a shortened state. If the angle of fixation is decreased, the muscle length increases. In the case of the rabbit the normal angle is 20-30^{o}, further bending of the leg stretches the soleus. In a previous experiment elevated

lysosomal proteinase activity was measured in the immobilized soleus muscle fixed in a shortened state. The extralysosomal proteinase activities did not change. A reduced redox potential, ATP and insulin content, and an increased number of glucocorticoid receptors were measured in this muscle. Direct connection between the high proteinase activity and these results require further studies. There was a correlation between the angle of fixation and the lysosomal proteinase activity. At normal position we could not measure higher activity during a two-month period of immobilization. In this case the cytosolic enzyme proteins were degraded faster than the total muscle. An enzyme protein specific for a certain muscle (that is, its activity is relatively high compared to that in other muscles) is damaged faster than the other proteins. After four weeks of immobilization a new protein distribution appeared. Discontinuing the cast fixation, proteinase activity increased further for a week, degrading the synthesized proteins characteristic of an other type of muscle, for these proteins were alien to the original muscle (Sohár et al., 1981).

These experiments raised the question: What happens to the muscle in overstreched position? We collected protein turnover data measuring the influence of stretch on protein degradation. Most of them originated from in vivo experiments. The results are summarized in Table 1.

Table 1

The influence of stretch on protein degradation in skeletal muscles

Author		Stretch	Protein degradation
Goldberg	1969	increase	decrease
Vandenburgh	1980	increase	decrease
Millward	1980	increase	increase
Etlinger	1980	increase	increase
		decrease	increase

Increased physical activity can be studied by different kinds of exercises. Table 2 shows some results published recently. Most of the exercises examined endurance training. We used high jumping as a dynamic force velocity training to study the changes in proteolytic activity during this type of training. The results demonstrated that proteinase activity began to increase when the load, i.e. number of jumping and the weight put on the rats's backs was too much for their muscles. They could carry out the task consuming the energy originating from muscle tissue, and the type of training with this load became equal with an endurance training (Sohár et al., 1982).

Table 2

Effect of exercise on protein catabolism

Author	Species	Training	Measurement
Shott 1979	rat	running	no alteration in free cath. D activity
Salminen 1980	mouse	running	acid proteinase acivity↑
Dohm 1980	rat	swimming	alkaline proteinase activity, CANP no changes in free cath. D activity↑
Dahlmann 1981	rat	swimming	alkaline proteinase activity ↑
Rennie 1981	man	treadmile	3-Me-His excretion↓
Dohm 1982	man man rat	running weight-lifting running	3-Me-His excretion↑
Kasparek 1982	rat	running	lysosome size↑
Sohár 1982	rat	high-jumping	proteinase activity↑

The combination of stretch and training is when the animals, rats in our experiments, are kept in a small cage which prevents them from moving. The muscles are in a stretched position and in the first period the rats try to escape from the cage. We call this the active stretch model. In the present paper the results of the experiments are discussed.

After examining passive and active stretch we measured the effect of permanent contraction on proteinase activity. A widely used weed killer, 2,4-dichlorphenoxyacetate (2,4-D) disturbs the functioning of skeletal muscles. The most striking effect of 2,4-D is the development of muscular rigidity. The activities of cathepsin D, cathepsin L-like and 5 mM calcium-activated neutral proteinases (CANP) were measured in the soleus and semimembranosus muscles of rats injected with 2,4-D in a period of two and three weeks (Sohár et al., 1981).

The energy requirement of protein degradation is an intensively investigated topic nowadays. Influence of ATP on protein metabolism in mammalian tissues are shown in Table 3. In skeletal muscle ATP plays a special role during the contraction and

Table 3

Effect of ATP on protein metabolism

Author		Observation
DeMartino	1979	ATP-dependent proteinase in the liver
Dell'Antone	1979	ATP-driven proton pump in the lysosomes
Decker	1980	cathepsin D activity increases in hypoxia
Morgan	1980	inhibition of proteolysis in hypoxia
Taegtmeyer	1980	ATP increases and decreases proteolysis
Seglen	1981	protein degradation is inhibited: ATP content increases or decreases
Etlinger	1981	ATP-dependent proteinase in skeletal muscle

relaxation. When its function is suspended, the ATP content decreases during the first four weeks. We could not measure directly the activity of ATP-dependent proteinase in muscle homogenate (this enzyme was identified in reticulocytes and rat liver). Rabbits were treated (Nagy et al., 1981) with 2,4-dinitro-sec-butyl-phenol, an uncoupler used as herbicide, for 3 weeks, and proteinase activity was measured in various

organs and in skeletal muscles. Lechner et al. (1970) published that the increase in the concentration of uncoupler can reduce the level of creatine phosphate, ATP and pyruvate in isolated atria.

The nutritional changes have no obvious or immediate effect on the protein metabolism in muscle, but on closer examination the effects of feeding and starvation can be observed in the skeletal muscle, too. During starvation, higher lysosomal proteinase activity can be measured in both types of skeletal muscles on the fourth or fifth day after the beginning of the experiment. The extralysosomal proteinase activity increases only slightly, similar to that in the immobilized skeletal muscle. The proteolytic activity returns to the normal level on the seventh or eigth day (Odobasic, 1981). Feeding and starvation change the hormonal concentration, first of all insuline and glucocorticoid levels. Insulin can inhibit protein degradation. Glucose and amino acids can diminish the proteolytic activity, too. In the present study the effect of the long-term protein-free diet on proteolytic enzyme activity in muscle is shown. The effect of inhibitor concentration during fasting was described by Noguchi (1974). The concentration of alkaline proteinase inhibitor decreased during starvation and the increased alkaline proteinase activity can be explained by this lower inhibitor level. Data about other proteinase inhibitors in skeletal muscles were also published (Table 4).

Table 4

Endogenous inhibitors of proteinases in skeletal muscle

Reference		Proteinase inhibited
Noguchi	1974	alkaline proteinase
Katunuma	1977	group-specific proteinase
Bird	1977	cathepsin B
Goll	1978	calcium-activated neutral proteinase
Lenney	1979	cathepsin B and H

The investigation of inhibitor capacity on cathepsin B and serine proteinase in immobilized and dexamethasone-treated muscle will be presented.

MATERIALS AND METHODS

Male CFY rats weighing 230 g and New-Zealand rabbits weighing 2300 g were used in groups of 10 animals each. The animals were kept under normal laboratory conditions (23°C, 12 hours light cycle). The food and water uptake was ad libitum.

Limitation of moving: each rat was kept in a small cage prevented from moving. The control group was kept in normal laboratory boxes.

Treatment with 2,4-D: 2,4-dichlorphenoxyacetate (Chemical Work, Fűzfő, Hungary), 100 mg per kg body weight, was administered intraperitoneally to rats for 2 or 3 weeks.

Uncoupler effect: rabbits were treated with dinitro-sec-butyl-phenol (DNSB),(Chemical Work, Fűzfő, Hungary) in an oral dose or 10 mg/kg/day for 3 weeks.

Protein-free diet: rats were kept on a protein-free diet for 10 days, 3, 6 and 9 weeks, respectively. Instead of protein the diet contained in terms of calory an equivalent amount of fat as the food given to the control animals.

Inhibitory experiments: a.) The right hind leg of rabbits was fixed in plaster cast in full extension. After two weeks of immobilization the soleus muscle was used for measurements. b.) The rabbits received intramuscular injections of dexamethasone (Oradexon®, Organon) 2 mg/kg every second day, for a three-week period.

Samples for enzyme and inhibitor measurements: After the animals had been bled, the soleus muscle, containing mainly slow-twitch oxidative fibres, and the semimembranosus muscle, containing mainly fast-twitch glycolytic fibres, were excised and cleaned of connective and fat tissues and homogenized in an Ultra-Turrax homogenizer (Janke Kunkel). 1.) To measure total enzyme activity, the muscles were homogenized in

10 volumes of 1 % KCl and 0.1 % Triton X-100 solution. 2.) For measurements of inhibitor the muscles were homogenized in 3 volumes of 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA-Na_2. 3.) To assay "free" activity of cathepsin D, the muscle was homogenized with 1 % KCl. 4.) The "bound" cathepsin activity was determined in the precipitate No. 3 homogenate obtained after centrifugation at 2000 g, for 20 min, and the pellet was homogenized in 0.1 % Triton X-100 solution and centrifuged again. Activity of cathepsin D was determined in the clear supernatant as described by Barrett (1972). Cathepsin L activity was measured by incubating the muscle samples at 40°C for 120 min in 1.0 ml 50 mM potassium phosphate, pH 6.0, containing 0.5 % azocasein and 5 mM 2-mercaptoethanol. Azocaseinase activity was measured with azocasein, pH 6.0, in the presence of 2-mercaptoethanol. The control contained 10^{-6} M Z-Phe-Phe-CH-N_2 and 10^{-6} M pepstatin. CANP activity was assayed with azocasein substrate at pH 7.5, in the presence of 5 mM $CaCl_2$ and 5 mM 2-mercaptoethanol as activators. Chymotrypsin-like activity was measured with acetyl-Tyr-p-nitroanilide (1 mM) as substrate at pH 8.0).

To measure the inhibitory capacity the supernatants were incubated with purified cathepsin B (Barrett, 1973) and trypsin (Sigma) for 10 min. Residual cathepsin B and trypsin activities were determined with benzoyl-D,L-Arg-p-nitroanalide at pH 6.0 and pH 7.5, respectively. Protein content was determined according to Goa (1953). Alanine aminopeptidase was assayed as described by Farr et al. (1968) using Co^{2+} ions (1 mM) for activation.

RESULTS

Specific enzyme activities are usually given in percentage compared to the control. The normal enzyme activities are demonstrated in Table 5.

The activity of cathepsin D was measured in the soleus muscle of rats kept in small cages. It can be seen in Fig. 1 that the "free" activity increase fell mainly in the first

period of the experiment. The increase in the "bound" and in the total activities is not evident till the end of the second week.

Table 5

Specific enzyme activities in muscles of rabbits.
Mean values ± SD for 10 animals

Enzyme	Semimembranous muscle	Soleus muscle	Unit
Cathepsin D	6.45±0.68	10.20±0.83	$\frac{\text{nkat}}{\text{g(protein)}}$
Azocaseinolytic activity	5.02±0.41	7.90±0.52	$\frac{\text{mg(azocasein)}}{\text{sec x g(protein)}}$
CANP	1.09±0.09	0.90±0.08	$\frac{\text{mg(azocasein)}}{\text{sec x g(protein)}}$
Chymotrypsin-like (Cathepsin G-like)	1.76±0.8	1.68±0.13	$\frac{\text{nkat}}{\text{g(protein)}}$

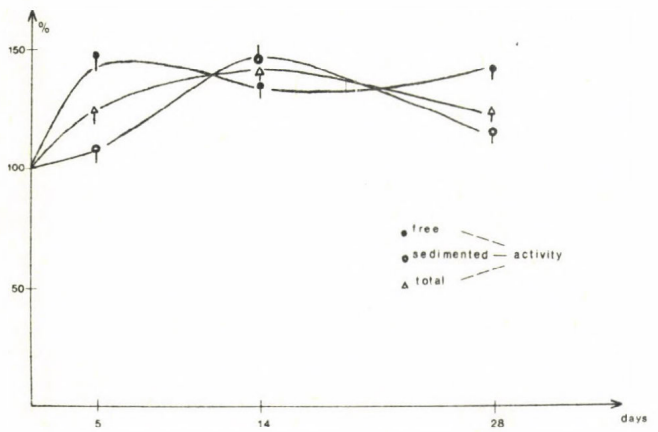

Fig. 1: Effect of active stretch on the activity of cathepsin D in the soleus muscle of rats. The values are percentages taking the controls as 100 percent.

The effect of 2,4-dichlorphenoxyacetate on proteinase activities in the semimembranous muscle of rats can be seen in Fig. 2. The activity of cathepsin D increased only in the second week. The cathepsin L-like activity increased significantly in the second week of the experiment and it further increased in the third week. The calcium-activated neutral proteinase activity was elevated in the second week, but it returned to the normal level in the third week. In the soleus muscle only moderate or negligible changes in proteinase activity could be observed.

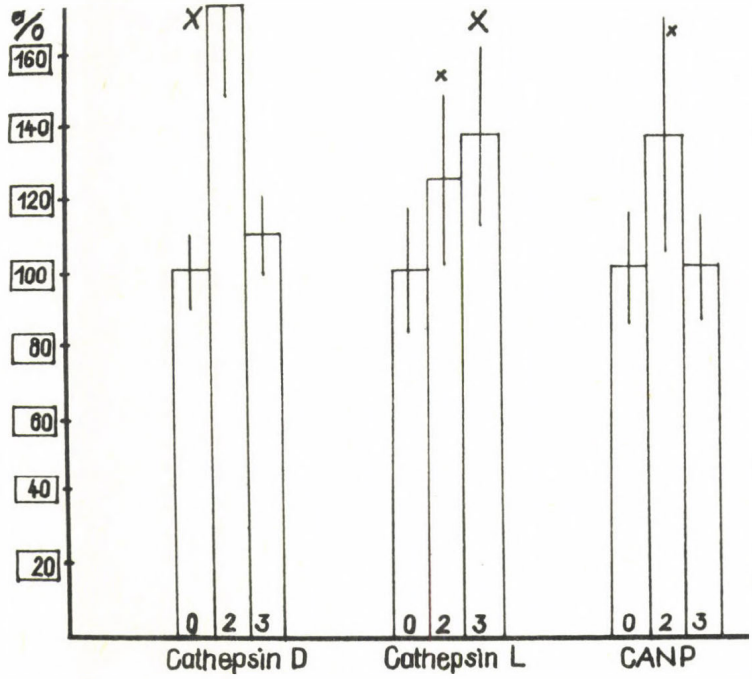

Fig. 2: Proteinase activities in the semimembranous muscle of rats treated with 2,4-dichlorphenoxyacetate. 0 - normal value, 2 - two weeks' treatment, 3 - three weeks' treatment, x - $p<0.05$

The activity of proteinases increased in the semimembranous muscle of rabbits treated with an uncoupler (Fig. 3). Cathepsin D showed the highest activity. The activities of

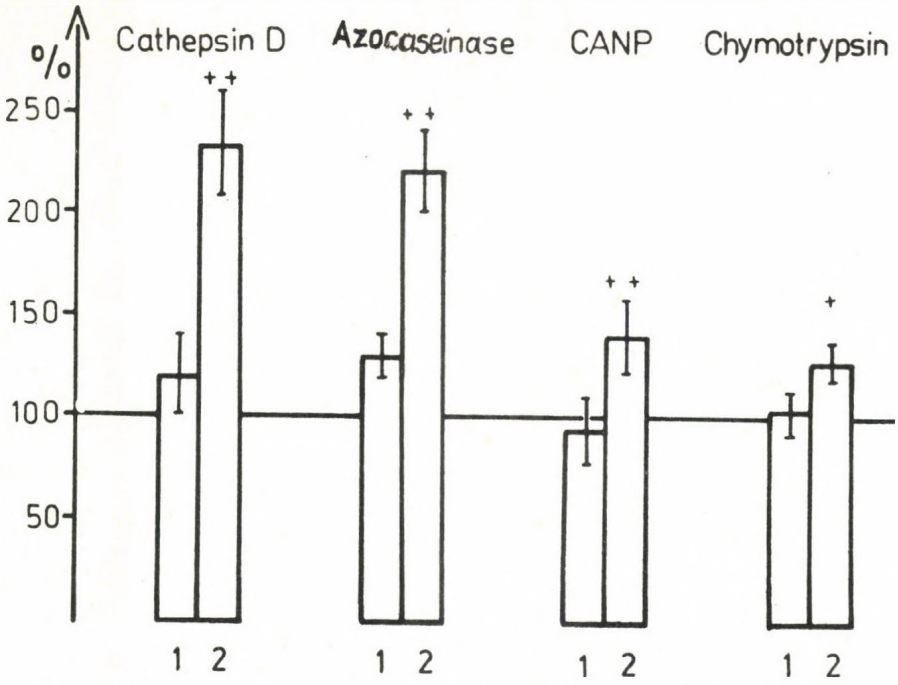

Fig. 3: Changes in the proteinase activities of skeletal muscles after 2,4-dinitro-sec-butyl-phenol administration. 1 - soleus muscle, 2 - semimembranous muscle + - $p<0.05$, ++ - $p<0.01$; CANP - calcium activated neutral proteinase.

the two extralysosomal enzymes increased less than the lysosomal enzyme activity. The proteinase activity in soleus muscle did not change significantly compared to the control.

During a long-term protein-free feeding both the lysosomal proteinase activity and the calcium-activated neutral proteinase activity increased in the soleus muscle. The alanine aminopeptidase and cathepsin G-like (chymotrypsin-like) activities slightly decreased. The cathepsin L-like activity was highest in the sixth week and the calcium-activated neutral proteinase activity was highest at the end of the experiment (Fig. 4)

Table 6 shows the effect of inhibitors. The inhibitor binding capacity of serine proteinases is higher in the immobilized soleus and in the steroid-treated semimembranous muscles. The cathepsin B inhibitor level increased slightly

in the immobilized muscles and in the steroid-treated soleus muscle.

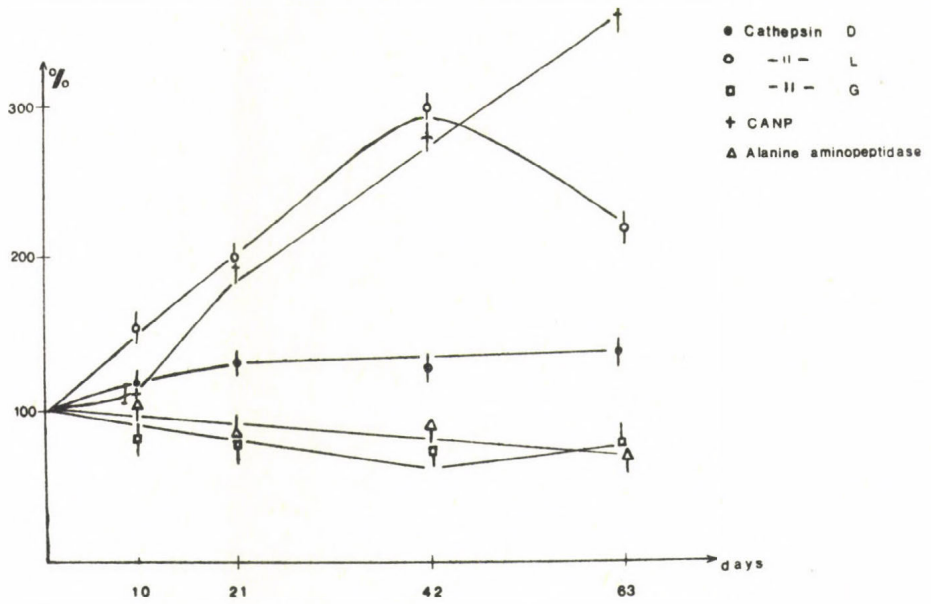

Fig. 4: Proteolytic activities in the soleus muscle of rats kept on long-term protein-free diet. The values are percentages of the control value.

Table 6

Changes in inhibitor binding capacities after immobilization and dexamethason treatment

Muscle	Trypsin inhibitor		Cathepsin B inhibitor	
	Control %	Immobilized %	Control %	Immobilized %
Soleus	100± 8.5	142 ±12.1	100± 5.6	122.6±6.2
Gastrocnemius	100± 7.6	109.6± 9.2	100± 9.1	119.3±8.3
	Control %	Steroid--treated %	Control %	Steroid--treated %
Soleus	100±10.6	104.6±11.2	100±10.1	118.1±9.8
Semimembranous	100±11.4	125 ±10.3	100±11.5	110.1±8.8

DISCUSSION

The increased proteolytic activity in skeletal muscles was studied in the present paper and the first question concerned the influence of stretch and immobilization. Till 1980 the general view had been that protein degradation increased with the decreased stretch and protein degradation decreased when the muscle was in a lenghtened position. Etlinger and Millward (1980) reported that the alteration of the normal length or sarcomer caused faster protein degradation. The effect of exercise assisted these effects. Vihko and Salminen (1980) observed higher cathepsin D activity in the muscle of running mice. They explained that the muscle in the running mouse is similar to the ischemic muscle. In our experiment the free part of cathepsin D activity increased in the first step. Dohm et al. (1980) found similar results in swimming rats. The stretched state promotes the degradation (or permeability) of the cell membrane. On the other hand, this increase in the proportion of free lysosomal proteinase activity resembles the alteration found by Decke and Wildenthal (1980) in ischemic heart where the lysosomal enzymes leak out from the lysosomes into the cytoplasm. Thus, leakage could play a role in the increased degradation during exercise. In the second step there is immobilization indeed and the proteinases activated earlier could activate the newly synthesized proteinases. Protein synthesis decreased during "shortened state" immobilization and the activated proteinases could not find new proteinases. The hypothetical process of activation in immobilized muscle is shown in Fig. 5. The experimental model of myotonia showed the same results as the proteinase activities increased in this muscle, too. However, the question remains the same: which is the first step in the activation process. The acidification, reduced redox potential, decreased insulin concentration, increased glucocorticoid content, etc., each can also cause higher proteinase activity in the skeletal muscle. In the immobilized or 2,4-dichlorphenoxy-acetate-treated muscle these causes are acting simultaneously. This is the problem with the

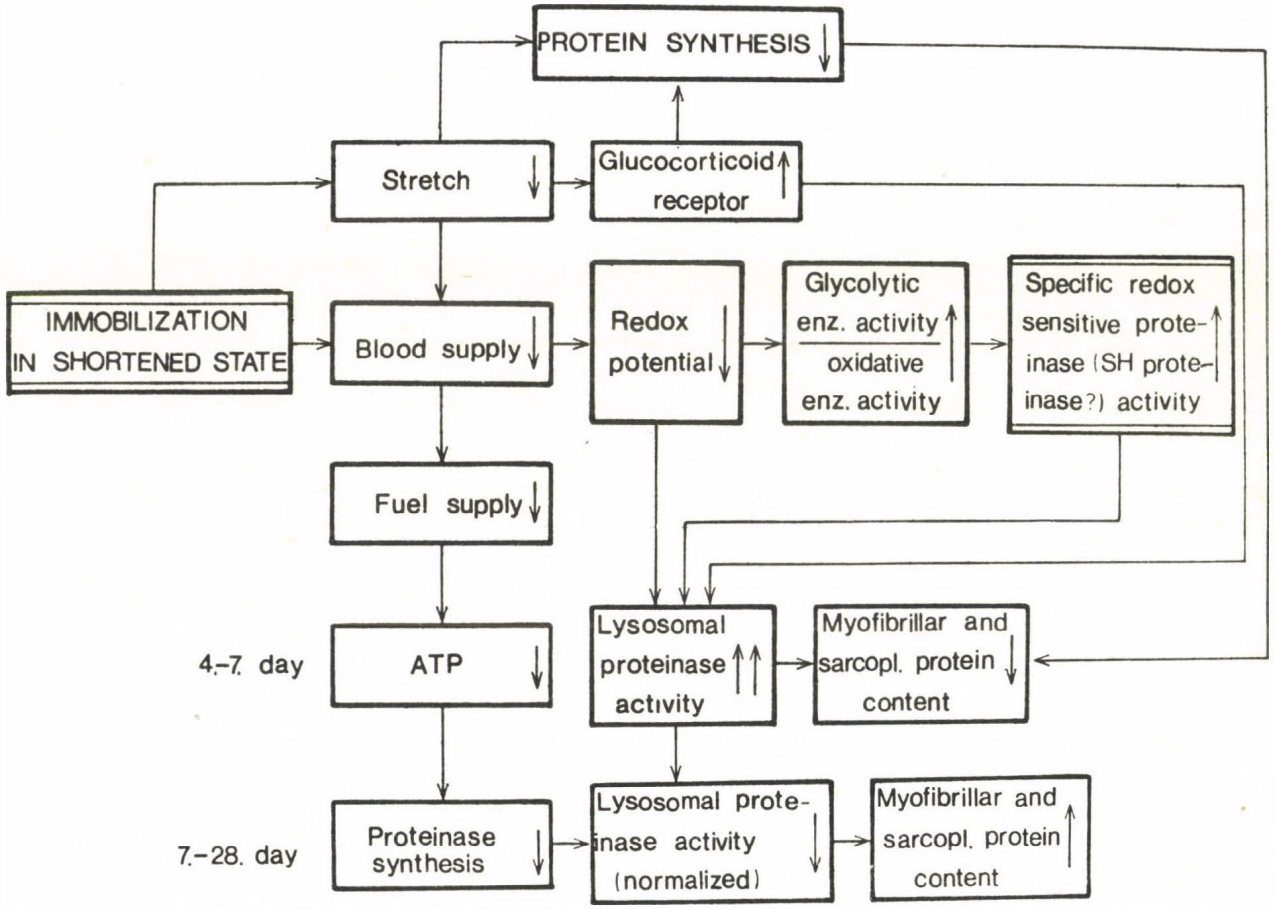

Fig. 5: Hypothetical processes during immobilization in shortened state.

uncoupler-treated animals, too, because the effect of uncoupler was shown in the isolated atria, but the uncoupler may cause local reduction in pH (by H^+/Ca^{2+} exchange) and thereby promote lysosomal breakdown (Duncan et al., 1980). In this case we can examine only simultaneous phenomena. The decrease in ATP concentration in the immobilized muscle is not an evidence for the role in proteinase activation. The various results are shown in Table 3.

During immobilization there is a reduced fuel supply in the muscles. Among other materials the protein supply also decreases. The protein-free diet could cause, beside the higher lysosomal proteinase activity, higher calcium-activated neutral proteinase, too. We may say that this factor also could play a role in proteinase activation in the immobilized skeletal muscle.

Many authors (see Table 4) found that endogenous proteinase inhibitors in the skeletal muscles play a role in the inactivation of proteinases, and their leakage allows the proteinase to act freely in the cell. We measured a total proreinase capacity with cathepsin B and trypsin. In both cases a lot of compounds can inhibit the same proteinase. During protein degradation amino acids and small peptides are produced in the muscle. For example, leucine was shown to be an inhibitor of protein degradation. In our experiments the protein-inhibitor capacities did not decrease. In immobilized muscles a higher level of trypsin inhibitor was measured, and this could explain why the serine proteinase activity did not increase in these muscles. In future specific proteinase inhibitors such as those reported by Lenney (1979) are to be determined, and they might be found to play a physiological role in proteinase activation in the skeletal muscle.

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DISCUSSION

WIEDERANDERS:

Is there anything known about the effect of hypoxic conditions on proteinases in other organs than muscle?

SOHÁR:

During starvation the lactate/piruvate ratio increases in the liver and it is known that in this case the protein degradation is faster in the liver. There are other papers on the changes of proteolytic activity in blood (enzymes of coagulation, leucocytic proteinases), when the animals are kept under hypoxic conditions. In our experiments skeletal muscle was studied, since these muscles have a similar function but their oxidative metabolism (red fiber content) differ from each other. Results on heart muscle are described in details in our paper.

PROTEINASE INHIBITORS

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THE PAPAIN SUPERFAMILY OF CYSTEINE PROTEINASES AND THEIR PROTEIN INHIBITORS

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The proteinases of the papain superfamily are of physiological, medical and nutritional importance, and in all of these connections it is necessary to know what controls exist over their activity in the human body. Four distinct human proteins have now been found to provide potent inhibition of these enzymes in the blood, tissues and cells. In this article we propose to start by commenting on some of the exciting results recently published by others on the membership of the papain superfamily of enzymes. We shall then briefly review knowledge of the protein inhibitors of these enzymes, with emphasis on work from our own laboratory, under three headings: alpha$_2$M, alpha-cysteine proteinase inhibitor, and the cystatin-like inhibitors.

THE PAPAIN SUPERFAMILY

Dayhoff et al. (1983) has defined a "superfamily" of proteins as a group for which statistical analysis of the amino acid sequences shows a less than one in a million probability of evolutionary relationship with other proteins. The best known of the cysteine proteinases (EC 3.4.22, previously called "thiol" proteinases) is papain, and the majority of the known cysteine proteinases fall into the superfamily of proteins homologous with papain. Sequence data shows that these include chymopapain, papaya peptidase, ficin, bromelain, the asclepains, actinidin, cathepsin B and cathepsin H (Fig. 1). Other evidence establishes beyond reasonable doubt that the superfamily also includes cathepsin L and calotropin. Thus, the molecular and enzymic properties of cathepsin L are very similar to those of the other lysosomal cysteine proteinases and papain (Barrett & Kirschke, 1981), and the tertiary structure of calotropin, from the madar plant Calotropus gigantea, determined by X-ray diffraction (Heinemann et al., 1982) shows the positions of the main-chain alpha-carbon atoms and of some residues that have been identified at the catalytic site so as to leave no doubt that this enzyme has essentially the same structure and catalytic mechanism as papain.

On the basis of sequence and other data, it seems probable that there are at least four other superfamilies of cysteine proteinases represented by the streptococcal cysteine proteinase(Tai et al., 1976), clostripain (Gilles et al., 1983), the calpains (Murachi, 1983), and the processing proteinase of poliomyelitis virus (Korant, in the present volume). If this is so, the cysteine proteinases are a great deal more diverse than the serine proteinases.

	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4
Papain	I P E - Y V D W R Q K - G A V T P - V K N - - - Q G S C G S
Cathepsin B (rat)	L P E S F - D A R E - Q W S N C P T I A Q I R D Q G S C G S
Cathepsin B (beef)	L P E S F - D A R E - Q W P N C P T I K E I R D Q G S C G S
Cathepsin H (rat)	Y P S S - M D W R K K - G N V V S P V K N - - - Q G A C G S
Cathepsin S/L(beef)	L P D S - V D W R E K - G G V T P - V K Y - - - Q G A C G S
Papaya Peptidase A	L P E N - V D W R K K - G A V T P - V R . . .
Papaya Peptidase B	L P E S - V D W R A K - G A V T P - V K . . .
Chymopapain B	Y P E S - I D W R A K - G A V T P - V K,R V P D - - S - G E
Asclepain A	L P N S - I D W R Q K N - V V F P - I K N - - - Q G G . . .
Asclepain B	L P N - F V D W R - K N G V V F P - I R N - - - Q G Q . . .
Actinidin	L P - S Y V D W R S A - G A V V D - I K S - - - Q G E C G G
Ficin	. . . P - I R Q - - - Q G Q C G S
Bromelain	V P Q S - I D W R D Y - G A V T - S V K N - - - Q N P C G A
Conserved	P D R Q C G

	5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2
Papain	C W A F S A V V T I E G I I K I R T G N L N Q - Y S - E Q E
Cathepsin B (rat)	C W A F G A V E A M S D R I C I H T - N/V N V E V S A E - D
Cathepsin B (beef)	C W A F G A V E A I S D R I C I H S - N . . .
Cathepsin H (rat)	C W T F S T T G A L E S A V A I A S G K M M T - L - A E Q Q
Cathepsin S/L(beef)	C W A F S A V V L A Q . . .
Chymopapain B	C W . . .
Actinidin	C W A F S A I A T V E G I N K I T S G S L I S - L S - E Q E
Ficin	C W . . .
Bromelain	C W G F A A I A T V E S V A S I Y K G I L Q P - L S - Q Q Q
Conserved	C W F I

	3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8
Papain	L L D C D R - - R S Y G C N G G Y P W S A L Q L V A Q - - Y
Cathepsin B (rat)	L L T C C G I Q C G D G C N G G Y P S G A G N F W T R K L V
Cathepsin H (rat)	L V D C A Q N F N N H G C Q G G L P S Q A F E Y I - - - L Y
Actinidin	L I D C G R T Q N T R G C D G G Y I T D G F Q F I - - - I N
Bromelain	V D D C A K . . .
Conserved	C G C G G

(Continued)

```
                          8                                                   9
                          9 0 1 2 3 4 5         6 7 8 9                       0 1 2 3

Papain                    - - G I H Y R N T - - - - - P Y Y E - - - - - - - - G V Q R
Cathepsin B (rat)         S G G V - Y - N S H I G C L P Y T I P P C E H H V N G S R P
Cathepsin H (rat)         N K G I M G E D S - - - - Y P Y - I - - - - - - - - G K N G
Actinidin                 D G G I - - - N T - - - D E N Y T A Q D - - - - - - G D - -

  Conserved                  G                       Y                       G


                                    10
                          4 5 6 7 8   9 0 1 2                 3 4 5           6 7 8 9

Papain                    Y C R S R - E K G P - - - - - - - - - Y A A - - - - - K T D G
Cathepsin B (rat)         P C T G E G D T P K C N K M C E A G Y S T S Y K E D K H Y G
Cathepsin H (rat)         Q C K F N P E K - - - - - - - - - V A F V K N V V N I - T L -
Actinidin                 - C D V A L Q D Q K - - - - - - - - - Y V T - - - - - - I D T

  Conserved                  C


                        11                          12                          13
                          0 1 2 3 4 5 6 7 8 9   0 1 2 3 4 5 6   7 8 9 0 1 2 3 4   5 6

Papain                    V R Q V Q P Y N Q G - A L L Y S I A - N Q P V S V V L - Q A
Cathepsin B (rat)         Y T S Y S V S E S E K E I M A E I Y K N G P V E G A F - T V
Cathepsin H (rat)         - - - - - - - N D E A A M V E A V A L Y N P V S F A F - E V
Actinidin                 Y E N V - P Y N N E W A L Q T A V T - Y Q P V S V A L - D A
Bromelain                 . K A R V P R N N E - S S M Y A V S - K Q P I T V A V V S A

  Conserved                                                       P


                              14                          15                    *16
                          7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2   3 4 5     6 7 8 9 0 1 2

Papain                    A G K D F Q L Y R G G I F V G P - C G N - - - K V D H A V A
Cathepsin B (rat)         F - S D F L T Y K S G V Y K H E A - G D V - - M G G H A I R
Cathepsin H (rat)         T - E D F M M Y K S G V Y S S N S C H K T P D K V N H A V L
Actinidin                 A G D A F K Q Y A S G I F T G P - C G T A - - - V D H A I V
Bromelain                 A - - N F(Z,L,Y)K S G V G D G Y - C K D - - - K L N H A V T

  Conserved                     F     Y     G                               H A
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(Continued)

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                                          17          *   *   18
                  3 4 5 6 7 8   9         0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8

Papain            A V G Y G P - N - - - Y I L I K N S W G T G W G E N G Y I R
Cathepsin B (rat) I L G W G I E N G V P Y W L V A N S W N V D W G D N G F F K
Cathepsin H (rat) A V G Y E - Q/N G L L Y W I V K N S W G S N W G N N G Y F L
Actinidin         I V G Y G T E G G V D Y W I V K N S W T T T W G E E G Y M R
Bromelain         A I G Y N K . .                     . . K A R W G E A G Y I R

 Conserved            G                               N S W       W G


                   19                        20                        21
                  9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1     2

Papain            I K R G T G N S Y G V C G L Y T S S F Y P V K - - N
Cathepsin B (rat) I L R G E N H - - - - C G I E S E I V A G I P R T Q
Cathepsin H (rat) I E R G K N M - - - - C G L A A C A S Y P I P Q V
Actinidin         I L R N V G G A - G T C G I A T M P S Y P V K Y N N
Bromelain         M A R D V S S S S G I C G I A I D P L Y P T E G

 Conserved            R                 C G
```

Fig. 1. Comparison of amino acid sequences of cysteine proteinases of the papain superfamily. The data are taken from the original publications cited below, and the sequences are numbered to correspond to that of papain. Cys-25 and the other residues thought to be intimately involved in catalytic activity (Lowe, 1976) are marked with asterisks. Dots indicate that a sequence continues. Oblique strokes show the points of limited proteolysis in cathepsins B and H. References: papain (Dayhoff, 1972), cathepsin B (rat) and cathepsin H (rat) (Takio et al., 1983), cathepsin B (beef) (Pohl et al., 1982), cathepsin L/S (beef) (Turk et al., 1983), papaya peptidases A and B (Lynn & Yaguchi, 1979), chymopapain B (Brocklehurst et al., 1981; Lynn & Yaguchi, 1979), asclepains A and B (Lynn et al., 1980), actinidin (Takio et al., 1983), ficin (Friedensohn & Liener, 1972; Brocklehurst et al., 1981) and bromelain (Goto et al., 1976; Goto & Murachi, 1980).

In general terms, one can say that the proteinases of the papain superfamily are proteins of about 25,000 molecular weight containing one essential thiol group (and sometimes, as in chymopapain, a non-essential one) as well as several disulphide bonds. Bromelain and the cathepsins are glycoproteins, whereas most of the other plant enzymes are not. There have been recent reviews of the properties of papain, chymopapain and papaya peptidase (Brocklehurst et al., 1981), bromelain (Cooreman et al., 1976), the cathepsins (Barrett & Kirschke, 1981) and the mechanism of action of the cysteine proteinases in general (Lowe, 1976; Polgar & Halasz, 1982).

In Fig. 1 we bring together all of the sequence data we are aware of for the enzymes homologous with papain. The sequences of papain, actinidin, and cathepsins B and H from rat liver are complete, and that of bromelain is over 80% complete, but the others are shorter N-terminal or active site sequences. In order to make the best matches, gaps have been inserted into some of the sequences, and these are not necessarily where they were indicated by the original authors cited in the legend, but where they appear most reasonable to us in view of all of the data available.

We have considered the sequence data of Fig. 1 in conjunction with a molecular model of papain constructed according to the coordinates determined by Drenth et al. (1968). Parts of the polypeptide chain that lie away from the active site cleft in the folded proteins show numerous differences in sequence, but there is a good deal of conservation of residues in the active site cleft. In this part of the molecules there are substitutions that have prompted us to speculate about the reasons for some of the differences in substrate and inhibitor specificity between the enzymes. These inevitably depend upon the assumption that all of the enzymes fold in very much the same way as papain, and this may not always be true.

With reference to the catalytic mechanism, it is notable that Asp-158 which was proposed by Angelides & Fink (1979) to play a significant role in activity of papain is replaced by glycine in cathepsin B, and is perhaps therefore not vitally important. Rat cathepsins B and H have sites of limited proteolysis towards the N- and C-terminals, respectively (Takio et al., 1983), and rat liver cathepsin L also is cleaved towards the C-terminus (Kirschke in the present volume). Since conserved residues, some of which are known to be important in the catalytic mechanism, tend to be clustered near both ends of the molecules, it would be surprising if any of the separated "heavy chains" were active.

The S_2 subsite of papain (in the nomenclature of Schechter & Berger 1968) is the dominant site in the specificity of the enzyme for low molecular weight substrates. The position of this site, which binds hydrophobic residues and consists of a fairly deep pocket binding the P_2 side chain of the substrate, has been identified by Drenth et al. (1976). The beta-carbon of the side chain is in contact with the residues Pro-68 and Ala-160. Val-133 and Val-157 form the end of the pocket. In cathepsin B, Val-133 is replaced by glycine and Val-157 probably by methionine (there is insufficient correspondence in this region to be absolutely certain). The replacement of valine by glycine may well create a gap at the end of the pocket. In the papain model the side-chain of Ser-205 is very close to the end of this pocket, whereas in cathepsin B, it is substituted by glutamic acid. Clearly, this substitution could explain why cathepsin B binds both X-Phe-Arg- and X-Arg-Arg- substrates well (Barrett & Kirschke, 1981). Unlike the S_1 subsite of trypsin, the end of the S_2 pocket of cathepsin B would be at the surface of the molecule, and hence it would not be essential that a basic residue be placed in this site, but it could be favourably accommodated with no severe size restriction. Rather more speculatively, the high activity of cathepsin B towards triple basic

substrates (e.g. X-Lys-Lys-Arg- substrates: MacGregor et al., 1979) could be explained by the fact that Tyr-61 of papain is replaced by aspartic acid. Drenth (1976) identifies Tyr-61 as a hydrophobic binding site for the P_3 side chain in papain.

In their paper on the sequence of cathepsins B and H, Takio et al. (1983) reasonably suggest that the change of Ala-162 to arginine in cathepsin B could explain the ability of cathepsin B to act as a peptidyl dipeptidase. The P_2' binding site of papain has not been identified, but it would certainly approach residue 162 which is within the active-site cleft. The side chain of Ala-162 in papain, however, is turned in towards the hydrophobic core of the protein.

Cathepsin H shows a substitution of Tyr-61 by histidine, which might give the enzyme a P_3 specificity towards acidic residues. The most notable feature of cathepsin H, however, is that it shows strong aminopeptidase activity. Gln-135 of papain is close to the P_1 peptide - NH of the substrate. In cathepsin H the equivalent residue seems to be glutamic acid, which could account for the specificity by binding the positively charged free amino terminus. Once again, however, the specificity would not be obligatory, as this residue is on the surface of the molecule.

Table 1 summarizes the extent of difference between the sequences, on the basis of which we have constructed the phylogenetic tree - Fig. 2. The numbers of PAM's between the enzymes indicate that papain and actinidin are the most closely related, cathepsin H is more distant from these plant enzymes, and cathepsin B is remote from the plant enzymes and from cathepsin H.

Table 1. Numbers of differences between pairs of sequences. The comparisons were made on the basis of the alignments in Fig. 1, 100% non-identity corresponding to 266 differences (the complete length of the alignment). The numbers of accepted point mutations (PAM's) were obtained from the numbers of differences by use of the table supplied by Dayhoff (1978).

Sequences compared		Non-identical	Difference (%)	PAM's
Papain	Cathepsin B	190	71	166
Papain	Cathepsin H	160	60	112
Papain	Actinidin	126	47	70
Cathepsin B	Cathepsin H	189	71	166
Cathepsin B	Actinidin	185	70	159
Cathepsin H	Actinidin	159	60	112

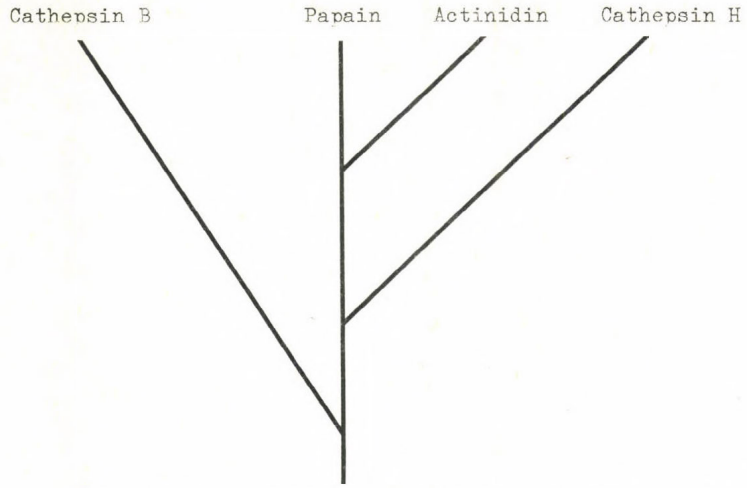

Fig. 2. Simple phylogenetic tree based on the differences in sequence between papain, actinidin and cathepsins B and H.

INHIBITORS

Many of the papain-type cysteine proteinases impinge upon human physiology and pathology. Thus, papain is used in the tenderizing of meat, and in other aspects of food processing. Bromelain has been used pharmaceutically (Cooreman et al., 1976), and chymopapain is an important agent for the treatment of herniated intervertebral discs. Several human parasites and pathogens contain cysteine proteinases that probably belong to the papain group: these include species of Leishmania (Coombs, 1982) and Schistosoma (Dresden et al., 1982). Cathepsins B, H and L are responsible for most of the proteolytic activity of lysosomes, which means that they mediate a significant proportion of intracellular protein degradation, and also are implicated by strong circumstantial evidence in the extracellular destruction of connective tissue matrices. For these reasons, it is important to understand the mechanisms that exist for regulation of the activity of proteinases of the papain superfamily in the human body.

ALPHA$_2$-MACROGLOBULIN

Alpha$_2$-macroglobulin (alpha$_2$M) was the first protein clearly characterized as an inhibitor of cathepsin B and related cysteine proteinases. An inhibitor of cathepsin B in human serum was found to inhibit the action of the enzyme on high molecular weight protein substrates more than that on small synthetic substrates. The inhibitor was purified and identified as alpha$_2$M (Starkey & Barrett, 1973). Working in an attempt to explain the curious effect of substrate molecular weight on the inhibition of cathepsin B and other proteinases by alpha$_2$-macroglobulin, we hit upon the "trap hypothesis" for the action of this protein (Barrett & Starkey, 1973; Starkey & Barrett, 1977). This idea has been modified in detail over the years, but we would now express it as follows. The alpha$_2$M molecule, a 725,000

molecular weight tetramer of identical subunits comprises two half-molecules, each independently capable of binding one proteinase molecule. The interaction is initiated by the active proteinase cleaving the sensitive "bait region" of one of the subunits (Fig. 3). For this reason, catalytically inactive forms of the proteinases do not interact, and in this respect alpha$_2$M differs sharply from all of the tight-binding protein inhibitors described below. Cleavage of the bait region in one subunit causes a rapid conformational change in it and its partner in the half-molecule, such as physically to trap the enzyme molecule within the macroglobulin. Large molecules, whether substrates, inhibitors or antibodies are then shut out, and the enzyme molecule is unable to react with them. The complexes have the distinctive characteristic of being active against low molecular weight substrates, however. The conformational change in the first half-molecule is transmitted to the second, which may "close" without trapping an enzyme molecule, but under favourable conditions a binding ratio of 2:1 is achieved. The very high rate of reaction of papain with alpha$_2$-macroglobulin is conducive to this (Howell et al., 1983).

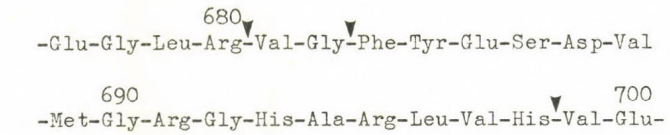

Fig. 3. The "bait region" of alpha$_2$M, showing the points susceptible to cleavage by papain.
Papain readily cleaves the native protein at any of these points, triggering the conformational change which traps the enzyme molecule (Mortensen et al., 1981; Sottrup-Jensen et al., 1983).

Some of the molecules of papain and other proteinases that have become trapped by the alpha$_2$M molecule also become covalently linked to the macroglobulin molecule (Salvesen & Barrett, 1980, 1981). The physiological significance of this is uncertain, but what almost certainly is of physiological significance is the very rapid clearance of alpha$_2$M-proteinase complexes from the circulation by the action of a macrophage cell-surface receptor specific for the special conformation of alpha$_2$M after it has complexed proteinase molecules (Starkey & Barrett, 1977). The concentration of alpha$_2$M in the plasma is about 3.4 micromolar, and this could inhibit up to 6.8 micromolar proteinase. The very high molecular weight of alpha$_2$M prevents it from escaping from the circulation except as a result of greatly increased vascular permeability at sites of inflammation.

Proteinases of the papain superfamily that are known to be trapped by alpha$_2$M include papain, ficin, bromelain, Schistosoma cathepsin B-like proteinase and cathepsins B, H and L. As yet, there is no reason to think that any proteinase of this group will be found not to be trapped by alpha$_2$M.

ALPHA-CYSTEINE PROTEINASE INHIBITOR

Alpha-cysteine proteinase inhibitor (alpha-CPI) of human plasma was first described by Sasaki et al. (1977), and further work by Ryley (1979) and Sasaki's group (Sasaki et al., 1981; Taniguchi et al., 1981) showed that the protein occurs in relatively low and high molecular weight forms in plasma, with alpha$_2$- and alpha$_1$- electrophoretic mobilities, respectively. Järvinen (1979) described the purification of two inhibitors of cysteine proteinases from human skin by use of affinity chromatography on a form of immobilized papain; the higher molecular weight form of these was almost certainly alpha-CPI derived from the blood.

In our work (Gounaris et al., 1983) alpha-CPI has been purified by a two-stage method: affinity chromatography on (S-carboxymethyl-papain)-Sepharose, and high resolution anion-exchange chromatography. The protein was obtained as a low molecular weight form (molecular weight about 64,000) and a high molecular weight form (molecular weight about 95,000). An antiserum was raised against alpha-CPI, and "rocket" immuno-assays showed the mean concentration in sera from 19 individuals to be 35.9mg/dl. Both forms of alpha-CPI were confirmed to be sialoglycoproteins by the decrease of electrophoretic mobility following treatment with neuraminidase. alpha-CPI was shown immunologically to be distinct from antithrombin III and alpha$_1$-antichymotrypsin, two serine proteinase inhibitors from plasma with somewhat similar molecular weight values. Neither these proteins nor haptoglobin showed any affinity for the (Cm-papain)-Sepharose column, but curiously kallikrein (or prokallikrein) was efficiently bound from plasma. alpha-CPI also was immunologically distinct from the two intracellular low molecular weight cysteine proteinase inhibitors from human liver.

Complexes of alpha-CPI with papain and S-carboxymethyl-papain were detectable in immunoelectrophoresis and in pore-limit gel electrophoresis, but dissociated to free enzyme and intact inhibitor in SDS polyacrylamide gel electrophoresis. The stoichiometry of binding of papain was close to 1:1 for both low and high molecular weight forms. alpha-CPI was found to be a tight-binding inhibitor of papain and human cathepsins H and L (see Table 2). By contrast, inhibition of cathepsin B was much weaker, and dipeptidyl peptidase I also was weakly inhibited.

Digestion of alpha-CPI with bromelain gave rise to an inhibitory fragment of molecular weight about 20,000, which was isolated. The properties of this fragment, and the nature of the high molecular weight form(s) of alpha-CPI are under further investigation.

CYSTATIN AND SIMILAR INHIBITORS

Cystatin. The protein from chicken egg-white that inhibits cysteine proteinases, and has been named "cystatin", was purified and characterized by Anastasi et al. (1983). The purification was by ovomucin precipitation, affinity chromatography on (S-carboxymethyl-papain)-Sepharose, and chromatofocusing. The final purification step separated two major forms of the protein (pI 6.5 and 5.6), with total

recovery of about 20% from the egg-white. By use of affinity chromatography and immunodiffusion it was shown that the inhibitor is also present at low concentrations in the serum of male and female chickens. Tryptic peptide maps of the separated forms 1 and 2 of egg white cystatin were similar except in one peptide, and each form had the N-terminal sequence Ser-Glx-Asx. The two forms showed complete immunological identity, and neither contained carbohydrate.

Enzyme	Inhibitor			
	Liver CPI-A	Liver CPI-B	Cystatin	alpha-CPI
Papain	1.9×10^{-11}	1.2×10^{-10}	$<5.0 \times 10^{-12}$	3.4×10^{-11}
Cathepsin B (human)	8.2×10^{-9}	7.3×10^{-8}	1.7×10^{-9}	3.5×10^{-7}
Cathepsin H (human)	3.1×10^{-10}	5.8×10^{-10}	6.4×10^{-11}	1.1×10^{-9}
Cathepsin L (human)	1.3×10^{-9}	2.3×10^{-10}	1.9×10^{-11}	3.7×10^{-11}
Dipeptidyl peptidase I (bovine)	3.3×10^{-8}	2.3×10^{-10}	2.0×10^{-10}	1.3×10^{-7}

Table 2. K_i values for CPI-A and CPI-B from human liver, egg white cystatin and human plasma alpha-CPI. CPI-A was the "5.2" form, and the CPI-B the "8.1" form (Green et al., 1983). Egg white cystatin was mixed forms 1 and 2 (Anastasi et al., 1983; M. J. H. Nicklin, unpublished results). alpha-CPI was the low molecular weight form (Gounaris et al., 1983).

K_i values for the inhibition of papain, cathepsins B, H and L, and dipeptidyl peptidase I by cystatin in comparison to the other protein inhibitors are given in Table 2. Some other cysteine proteinases, and several non-cysteine proteinases were found not to be significantly inhibited by cystatin. Cystatin complexes with active cysteine proteinases and with the inactive derivatives formed by treatment of papain with iodoacetate, E-64 [L-trans-epoxysuccinyl-leucyl-amido(4-guanidino)butane] and Z-Phe-Ala-CHN_2 were demonstrated by isoelectric focusing and cation exchange chromatography. The complexes dissociated in SDS gel electrophoresis (with or without reduction) with no sign of fragmentation of the inhibitor. Cystatin was found not to contain a free thiol group, and there was no indication that disulphide exchange plays any part in the mechanism of inhibition.

The complete amino acid sequence of cystatin was established by Schwabe et al. (1983). As is shown in Fig. 4, cystatin is composed of 116 amino acid residues, and the molecular weight can be calculated as

13,143. No significant similarity to any other known sequence has been detected, and cystatin probably represents a new superfamily. The four cysteine residues form two disulphide bonds. The results of computer analysis of the sequence and circular dichroic spectrometry indicate that the secondary structure includes relatively little alpha-helix (about 20%) and that the remainder is mainly beta-structure (Fig. 4).

```
                    5                   10                  15
H-Ser-Glu-Asp-Arg-Ser-Arg-Leu-Leu-Gly-Ala-Pro-Val-Pro-Val-Asp-

                   20                  25                  30
-Glu-Asn-Asp-Glu-Gly-Leu-Gln-Arg-Ala-Leu-Gln-Phe-Ala-Met-Ala-

                   35                  40                  45
-Glu-Tyr-Asn-Arg-Ala-Ser-Asn-Asp-Lys-Tyr-Ser-Ser-Arg-Val-Val-

                   50                  55                  60
-Arg-Val-Ile-Ser-Ala-Lys-Arg-Gln-Leu-Val-Ser-Gly-Ile-Lys-Tyr-

                   65                  70                  75
-Ile-Leu-Gln-Val-Glu-Ile-Gly-Arg-Thr-Thr-Cys-Pro-Lys-Ser-Ser-

                   80                  85                  90
-Gly-Asp-Leu-Gln-Ser-Cys-Glu-Phe-His-Asp-Glu-Pro-Glu-Met-Ala-

                   95                 100                 105
-Lys-Tyr-Thr-Thr-Cys-Thr-Phe-Val-Val-Tyr-Ser-Ile-Pro-Trp-Leu-

                  110                 115
-Asn-Gln-Ile-Lys-Leu-Leu-Glu-Ser-Lys-Cys-Gln-OH
```

Fig. 4. Amino acid sequence of egg white cystatin. The sequence of chicken egg white cystatin form 1 as determined largely by automatic sequencing methods (Schwabe et al., 1983).

Recent work on the interaction of cystatin with cysteine proteinases and dipeptidyl peptidase I (M. J. H. Nicklin and A. J. Barrett, unpublished results) has shown that cystatin is a tight-binding but reversible inhibitor of these enzymes, that binds to the catalytic site competitively with substrate, so as to mask the chemical reactivity of the active site thiol and to quench the fluorescence of a nearby tryptophan residue (Trp-177) in papain. Contrary to what has been thought, the inhibitory site for dipeptidyl peptidase I is very close to, or identical with, that for the proteinases, so that they compete. Work is in progress to identify the "active site" region in the sequence of cystatin.

Human liver cellular cysteine proteinase inhibitors. There have now been many reports of low molecular weight (12,000-15,000) proteins that inhibit cysteine proteinases being found in tissues and cells from man and other species (reviewed by Green et al., 1983).

In the work of Green et al. (1983) cysteine proteinase inhibitor (CPI) forms from human liver have been purified from the tissue homogenate by alkaline denaturation of complexing cysteine proteinases, acetone fractionation, affinity chromatography on (S-carboxymethyl-papain)-Sepharose and chromatofocusing. The multiple forms of CPI were shown immunologically to be forms of two proteins, referred to as CPI-A (comprising the forms of relatively acidic pI) and CPI-B (comprising the more basic forms). CPI-A and CPI-B are similar in their molecular weight of about 12,400, considerable stability to pH 2, pH 11 and 80°C, and tight-binding inhibition of papain, several related cysteine proteinases and dipeptidyl peptidase I. In all these respects they also resemble cystatin. K_i values were estimated for papain, human cathepsins B, H and L, and dipeptidyl peptidase I (Table 1). The affinity of CPI-A for cathepsin B was approximately 10-fold greater than that of CPI-B, whereas CBI-B showed approximately 100-fold stronger inhibition of dipeptidyl peptidase I. For all four enzymes, the liver inhibitors were less tight binding than cystatin. On the basis of antigenicity and pI, CPI-A seems to correspond to the epithelial inhibitor described previously, and CPI-B to the inhibitor from other cell types (Järvinen & Rinne, 1982).

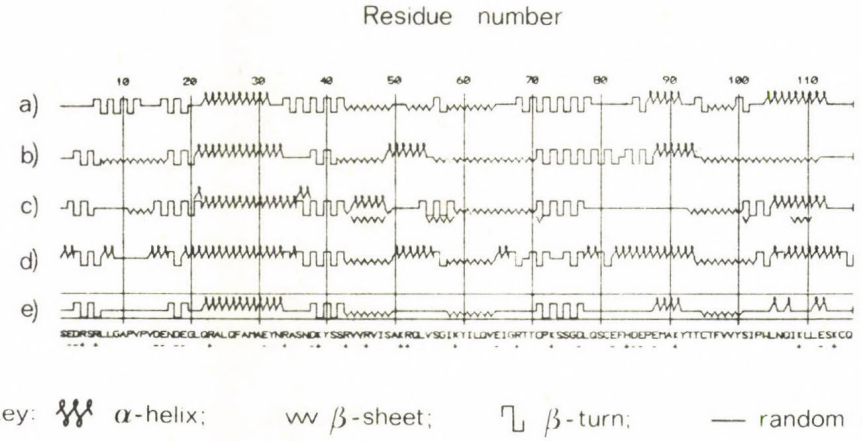

Fig. 5. Computer prediction of secondary structure from the sequence of cystatin. The algorithms used to make the four predictions were those of (a) Burgess et al., (b) Chou & Fasman, (c) Nagano, and (d) Robson & Suzuki. Line (e) shows those parts of the structure for which three of the four predictions agree. (See Schwabe et al., 1983, for full details.)

CONCLUSIONS

The clear knowledge that is now emerging about the evolutionary relationship and close structural similarities of the lysosomal cysteine proteinases to some well known plant enzymes is providing a valuable basis for an understanding of their distinctive reactivities with substrates and inhibitors.

Until recently, the protein inhibitors of cysteine proteinases have been almost unstudied, in contrast to the decades of detailed investigation that have been devoted to the serine proteinase inhibitors. This imbalance is now being adjusted, and already we seem to be able to identify the inhibitors that are likely to restrict the activities of cysteine proteinases in the body. In the blood, $alpha_2M$ is likely to be particularly important, rapidly trapping the enzymes to form complexes that are efficiently cleared from the circulation. No doubt alpha-CPI is also active in the plasma, but being slightly smaller than albumin it is able to escape into the urine and almost certainly into extravascular tissue fluids. Cysteine proteinases secreted by tissue cells are likely to encounter alpha-CPI before the other inhibitors. Inside human cells there are two low molecular weight inhibitors with properties reminiscent of those of cystatin. Probably these serve to mop up minor leakages of lysosomal cysteine proteinases which would otherwise be most destructive in the cell. They may also provide defence against some intracellular parasites, and some larger ones, but we have yet to learn the full significance of the existence of two proteins of this type with different cellular distributions.

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THE PROPERTIES AND PHYSIOLOGICAL ROLE OF ENDOGENOUS INHIBITORS OF CYSTEINE AND SERINE PROTEINASES

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INTRODUCTION

Studies from several laboratories report that leucocyte cells contain many types of proteinases (Havemann and Janoff, 1978; Barrett, 1977). These enzymes are known to be potent mediators of inflammatory response (Weissmann et al., 1978; Rossi and Patriarca, 1982). Regulation of their proteolytic activity is cellularly and extracellularly controlled by various inhibitors of proteinases (Kopitar et al., 1981, 1982b; Fritz et al., 1974). Recent investigations showed that leucocytes contain inhibitors of elastases, cathepsin G, plasminogen activator - urokinase type, and cysteine proteinases (Kopitar et al., 1981; 1982a, b).

The present communication describes the biochemical and biological properties of endogenous proteinase inhibitors isolated from peripheral pig and human leucocytes.

Abbreviations: Bz-Arg-Nap: benzoyl-arginine-p-nitroanilide
DTT: dithiothreitol
GSSG: glutathion, oxydized
NEM: N-ethylmaleimid
pNA: -p-nitroanilide

Inhibitor of elastase and cathepsin G

A protein inhibitor of elastase and cathepsin G, designated I-2, was purified to apparent homogeneity from the cytosol of pig peripheral blood (Kopitar and Lebez, 1975). Its molecular weight is about 40 000, its isoelectric point is 5.6-5.7. It is stable in buffer solutions in the pH region between pH 3.0-8.0, but is not resistant to heat. This inhibitor diminished the hydrolysis of haemoglobin, fibrinogen and elastin by pig elastase, as well as of human elastase (the experiment was performed by M. Jochum, Münich) with Succ-Ala-Ala-Val-pNA substrate. The K_i determined according to Green and Work (1953) for pig elastase (Hb pH 7.5) was 7 nM. Cathepsin G is also inhibited with the I-2 protein inhibitor, whereas cysteine proteinases, chymotrypsin, trypsin and urokinase are not. It was found that it could be inactivated by cathepsin D, by hydrolysis of the inhibitory molecule (Kopitar et al., 1980; 1982a). A similar endogenous elastase inhibitor of 36 kDa was isolated from horse peripheral leucocytes by Dubin (1977), and from bovine pulmonary macrophages by Valentine et al. (1981).

Inhibitor of plasminogen activator - urokinase type

Two inhibitors of urokinase were isolated from the cytosol and the extract of the nuclei of peripheral pig leucocytes (Kopitar, 1981). Only the inhibitor of higher molecular weight was isolated in the homogenous form, as a single polypeptide chain with a molecular weight of 68 000, designated I-3.

The latter one has an isoelectric point from 4.4 to 4.5 and it is stable in buffer solutions between pH 3.0-8.0, and belongs to the so called fast reacting inhibitors.

The inhibitor was effective only with plasminogen activators of urokinase type, but not with tissue plasminogen activator (test made in the laboratory of Prof. Rifkin, New York). It does not inhibit plasmin, trypsin, chymotrypsin or intracellular proteinases of serine and cysteine type.

In some of our recent studies we have already reported that the inhibitor of plasminogen activator can also be inactivated by hydrolysis with cathepsin D (Kopitar et al., 1980; Drobnič Košorok et al., 1981).

Recently a similar inhibitor acting against urokinase was also found in rabbit endothelial cells (Loskutoff and Edgington, 1981), human placenta (Kawano et al., 1970) and in human cornified cells (Hibino et al., 1981).

Inhibitor of cysteine proteinases

During the last five years, the inhibitors studied most intensively were those for cysteine proteinases. In our previous publications the isolation and characterization of a proteinase inhibitor of cysteine proteinases, designated I-1A and B or sericystatin A and B from pig leucocytes were described. This inhibitor occurs in the cell in various molecular forms (Kopitar et al., 1978, 1981, 1982a, b).

Table 1

Properties of pig leucocyte sericystatin A and B

Characteristics	A	B
Molecular weight	13 000	11 000
pI	4.6 - 5.6	5.8 - 7.0
Location in nuclear fraction	+	+
Cytosol	+	-
K_i (mol/l) of the complex of the inhibitor with:		
Papain	4×10^{-9}	1.2×10^{-9}
Cathepsin B	n.d.	7.0×10^{-8}
Cathepsin H	n.d.	5.0×10^{-8}
Cathepsin G	1.0×10^{-5}	no inhibition

n.d.: not determined

Table 1 shows the main characteristics of these two isoinhibitors isolated from pig leucocytes. In Fig. 1 their homogeneity, as well as their isoelectric points determined by gel isoelectric focusing are seen. As shown by Table 1 and Fig. 1, the samples differ not only in isoelectric point but also in

their inhibitory activity towards the target enzymes.

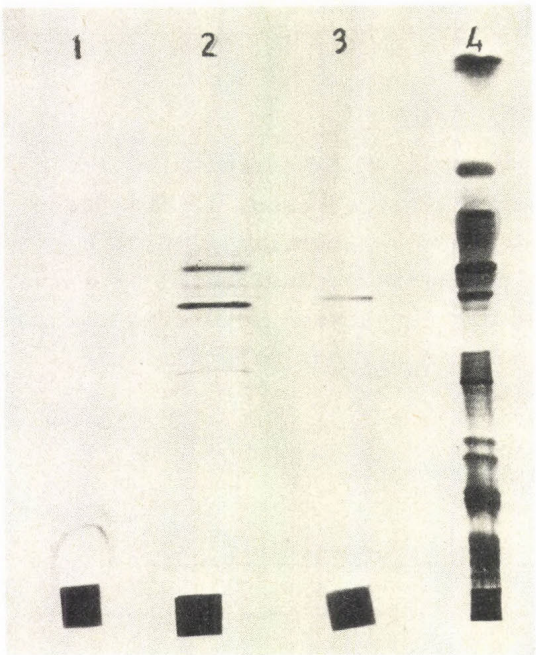

Fig. 1:
Isoelectric focusing of sericystatin A and B. Sericystatin A (1), proteins eluted in third protein peak from CM-cellulose, containing sericystatin B (2), sericystatin B (3), standards (4) (Ip 3.0-10.4).

As it was reported recently (Babnik et al., 1983) these isoinhibitors differ also in their antigenic structure. As shown in Fig. 2 intracellular inhibitors belong to specific types and they are unrelated proteins.

Inhibitors of cysteine proteinases are characterized by their high thermostability (Järvinen et al., 1978; Kopitar et al., 1978; Lenney et al., 1979). More detailed studies showed that their stability is time- and temperature-dependent. Pig inhibitor of cysteine proteinases, types A and B, were stable in buffer solution at pH 7.5, -20°C for a longer time; up to 1 year the specific inhibitory activity decreases by less than 10 %. Similar stability was observed when these inhibitors were incubated at 37°C up to 3 h (0.25 nmol in 0.25 ml of 0.03 M phosphate buffer, pH 6.5). Further, they were rather stable at room temperature (20-24°C); during 24 h incubation their activity was diminished only by about 10 %, but after

5 days of incubation the inhibitory activity was completely lost.

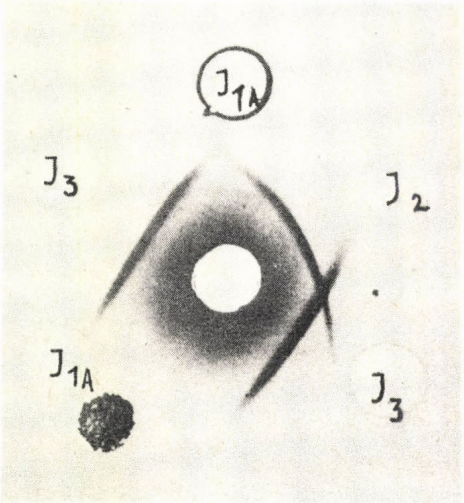

Fig. 2:
Double immunodiffusion of inhibitor I-1A, I-2 and I-3 against antiinhibitor (I-2 and I-3) antiserum. Antiinhibitor antibodies (10 µl) were placed in the middle well and all three inhibitors (10 µl) were put into the separate outer wells as indicated (published in Mol. Immunol. 1983, Babnik et al.).

Complexes of cysteine proteinase - inhibitor were stable in sodium phosphate buffer, pH 6.5, up to 1 h at 37°C. Under these conditions there was no evidence for reappearance of enzyme activity. As it was observed (Kopitar et al., 1981) the effect of these endogenous inhibitors on cysteine proteinases is reversible. However, the nature of the bond formed between the enzyme and the inhibitor is still obscure. The cysteine proteinases can be displaced from the complex by disulphide containing agents, such as oxydized glutathione and cystine with subsequential reactivation of the enzyme activity. The rate of reactivation depends on time, temperature and disulphide concentration.

Activation of the inhibitor of cysteine proteinases

As shown in Fig. 3, the activity of the inhibitor was increased by 15-20 % by incubating 0.15 nmol inhibitor in 0.25 ml of 0.03 M sodium phosphate buffer, pH 6.5, containing 100 nmol DTT at 37°C for 20 min (curve a). In the absence of DTT 30 min

incubation was required to achieve 20 % enhancement. As both curves show, activation at 37°C is faster in the presence of DTT.

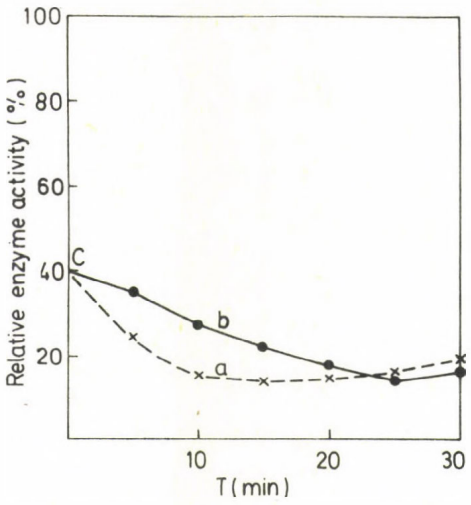

Fig. 3:
Effect of temperature and DTT on sericystatin A activity. a: --- with DTT; b: —— without DTT.

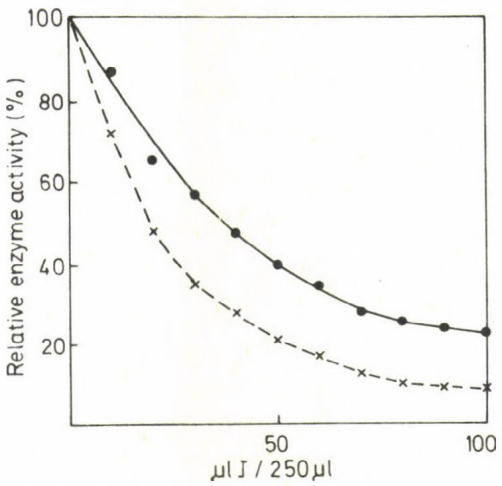

Fig. 4:
Inhibition of bovine cathepsin B (0.15 nmol) by adding various quantities of pig sericystatin B (10-100 µl i.e. 0.3-3 nmol). Full line represents unactivated-- control inhibitory tests. Enzyme was preincubated with inhibitor used in different concentrations, in buffer solution, at room temperature for 5 min. Dotted line represents DTT activated inhibitor sample (100 nmol for 20 min).

Titration of bovine spleen cathepsin B with activated and non-activated pig sericystatin B (Fig. 4) shows a significant increase in inhibition of activated inhibitor, thus inhibitor activated by DTT was more potent. The K_i value was calculated

according to Green and Work (1953) and in the case of cathepsin B it decreases from 5.5×10^{-7} M to 7.0×10^{-8} M. A control experiment with cathepsin B was performed in the presence of same amount of DTT for 5 min incubation at room temperature (20-24°C). During the complex formation only the enzyme was in contact with DTT. There was no decrease in enzyme activity. The titration experiments (Table 1) show that this inhibitor strongly inhibits cysteine proteinases, the K_i of the complex being 10^{-9} M to 10^{-8} M, whereas it appeared to be much weaker against cathepsin G (K_i 10^{-5} M). This inhibitor does not affect trypsin, chymotrypsin, elastase, urokinase and collagenase (the experiment was performed by D. Wooley, Manchester).

Inactivation of the inhibitor of cysteine proteinases

The effect of GSSG in increasing doses on the inhibitory activity of pig inhibitor type A was studied at two different times and temperatures. Fig. 5 shows the effect of 3-75 nmol GSSG on the activity of 0.5 nmol inhibitor. As it can be seen the inactivation of the inhibitor is reversible, time- and temperature-dependent. At higher concentrations of GSSG, the inactivated inhibitor was reactivated. Papain was also incubated with the same amount of GSSG for 10 min at 22°C and the enzyme was in contact with GSSG only for this period (during the activity assay). As shown in curve d, under these conditions GSSG did not inactivate papain.

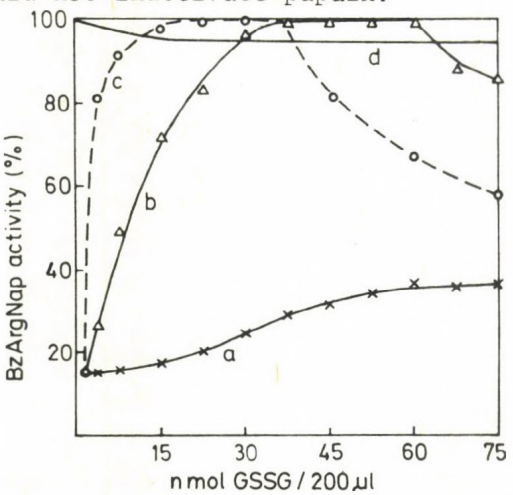

Fig. 5:
Effect of GSSG on the activity of pig sericystatin A. 0.5 nmol of inhibitor could inhibit 0.35 nmol of papain to produce 85 % inhibition. a) 15 min at room temperature, b) 1 h at 37°C, c) 3 h at 37°C. After incubation the tubes were cooled and papain (0.35 nmol) was added. After 5 min papain activity was assayed on Bz-Arg-Nap substrate.

The results of inactivation with GSSG presented in Fig. 5 have been confirmed by simultaneously performed gel electrophoretic studies . The enzyme and inhibitor were shown to be complexed, but adding GSSG to the inhibitor prior to cathepsin B, the complex formation was prevented (Fig. 6).

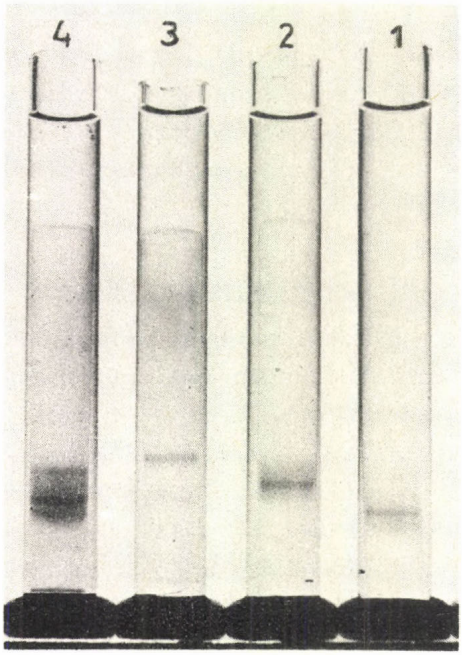

Fig. 6:
PAGE demonstrating the interaction between cathepsin B: sericystatin B and GSSG. 1) Sericystatin B (40 µg); 2) cathepsin B (8 µg); 3) complex of cathepsin B and inhibitor; 4) inactivation of inhibitor by prior preincubation with GSSG resulted in prevention of complex formation.

Preliminary analyses of the amino acid composition revealed 2 Cys and 2 Met residues in pig sericystatins (A and B).

The free -SH group content of pig inhibitor type A and B was determined. In the case of type A at a protein concentration of 0.7×10^{-6} M, the sulfhydryl group concentration was determined as 0.15×10^{-6} M. In the case of type B, however, the titration result indicated approximately 0.1 mol SH/mol of inhibitor. Alkylation of the free SH groups of the inhibitor with iodacetamide did not affect inhibitory activity, only by about 15-20 %. A similar effect was obtained with the sulfhydryl reagent N-ethylmaleimide (NEM). Our experiments in vitro on the treatment of the inhibitor with NEM disagree with the recent finding of Morgan et al. (1981), who showed that NEM can inactivate cytosolic inhibitor of cysteine proteinases of NIL

and PyNIL hamster fibroblasts when these cells are incubated in a culture medium containing NEM. These results indicate that this inhibitor is present in cells (cytoplasm) in an active SH form, and that its formation is regulated by enzymes coupled to the redox potential of the glutathione cycle.

The results of the present paper contribute to our recent study on the inhibitory mechanism of inhibitor types A and B, and the inactivation effect of GSSG on these inhibitors (Kopitar et al., 1981). The inactivation of the inhibitor by GSSG could be reversed with the subsequent regeneration of inhibitory activity, and the effect of GSSG on inhibitor is concentration-time- and temperature dependent. The equilibrium between inactivated - activated states could be ascribed to the ratio oxidized (GSSG) and the reduced form (-GSH) of glutathione. The activation experiments with DTT contribute to the redox potential dependent regulatory mechanism of inhibitory activity (Fig. 2 and 3).

Effect of the inhibitor in systems in vivo and in vitro

Pig leucocyte inhibitor type I-1A was investigated for V-79-379A cell proliferation ability in vitro. It was found that the inhibitor at doses of 10-80 µg per ml cell culture medium showed a profound cytocydal effect on the colony forming ability of these cells (Kopitar et al., 1981).

The regressive effect of pig leucocyte inhibitors type I-2 and I-1A and B has already been reported (Giraldi et al., 1977; Kopitar et al., 1982a). It was found that leucocytes inhibitors significantly diminished the growth of the primary tumour in mice bearing Lewis lung carcinoma. The number of lungmetastases found at sacrifice was also markedly reduced to about 64 %. A similar regressive effect on metastases formation was also obtained when animals were treated with spleen intracellular inhibitors (Giraldi et al., 1980).

Since lysosomal cathepsin B converts proinsulin to insulin (Ansorge et al., 1977; Bansal et al., 1980), the effect of pig sericystatin A was tested on an insulin dependent tumour - murine myeloid leukemia (Pavelič et al., 1983). The

experiments showed that inhibitor administered intraperitoneally to mice bearing myeloid leukemia diminished insulin in blood and prolonged their mean survival time . Simultaneous application of insulin could abolish these effects.

Studies on the inhibitory ability of cytosol obtained from leucocytes of synovial fluid of patients with rheumatoid arthritis against neutral proteinase and trypsin showed very pronounced differences (Rozman et al., 1983). In three samples (patients) no inhibitory activity was found against neutral proteinases and only a slight effect was observed on trypsin. In four samples a low (0-20 %) inhibitory activity was found against neutral proteinases and trypsin. The other ten specimens showed rather evident inhibitory activity on the tested enzymes. The absence of proteinase inhibitory activity in some samples was explained by the formation of inhibitor - proteinase complexes, because the inhibitory activity generated after the activation. An interesting finding is that in the absence of cytoplasmic inhibitory activity the negative latex test rheumatoid factor was more frequent and the cartilage and bone of the affected joint was preserved.

What is the role of these cellular proteinase inhibitors, which are located in cytoplasm and nuclei? The presented results indicate that these inhibitors have an important protective function against inappropriate proteolysis.

ACKNOWLEDGEMENTS

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*

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DISCUSSION

GRÁF:

Why do you think that the in vitro inhibitory effect of your leukocyte inhibitor on lysosomal cathepsin B and the decrease of insulin level in blood of mice are related phenomena? Is it possible that the inhibitor directly affects insulin release by an unknown mechanism instead of inhibiting the intracellular processing of proinsulin to insulin, in which phenomenon the secretory granules rather than lysosomal proteinases are involved?

KOPITAR:

It is known that cathepsin B and some other cysteine proteinases convert proinsulin to insulin (Bansal et al., 1980). In our paper (Pavelič et al., Eur. J. Cancer Clin. Oncol., 19, 123. 1983) we reported that sericystatin A reduces the supranormal blood levels of insulin detectable by insulin-specific radioimmunoassay in mice (with murine myeloid leukemia). It was concluded that sericystatin A reduces tumor proliferation indirectly i.e., by reducing the circulating insulin level.

HALÁSZ:

What kind of papain did you use in your experiments? Was it purified or not?

KOPITAR:

In our experiments we used 2x crystallized papain (type III. and IV) from Sigma.

CHRISTENSEN:

Can the sericystatin A be a substrate for cathepsin G? Is it possible that the inhibition is caused competitively by the degradation products of sericystatin A?

KOPITAR:

Theoretically this possibility may exist. In these experiments we used 10 min preincubation time, at 25°C, for the complex formation of cathepsin G and the inhibitor sericystatin A. The residual cathepsin G activity was assayed with a specific substrate (Succ-Ala-Ala-Pro-Phe--pNA) for 5 min. The difference in the activity (ΔA/min) was constant.

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INTERACTION OF HORSE LEUCOCYTE PROTEINASES WITH SOME NEUTRAL INHIBITORS FROM BLOOD PLASMA AND LEECHES

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ABSTRACT

The granules from horse blood leucocytes contain three serine proteinases: 1, 2A and 2B. Unlike proteinases 2, native proteinase 1 occurs in a form consisting of two proteins: catalytic and regulatory. It is known that the action of neutral proteinases from blood leucocytes is responsible for acute inflammatory states e.g. acute arthritis or lung emphysema. For this reason the interaction of these enzymes with alpha-1-antiproteinases from horse and rabbit blood and the inhibitor from leeches, called eglin, was investigated. The stoichiometry of inhibition and interaction rate of the enzymes and dissociation constant of the complexes were determined, attention being paid to accurate determination of the amount of active enzymes in the preparations by titration of their active centre with ^{3}H--di-isopropylphosphofluoridate. From the obtained results it was concluded that the alpha-1-proteinases inhibitor from rabbit blood is more efficient than the one from horse blood, and that eglin is a fairly effective inhibitor of horse leucocyte proteinases: this finding may have potential therapeutical application during treatment of horse emphysematous diseases.

INTRODUCTION

Resent studies indicate that azurophil granules from horse polymorphonuclear leucocytes contain three groups of proteinases:

elastase-like (EC 3.4.21.11), chymotrypsin-like (cathepsin C, EC 3.4.21.20) and collagenase-like (EC 3.4.24.7) (Starkey, 1977). A comparison of the neutral proteinases from polymorphonuclear leucocytes of several experimental animal species was given by Ashe and Zimmerman (1982). In distinction to human (Starkey, 1977), bovine (Marossy, 1980) and pig (Kopitar and Lebez, 1975) polymorphonuclear leucocytes, horse granules contain only elastase-like neutral proteinases (Dubin et al., 1976). Three different serine proteinases are responsible for hydrolysing casein, N-benzyloxycarbonyl-L-alanine 4-niteophenyl ester (Z--Ala-ONp), acetyl-tri-alanine methyl ester (Ac-Ala-Ala-Ala-OMe) (Koj et al., 1976), and N-butyloxycarbonyl-L-alanine 4-nitrophenyl ester (Boc-Ala-ONp) (unpublished results). These serine proteinases, designated 1, 2A and 2B, possess isoelectric points 5.3; 8.8 and over 10, respectively (Dubin et al., 1976) and relative molecular mass M_r 49 000 (Potempa, 1982a), 24 500 and 20 500, respectively (Dubin et al., 1976). Native proteinase 1 occurs in the leucocyte granule extract in a form consisting of two proteins, one of which is catalytically active (M_r 30 000, pI 8.2 and 9.15) and the other acidic protein has no proteolytic activity (M_r 20 000, pI 4.5) (Potempa, 1982b). The complex can be completely dissociated on Cibacron-Blue-Sepharose or by sodium dodecyl sulphate during electrophoresis even in the absence of a reducing agent. It is known that the neutral proteinases of granulocytes are involved in the degradation of connective tissue components in inflammatory diseases (Jannof, 1975; Baggioloni et al., 1979; Zimmerman, 1979). Proteinase inhibitors might therefore be valuable therapeutical tools in the treatment of this condition (Baugh and Schnebli, 1980; Janoff and Dearing, 1980). Hence, it was decided to investigate the interaction of horse leucocyte proteinases with some neutral inhibitors from blood plasma and leeches.

MATERIALS AND METHODS

Enzymes

Crystalline bovine trypsin and alpha-chymotrypsin were from Koch-Light (Colnbrook, England), while neutral proteinases from horse leucocytes were purified from granule extract: proteinase 1 by the method of Potempa (1982b) and proteinases 2A and 2B by the method of Dubin et al. (1976). The amounts of active enzymes were independently determined by titration with p-nitrophenyl guanidinobenzoate (for trypsin according to Chase and Shaw, 1970), with p-nitrophenyl acetate (for chymotrypsin, according to Kézdy and Kaiser, 1970), or by using tritiated diisopropylphosphofluoridate (Dip-F) (6.5 Ci/mmol , Radiochemical Centre, Amersham, Bucks, UK) for neutral proteinases in the following procedure, using trypsin and chymotrypsin as standards: tritiated Dip-F was diluted with fresh 8 mM carrier Dip-F in dry propylene glycol to obtain approximately 5 µCi in 15 µl. The tested enzymes (2 to 8 nmole) were diluted with 0.05 M Tris-HCl pH 8.8 to 0.4 ml in plastic tubes (Eppendorf type) of a microcentrifuge and immediately 15 µl ^{3}H-Dip-F solution was added with vigorous shaking. Shaking was continued for 30 min and then 50 µl of bovine serum albumin solution (10 mg/ml 0.05 M Tris-HCl, pH 8.8) was pipetted, followed 10 min later by 0.5 ml 10 % trichloroacetic acid. The precipitate was centrifuged, dissolved in 0.2 ml 0.1 N NaOH, reprecipitated with 1 ml 10 % trichloroacetic acid, washed 2 times in 1 ml 5 % trichloroacetic acid, and finally dissolved in 0.1 ml Protosol (New England Nuclear, Boston, MA USA). The tube contents were transferred quantitatively to a scintillation vial, made up to 10 ml with the Bray scintillation cocktail, and radioactivity was determined in a Packard spectrometer. The values of radioactivity found in samples containing known amounts of trypsin or chymotrypsin were used as standards for calculation of the concentration of active leucocyte proteinases run in a parallel assay. In the described conditions a linear relationship was found between incorporated radioactivity and the amount of serine proteinases ranging between 2 and

8 nanomoles. At lower concentrations of the enzymes the accuracy was not satisfactory.

Inhibitors

Rabbit alpha-1-proteinase inhibitor (alpha-1-PI) was purified as the F and S forms by the procedure described elsewhere (Koj et al., 1978), and horse alpha-1-proteinase inhibitor by the method of Kurdowska et al., 1982). Eglin C, the inhibitor from leeches, was obtained by the procedure of Seemüller et al., (1977).

Assay of enzymatic activity

The activity of trypsin was determined at pH 8.0 with p-toluene-sulphonyl-L-arginine methyl ester (Tos-Arg-OMe, Koch-Light, Colnbrook, England) (Hummel, 1959), chymotrypsin at pH 6.5 with N-benzyloxycarbonyl-L-tyrosine-p-nitrophenyl ester /CBZ-Tyr-ONp, Sigma Chemical Co., St.Louis, MO, USA) (Aubry and Bieth, 1977), and leucocyte proteinases at pH 6.5 with N-tert-butyloxycarbonyl-L-alanine-p-nitrophenyl ester (BOC--Ala-ONp, Fluka AG, Buchs, Switzerland) (Visser and Blout, 1972).

The stoichiometry of the reaction between the tested proteinases and inhibitors as well as the dissociation constants (K_i) were determined either by the method of Green and Work (1953) or of Bieth (1980), depending on the shape of the reaction curves, and the association rate constants (k_{on}) according to Bieth (1980).

In some experiments inhibition of leucocyte proteinases by eglin was tested with protein substrates - azocasein (prepared according to Hazen et al., 1965) and bovine fibrinogen (Miles Lab., Elkhart, USA) - using the following procedure: 0.1-0.2 nmol of enzyme was incubated for 5 min at 25°C with variable amounts of eglin in 0.3 ml 0.1 M sodium phosphate pH 7.4. Then 0.3 ml of the protein substrate in the same buffer was added to the final substrate concentration of 1.5 % (w/v). After 60 min at 37°C the reaction was stopped by addition of 0.3 ml 12 % (w/v) trichloroacetic acid and the mixture

was centrifuged. The A_{360} (for azocasein) or A_{280} (for fibrinogen) of the acid soluble reaction products were determined.

RESULTS AND DISCUSSION

The final preparation of horse plasma alpha-1-proteinase inhibitor (alpha-1-PI) purified by the method of Kurdowska et al., (1982) always showed a considerable degree of heterogeneity during electrophoresis on polyacrylamide gel at pH 8.3, while it gave a single precipitin arc on immunoelectrophoresis in agarose. This is an agreement with a recent report of Laegreid et al. (1982). As shown by Juneja et al. (1979) by two-dimensional electrophoretic analysis the horse serum contains two groups of alpha-1-globulin (designated PI1 and PI2) proteinase inhibitors. Preliminary studies indicated that PI1 and PI2 proteins differ from each other in relative molecular mass and in proteinase inhibiting spectra. The PI1 fractions showed a strong inhibition for both trypsin and chymotrypsin while the PI2 fractions had an equally strong inhibition for trypsin but showed a much weaker one for chymotrypsin. A similar phenomenon was found by Kobayashi and Nagasawa (1974) for proteinase inhibitors from guinea pig serum. It was very difficult to purify these two inhibitors on a preparative scale, but by using the method of Kurdowska et al. (1982) it was sometimes possible to separate the PI2 proteinase inhibitor from PI1. As shown in Fig. 1, the PI2 fraction effectively inhibited trypsin (approximately 1:1 molar ratio of the inhibitor to enzyme) and the proteinase-inhibitor complex was rather stable (dissociation constant K_i=17 nM). On the other hand, this fraction inhibited only about ten per cent of the esterolytic activity of chymotrypsin, even with a five-fold molar excess of the inhibitor, and no inhibition was observed either in the case of pancreatic elastase or three horse leucocyte neutral proteinases. In most cases, however, the tested alpha-1-PI isolated from horse plasma was a mixture of PI1 and PI2 (Table 1). The reaction of this preparation with trypsin was prompt, the stoichiometry in most experiments being close to a 1:1 molar ratio and the complex enzyme inhibitor fairly

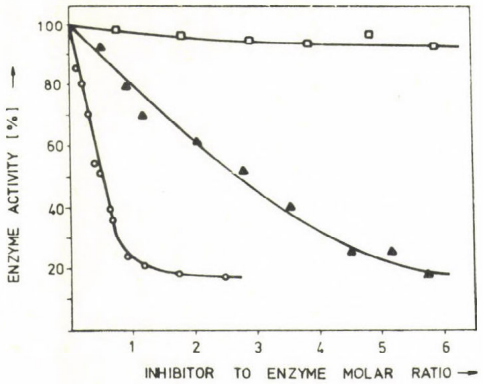

Fig. 1: Inactivation of bovine trypsin (circles) and chymotrypsin (triangles and squares) by horse alpha-1-proteinase inhibitor: PI1 + PI2 (full symbols) or PI2 (open symbols).

stable (K_i=11 nM). In the case of chymotrypsin the enzyme also reacted quickly with the inhibitor (rate constant $k_{on}=1.45\times10^5$ $M^{-1}s^{-1}$) but the molar ratio could not be determined at all, and even with a five-fold molar excess of alpha-1-PI the proteinase retained approximately 30 per cent of its initial activity (Fig. 1). A somewhat similar picture was observed with three elastase-like proteinases from horse leucocytes but the reaction was much slower. A high dissociation constant (Table 1) of the proteinase-inhibitor complex cannot alone explain the peculiar stoichiometry of the reaction between alpha-1-PI and chymotrypsin, but it represents an additional factor responsible for the low inhibitory capacity of horse alpha-1-PI against both chymotrypsin and leucocyte proteinases. Hence, in horse blood alpha-1-PI are probably not involved in the inactivation of chymotrypsin and leucocyte proteinases in vivo.

Table 1

Comparison of inhibitory constants (K_i) and reaction rates (k_{on}) of serine proteinases and inhibitors

Enzyme	Alpha-1-PI horse		Alpha-1-PI rabbit				Eglin C	
			Form F		Form S			
	K_i (nM)	k_{on} $10^5 M^{-1}s^{-1}$	k_i (nM)	k_{on} $10^5 M^{-1}s^{-1}$	k_i (nM)	k_{on} $10^5 M^{-1}s^{-1}$	k_i (nM)	k_{on} $10^5 M^{-1}s^{-1}$
Trypsin	11.0	1.33	0.81	5.05	2.69	0.21	n.d.	n.d.
Chymotrypsin	190.0	1.45	0.22	14.50	1.02	11.60	0.23	30-90
Proteinase 1	145.0	0.07	1.02	39.30	1.14	0.27	0.63	12.8
Proteinase 2A	172.0	n.d.	0.68	>100.00	1.93	0.53	0.47	9.5
Proteinase 2B	262.0	n.d.	0.38	>100.00	3.82	58.60	25.50	8.9

n.d.: not determined

In rabbit blood alpha-1-PI occurs in two principal immunologically identical forms, designated according to their electrophoretic mobility as F (fast) and S (slow) (Koj et al., 1978). As is shown in Fig. 2 and Table 1, these two forms of rabbit alpha-1-PI have differential reactivity with various proteinases. The F form reacted much faster with trypsin or horse leucocyte proteinases 1 and 2A than the S form, whereas

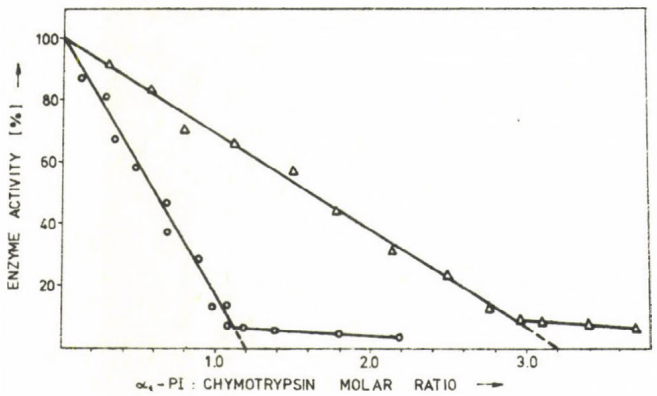

Fig. 2: Inactivation of bovine chymotrypsin by rabbit alpha-1-PI F (circles) or S (triangles) with CBZ-Tyr-ONp as substrate

with chymotrypsin or proteinase 2B the values of the inactivation rates were faintly similar for the two forms. When these differences in reaction rates were taken into account during determination of the stoichiometry of enzyme inactivation by alpha-1-PI-F and S, the curves extrapolated to approximately 1:1 molar ratio of the inhibitor to the enzyme, with the exception of the chymotrypsin and alpha-1-PI-S interaction. In this case an approximately three-fold molar excess of alpha--1-PI-S was required for almost total inhibition of enzymatic

activity (Fig. 2). Such a mixture of a three-fold excess of alpha-1-PI-S and chymotrypsin was totally inactive against trypsin, this indicating that all the inhibitor reacted with chymotrpysin to form a stable complex or inactivated inhibitor. On this basis, the supposition that the native alpha-1-PI-S contains only third of the binding sites for chymotrypsin while being fully active with trypsin appears not to be justified. In agreement with reports of other authors on human alpha-1--PI (Beatty et al., 1980) it was found that also rabbit inhibitor F reacted much faster with leucocyte proteinases than with trypsin or chymotrypsin (Table 1). On the other hand, the S form showed much lower inactivation rate constants with trypsin and leucocyte proteinases 1 and 2A than with chymotrypsin and proteinase 2B, thus resembling chemically modified human alpha-1-PI (Beatty et al., 1980). All these findings indicate structural differences in the reactive centre of the two forms of rabbit alpha-1-PI, but the problem requires further studies. From the K_i values given in Table 1 it was concluded that alpha-1-proteinase inhibitors from rabbit blood are more efficient than that from horse blood.

The low molecular mass inhibitor (M_r 6 000) isolated from leeches as described by Seemüller et al. (1977) was fairly effective inhibitor of horse leucocyte proteinases in vitro as is shown in Table 1. The interaction of proteinase 1 and 2A gave typical rectilinear curves (Fig. 3a and 3b) which were not altered by changes in BOC-Ala-ONp concentrations within the range $1\text{-}3\times10^{-4}$ M. Hence, the K_i value could be calculated by the method of Green and Work (1953). At higher substrate concentrations (e.g., 5×10^{-4} M) the curves became slightly concave although the method of Bieth (1980) could not be applied for calculation of K_i. In the case of proteinase 2B the inactivation curve with eglin always yielded a concave curve, even at the lowest tested BOC-Ala-ONp concentration (10^{-4} M, Fig. 3c). The apparent K_i values calculated by the method of Bieth (1980) at 3 different substrate concentrations could be corrected for substrate-induced complex dissociation assuming a K_m value for BOC-Ala-ONp equal to 0.24 mM. The

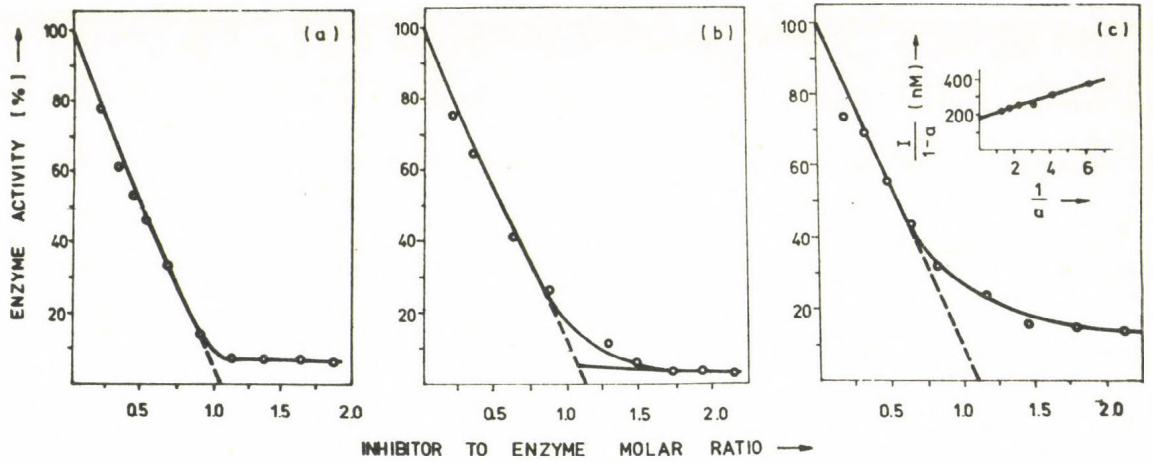

Fig. 3: Interaction of eglin with proteinase 1 (a), 2A (b) and 2B (c) with BOC-Ala-ONp as substrate. Insert shows replot of the curve c according to Bieth (1980) with the apparent K_i)32.5 nM.

corrected K_i values for proteinase 2B ranged between 28.2 and 22.9 nM with the mean value given in Table 1. Further experiments indicated that the complex of proteinase 2B with eglin was also dissociated with one of the protein substrates-azocasein (Fig. 4c). The inhibition of proteinase 2B by equimolar concentration of eglin was only slight. The other two proteinases (1 and 2A) were fairly effectively inhibited by eglin with azocasein, although the curvature of the lower portion of the curves and extrapolated equivalence points suggest the occurrence of equilibrium between free and complexed enzymes (Fig. 4a and 4b). On the other hand, the results shown in Fig. 4 indicate that with fibrinogen as substrate all three proteinases are almost completely inhibited by eglin at nanomolar concentrations. From the inactivation rate constants given in Table 1 it may be concluded that the reactions with both chymotrypsin and neutral proteinases are very fast, inactivation of proteinases being practically completed within less than 30 seconds. It remains to be explored whether eglin is also an effective inhibitor of horse leucocyte proteinases in vivo.

The action of neutral leucocyte proteinases on the lung elastin tissue is responsible for lung emphysema. Although the ethiopathology of such a condition is best documented for man, certain data suggest that the horse is also a sensitive species (Gerber cited after von Fellenberg and Pellegrini, 1979). Three very active elastase-like neutral proteinases from horse leucocyte (Dubin et al., 1976; Koj et al., 1976) can be rather inefficiently inhibited by homologous alpha-2-macroglobulin (von Fellenberg and Pellegrini, 1979) and by alpha-1-proteinase inhibitor (this study). Moreover, homologous antithrombin III is completely inactive against these enzymes but a prolonged exposure of inhibitor to leucocyte proteinases reduces the antitryptic activity of the inhibitor (Koj and Kurdowska, 1981). Exogenous proteinase inhibitors may therefore have some potential practical significance in the prevention and treatment of horse lung emphysema. On the other hand, the horse leucocyte cytosol contains very active inhibitor against

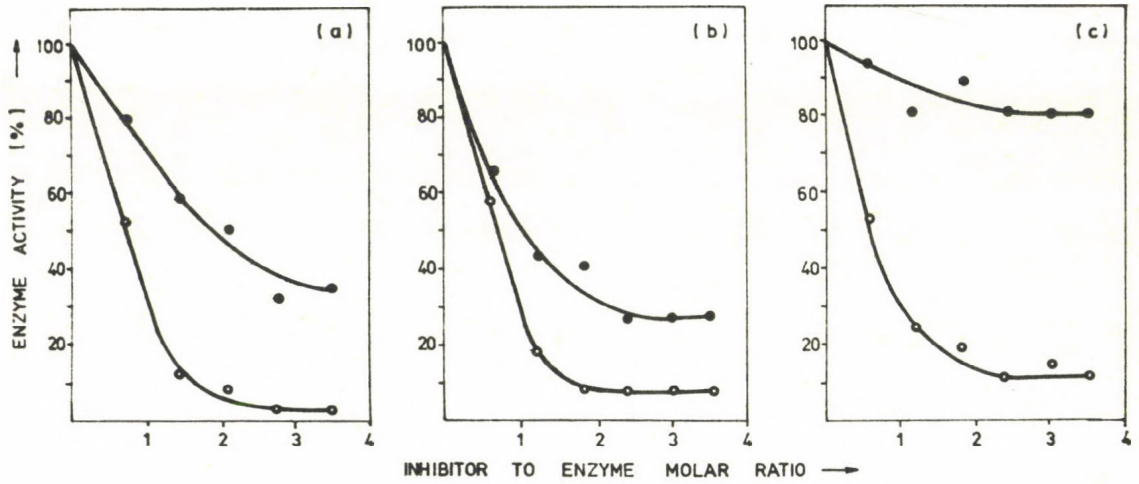

Fig. 4: Curves of inhibition of three leucocyte proteinases by eglin with azocasein (full circles) or fibrinogen (open circles) as substrates. a - proteinase 1; b - proteinase 2A; c - proteinase 2B.

chymotrypsin, pancreatic alastase, and horse leucocyte neutral proteinases (Dubin, 1977). Detailed kinetic studies of the interaction between this inhibitor and proteinases are now in progress.

ACKNOWLEDGEMENTS

We would like to thank Dr. U. Seemüller (University of München, FRG) for providing a sample of eglin C. This study was supported by a grant within the Research Project no. MR II. 1.2.2. co-ordinated by The Nencki Institute of Experimental Biology, Polish Academy of Sciences.

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DISCUSSION

KORANT:

Which pathological conditions in horses do you propose to treat with your inhibitors?

DUBIN:

We suggest to use eglin to treat horse-lung emphysema.

Symposia Biologica Hungarica 25 (1984)

GRANULOCYTE ELASTASE MEDIATED FIBRINOLYSIS

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Neutral proteinases from neutrophil granulocytes are released at inflammatory sites by phagocytic stimuli (Baggiolini, 1982) and during clot formation (Plow, 1982) from the involved granulocytes. They are able to degrade a variety of substrates and may therefore play a role in pathological protein break down (Havemann and Janoff, 1978). Elastase, the most important granulocyte proteinase, is known to degrade beside fibrinogen and other clotting factors (Schmidt et al., 1974) and the coagulation inhibitors (Jochum et al., 1981) also the inhibitor of plasmin fibrinolysis, α_2-plasmin inhibitor (Klingemann et al., 1981).

α_2-plasmin inhibitor is known to exist in three forms (Clemmensen et al., 1981), the plasminogen binding (69 kDa), the non-plaminogen binding still active (65 kDa) and the inactive 58 kDa from. α_2-plasmin inhibitor is inactivated by elastase as measured by the inhibitory activity of α_2-plasmin inhibitor against plasmin (Fig. 1).

The α_2-plasmin inhibitor, used, consisted mainly of the 69 kDa plasminogen binding inhibitor as shown in Fig. 2 by SDS-polyacrylamide gel-electrophoresis. During inactivation this band disappeared and a 65 kDa and a 58 kDa band became visible, representing the non-plasminogen binding active and

Abbreviations: BOC: t-butyloxycarbonyl-
Nan: p-nitroanilide
Np : nitrophenyl ester
Plg: plasminogen

later on (24 h) inactive inhibitor molecule and the inactive plasmin inhibitor with a lower molecular mass. While unchanged after 24 h incubation in buffer, no residual plasminogen binding α_2 plasmin inhibitor was visible in the 24 h digest with elastase.

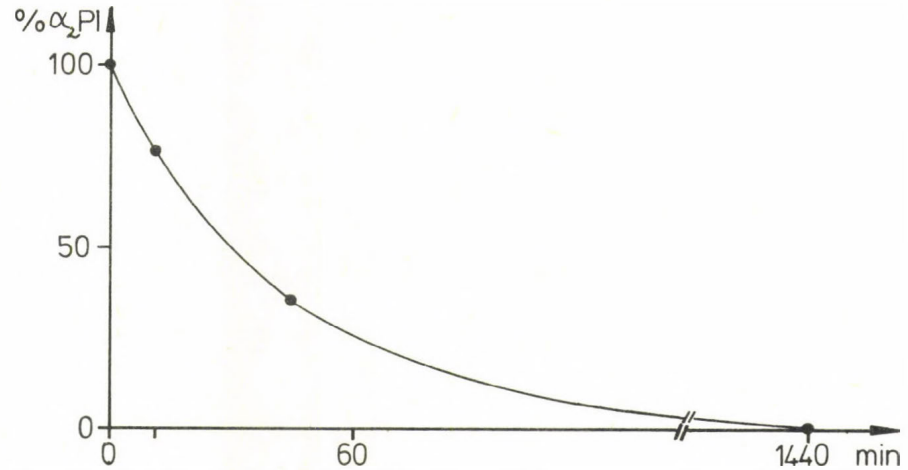

Fig. 1: Elastase induced inactivation of α_2 plasmin inhibitor. α_2 plasmin inhibitor activity was estimated by its capacity to inhibit plasmin action on the chromogenic substrate D-Val-Leu-Lys-Nan. Molar ratio elastase to α_2 plasmin inhibitor = 0.01

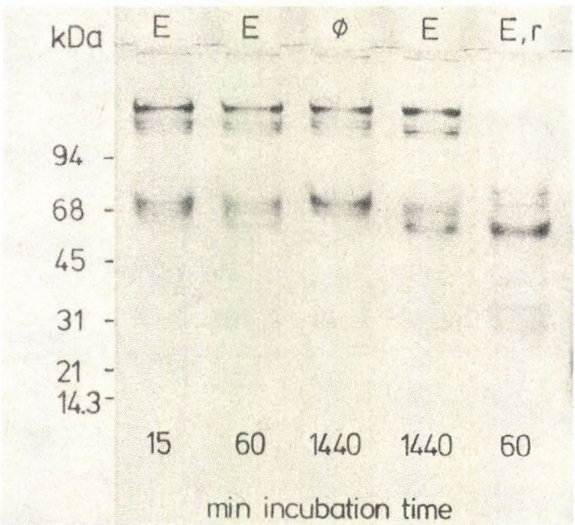

Fig. 2:

SDS-polyacrylamide gel-electrophoresis of α_2-plasmin inhibitor samples, incubated with buffer (ø) or elastase (E) 15 or 1440 min. The 60 min incubation with elastase was also applied to the gel following reduction (Er). The molecular mass of the marker proteins in kDa are indicated on the left.

Following reduction the 69 kDa band remained unchanged, the 65 kDa band, however, disappeared. Judging by the staining intensity, the 58 kDa band may exist of the unchanged inactive 58 kDa molecular and of the reduced non-plasminogen binding α_2 plasmin inhibitor. Obviously elastase splits an intramolecular loop of α_2 plasmin inhibitor which leads to the loss of plasminogen binding capacity of the plasmin inhibitor.

The plasminogen binding and the non-binding α_2 plasmin inhibitor forms can be demonstrated by two-dimensional immunoelectrophoresis, if separation in the first dimension is performed in the presence of plasminogen (Fig. 3).

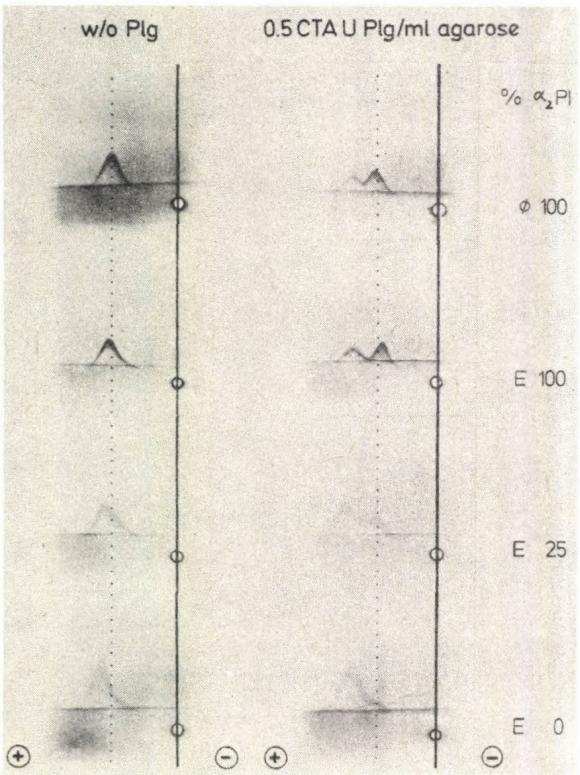

Fig. 3:
Two-dimensional immunoelectrophoresis of plasma, incubated with buffer (Ø) or elastase (E) to give residual α_2 plasmin inhibitor activities as indicated on the right. Separation during the first dimension was performed without (left plates) or with 0.5 CTA U plasminogen/ml agarose (right plates), anti-α_2 plasmin inhibitor serum was used for precipitation in the second dimension.
The appearance of two precipitation arcs only is due to the presence of plasminogen, as without plasminogen only one arc became visible.

Plasma was incubated with varying concentrations of elastase to give the residual α_2 plasmin inhibitor activities as indicated. The inhibition was separated with and without plasminogen in the first dimension and precipitated with anti-

α_2 plasmin inhibitor serum in the second dimension. Only in the presence of plasminogen the two inhibitor forms are separated: The plasminogen-binding form is retarded. The concentration of the faster migrating non-plasminogen binding form increases with inactivation.

It may be concluded by the results, that elastase or a similar reacting proteinase induce the conversion which was hitherto described to occur spontaneously (Clemmensen et al., 1981).

Inactivation of α_2 plasmin inhibitor in plasma required physiologically unexpected high elastase concentrations (Fig. 4).

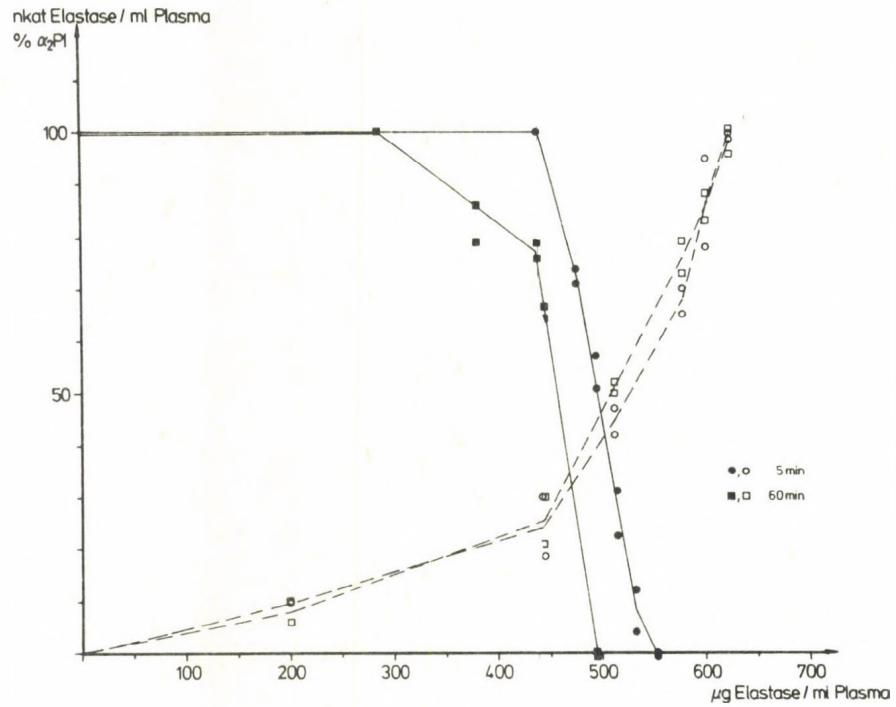

Fig. 4: % α_2 plasmin inhibitor activity (——) following 5 and 60 min incubation of human plasma with 0 to 630 µg elastase per ml. The elastase activity (nkat/ml) of identical samples was measured with the chromogenic substrate < Glu-Pro-Val-Nan (---).

Following 5 min incubation with 550 µg elastase per ml plasma α_2 plasmin inhibitor was totally inactivated, following one hour incubation still 500 µg elastase/ml plasma were required. Elastase below 100 µg/ml concentration did not inactivate α_2 plasmin inhibitor even after prolonged incubation time (two days).

Elastase activity was simultaneously measured with the chromogenic substrate (<Glu-Pro-Val-Nan). The beginning of α_2 plasmin inhibitor inactivation at 450 µg elastase/ml plasma corresponds with a steeper increase in elastase activity. The initial flat increase of elastase activity with growing concentrations represents the low residual activity of elastase, which is saturated by numerous protein substrates, among them α_2 macroglobulin. α_2 macroglobulin bound elastase is known to react with peptide substrates. In experiments with α_2 macroglobulin/elastase complexes and purified α_2 plasmin inhibitor, however, no inactivation of the plasmin inhibitor was to observe.

Degradation of purified fibrinogen, leeding to split products with anti-clotting properties is well described (Gramse et al., 1981). If elastase induced fibrinogen degradation was performed in human plasma and measured by thrombin clotting time also an unphysiologically high concentration was required to make fibrinogen uncoagulable (Fig. 5). But, thrombin time was prolonged already in a concentration range, where mainly α_2 macroglobulin bound elastase yielded the low residual activity against the chromogenic substrate. Moreover, thrombin time measurement is not a highly susceptible indicator for fibrinogen degradation, as the high molecular early X-like degradation product of fibrinogen yet can be coagulated by thrombin. It was therefore of interest to know, if α_2 macroglobulin bound elastase is able to split fibrinogen. This was tested using FITC-labelled fibrinogen as substrate (Fig. 6).

The release of trichloroacetic acid soluble peptides, which served as criterion for fibrinogen degradation by elastase, was measured by fluorometer. Elastase was preincubated with increasing molar excess of α_2 macroglobulin before incubation with FITC-fibrinogen. For comparison, the activity of the

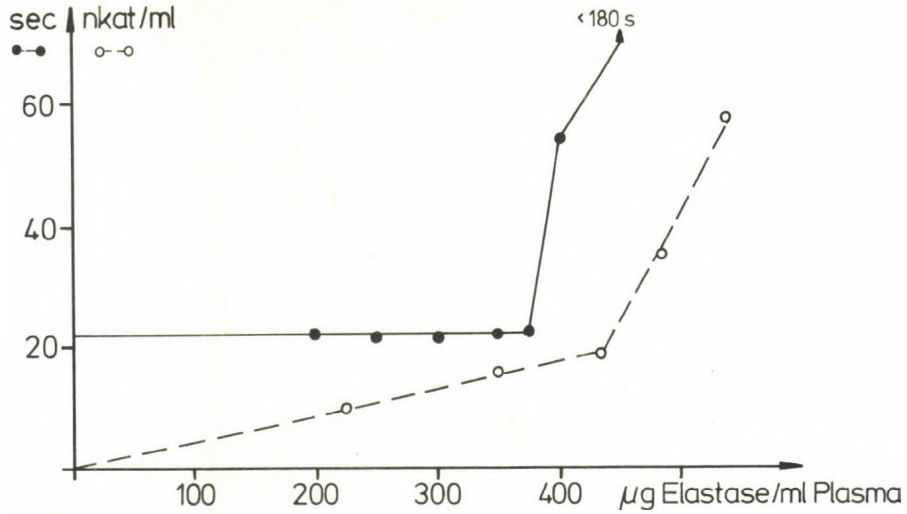

Fig. 5: Thrombin time of plasma, measured after 5 min incubation with 0 to 500 µg elastase/ml. Measurement of elastase activity see Fig. 4.

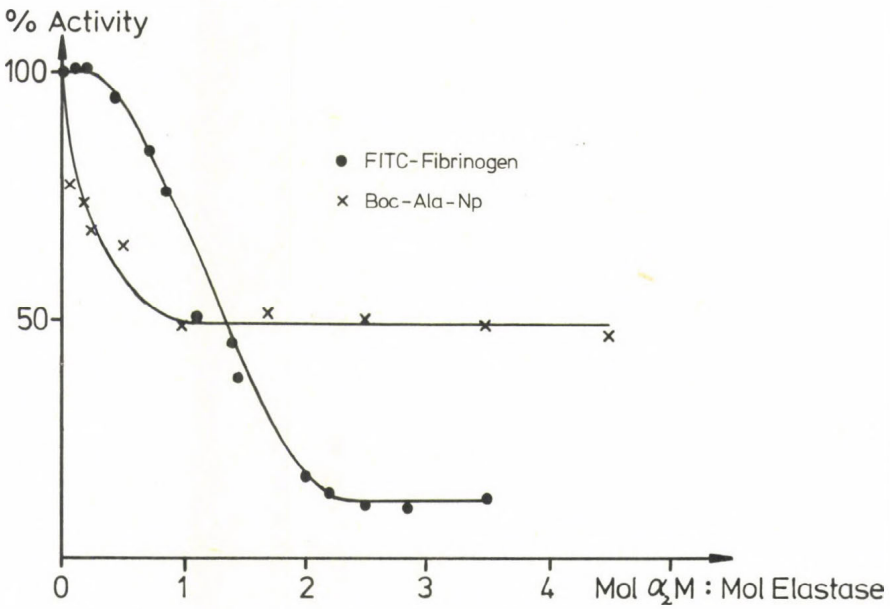

Fig. 6: Esterase activity of granulocytic elastase against Boc-Ala-Np and proteinase activity against FITC-fibrinogen (%) in the presence of 0 to 5 mol α_2 macroglobulin per mol elastase. For each test, 1.5 µg elastase had been preincubated for 30 min with different amounts of α_2 macroglobulin.

α_2 macroglobulin/elastase samples was measured using a chromogenic substrate. Despite the proteinase activity against FITC-fibrinogen was much stronger inhibited by α_2 macroglobulin than the esterase activity against the dipeptide, it also reached a plateau of residual activity and was not fully inhibited. Despite some denaturation of fibrinogen by FITC-labelling one may therefore speculate, that α_2 macroglobulin bound elastase is able to split fibrinogen.

Heparins which are widely used for anticoagulation, are also known to inhibit serin proteinases (van Haeringer, 1963). We therefore tested the influence of this anticoagulant on elastase, on its esterolytic and especially on its fibrinolytic properties. Elastase, a strongly cationic protein, cannot be demonstrated in two-dimensional immunoelectrophoresis because of its cathodic migration. But if heparin is present in the first dimension, the formed heparin/elastase complex migrates towards the anode and can be precipitated with anti-elastase serum in the second dimension (Fig. 7).

Fig. 7:
Two-dimensional immunoelectrophoresis of purified granulocyte elastase: upper plate: with 2 USP U heparin/ml agarose in the first dimension, precipitated with anti-elastase serum in the second one. The elastase for the upper plate had been preincubated with heparin.
lower plate: heparin was omitted.

The bond between elastase and heparin, however, is dissociated when the complex migrates in electrophoresis without heparin.

To show the influence of heparin on complex formation of elastase with its plasma proteinase inhibitors - α_2 macroglo-

bulin and α_1 proteinase inhibitor - two-dimensional immunoelectrophoresis with plasma and elastase/plasma were carried out in the presence or absence of heparin. Neither α_2 macroglobulin alone nor its complex with elastase; neither active α_1-proteinase inhibitor nor its proteolytic degraded, inactive form were modified in their electrophoretical behaviour by heparin. In contrast, the elastase/α_1 proteinase inhibitor complex, without heparin migrating more slowly than α_1 proteinase inhibitor alone, is shifted towards the anode. This is demonstrated in two-dimensional immunoelectrophoresis in Fig. 8 with anti-α_1 proteinase inhibitor serum and anti-elastase serum, respectively.

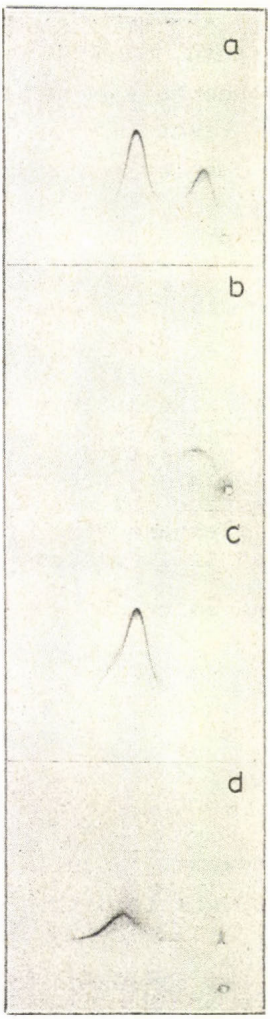

Fig. 8:
Two-dimensional immunoelectrophoresis of plasma preincubated with 150 µg elastase per ml.
The first dimension on plates c and d was performed in the presence of 2 USP U heparin/ml agarose. Anti-α_1 proteinase inhibitor serum was used for precipitation in the second dimension on plates a and c, anti-elastase serum on plates b and d.

Nevertheless, the binding of heparin to elastase or its complex with α_1 proteinase inhibitor is not as strong as heparin binding to antithrombin III. This was proven by gelfiltration experiments with protein/^{3}H-heparin complexes. While the antithrombin III/heparin complex was stable even in a strongly chaotropic buffer, elastase was separated from ^{3}H-heparin by gel filtration under identical conditions.

The influence of heparin on the elastase activity was assayed with various substrates. While elastase activity against the chromogenic substrate (<Glu-Pro-Val-Nan) was not changed by heparin, elastase activity against the dipeptide Boc-Ala-Np was inhibited to about 60 % by 50 to $5x10^{-5}$ USP U/ml. Lower and higher heparin concentrations had no effect (Fig. 9).

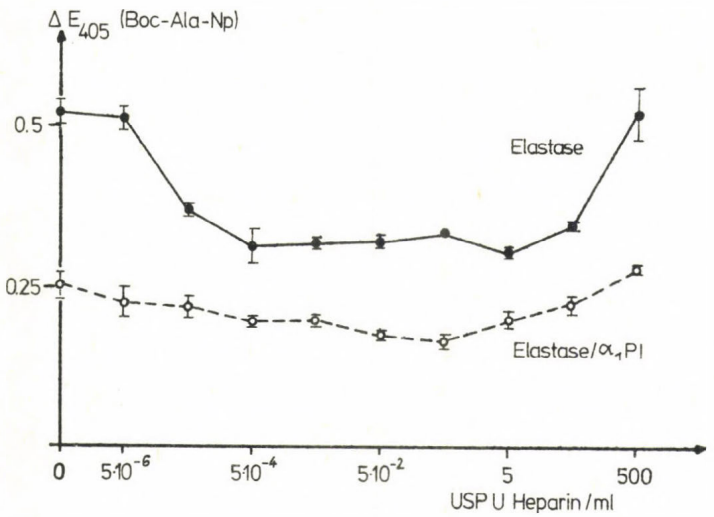

Fig. 9: Activity of elastase or elastase/α_1 proteinase inhibitor mixture (molar ratio 2:1) against Boc-Ala-Np in the presence of 500 to $5x10^{-6}$ USP U heparin per ml test volume.

The inactivation of elastase by α_1 proteinase inhibitor was not affected by heparin. Elastase/α_1 proteinase inhibitor mixtures in the molar ratio of 2 to 1 showed the same susceptibi-

lity of the non-inhibited elastase like free elastase to heparin.

When FITC-fibrinogen was used as a substrate for elastase, a strong enhancement of fibrinogen degradation by heparin was observed (Fig. 10). Incubation in 5 to $5x10^{-6}$ USP U heparin/ml nearly doubled the amount of trichloroacetic acid-soluble split products of fibrinogen. Still higher heparin concentrations abolished this effect. Incubation in 5 mmol/l EGTA, a strong Ca^{++} chelator, yielded the same degree of fibrinogen splitting as low heparin concentrations. The behaviour of the elastase/α_1 proteinase inhibitor mixtures again is in accordance with free elastase.

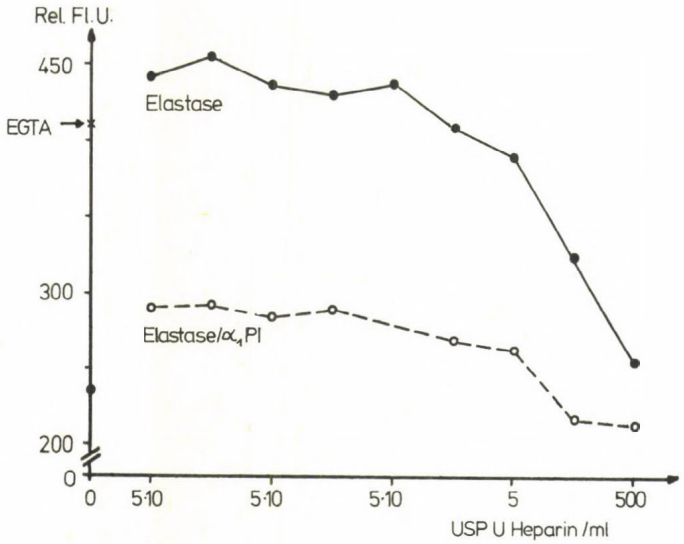

Fig. 10: Degradation of FITC-fibrinogen by elastase in the the presence of 500 to $5x10^{-6}$ USP U heparin/ml or 5 mmol/l EGTA in the test volume. 1 µg elastase/mg FITC-fibrinogen was incubated for 60 min at 37°C. High molecular weight fragments were precipitated by trichlorocetic acid and the acid-soluble fibrinopeptides were measured by its fluorescence relative to a standard by an Eppendorf-Fluorimeter expressed in relative fluorescence units (Rel.Fl.U.)

The results show, that beside the direct fibrinogen degradation by granulocytic elastase and its enhancement by heparin fibrinolysis may also be induced indirectly by α_2 plasmin inhibitor inactivation, which results in an increase in plasmin-mediated fibrinolysis. By our experiments in plasma the degradation of α_2 plasmin inhibitor in systemic fibrinolytic events seems unlikely as more than 450 µg elastase/ml plasma were required to inactivate the plasmin inhibitor. Fibrinogen degradation by elastase, however, which already occurred at lower elastase concentrations and probably also by α_2 macroglobulin-bound elastase may contribute to the bleeding tendencies observed in patients with septicemia and leukemia (Egbring et al., 1977; Deswald et al., 1982).

Nevertheless, following elastase release at inflammatory sites or during clot formation, fibrinolysis may be also indirectly mediated by α_2 plasmin inhibitor inactivation. Within such a microenvironment, heparin - released from basophils or mast cells (Camussi et al., 1978) - may further enhance the elastase induced fibrinogen degradation in concentrations, up to now considered as to low for anticoagulation.

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DISCUSSION

JOCHUM:

Have you ever used pathological plasma in your experiments? Probably in this case a part of α_1-proteinase inhibitor might be oxidized and the elastase could be able to degrade much earlier the α_2-antiplasmin present in plasma.

GRAMSE:

That is true, but even the oxidized α_1-proteinase inhibitor is very efficient with elastase.

JOCHUM:

Oh, no. That is not the case. Oxidized α_2-proteinase inhibitor is not at all effective with leukocytic elastase. There are some data published by Dr. Travis from Athens, Georgia, and he has shown that the oxidized inhibitor reacts with the elastase by a factor of about 2000 times less than the non-oxidized protein.

GRAMSE:

I think that the factor was 10 000. But it depends on the substrate you use and Dr. Travis used elastin, which has much higher affinity for elastase than α_2-plasmin inhibitor. And the other important thing is that the affinity of the substrate for elastase is different.

JOCHUM:

That is true, but the results I have mentioned were obtained with synthetic substrates, not with elastine.

GRAMSE:

For synthetic substrates it is true, but α_2-plasmin inhibitor is not very highly susceptible, and it has not such high affinity for instance for synthetic substrates, as for example elastase to elastin or fibrinogen or some complement factors.

ELŐDI:

Have you any evidence for the biphasic property of the binding plasmin inhibitor to elastase, since your Fig. 1 reminds me very much of a simple inactivation curve of an enzyme? This phenomenon follows usually apparently first order kinetics. Have you ever tried to plot semilogarithmically the time dependent data as a function of time?

GRAMSE:

It depends on the test system and our system measured not first order but second order reaction.

ELŐDI:

Have you tried a semi-logarithmic plot?

GRAMSE:

No, we didn't try to do so, but the authors who described this inactivation did so, and they made experiments also with ethylene amino caproic acid to show the difference between binding and inactivation. They could show the difference between the binding capacity of other two plasmin inhibitors.

ELŐDI:

Concerning the inhibitory effect of α_2-macroglobulin one would expect an opposite situation i.e. the inhibitory effect to be less pronounced with low molecular weight substrate and stronger with a protein. Besides, is there any explanation for the fact that even at high inhibitor concentration the enzyme is only partially inhibited?

GRAMSE:

I have no correct idea, but I believe it must be dependent on the competition between α_2-macroglobulin and fibrinogen as two protein substrates, and perhaps therefore the inactivation occurs at a higher molar excess of other two macroglobulins than with low molecular weight substrates.

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BENZAMIDINE DERIVATIVES AS INHIBITORS OF SERINE PROTEINASES SPECIFIC INHIBITION OF TRYPSIN AND THROMBIN

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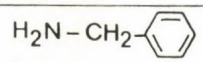
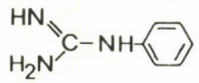
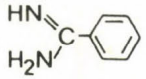
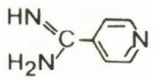
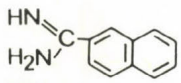
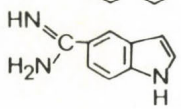
INTRODUCTION

Trypsin and trypsin-like serine proteinases cleave C-terminal bindings of basic amino acids, particularly those of arginine. Consequently, the first synthetic inhibitor of these enzymes, tosylagmatine (Lorand and Rule 1961), has been developed from the substrate tosylargininemethyl ester. Further basic structures of inhibitors are aromatic amines, guanidines and amidines (Table 1). Our group is engaged mainly in the development of competitive reversible inhibitors of the benzamidine type. However, in most cases benzamidine derivatives and likewise inhibitors of other type are no specific inhibitors of

Table 1. Inhibition of thrombin and trypsin by basic compounds

	K_i, µmol/l		
	Thrombin	Trypsin	References
	12300	300	Markwardt et al. 1968
	9000		Markwardt and Walsmann 1968
	220	35	Markwardt et al. 1968
	550	485	Geratz et al. 1979
	85	15	Markwardt et al. 1968
	7.7	29	Geratz et al. 1979

one of these enzymes due to the similarity of the active centres of the trypsin-like serine proteinases. They were found to possess inhibitory activity towards thrombin, factor Xa and other coagulation factors, kallikreins, C1-esterase, plasmin, urokinase, acrosin and other enzymes of this type besides trypsin.

Particular attention was directed to the development of specific inhibitors of thrombin. Thrombin is one of the key enzymes of blood coagulation. It initiates the conversion of fibrinogen into fibrin, it activates coagulation factors (V, VIII) and the fibrin-stabilising factor XIII and it triggers aggregation of blood platelets. Therefore, the inhibition of the enzyme thrombin represents an effective interference in blood coagulation. Synthetic, low molecular weight inhibitors of thrombin have stimulated growing interest since such substances could find potential use as orally active anticoagulants with a rapid action (Markwardt 1980). In order to avoid interactions of thrombin inhibitors with other serine proteinases of the blood in their in vivo use, particular attention was directed to the development of inhibitors with specific antithrombin activity. Comparison of the antitrypsin and antithrombin activity of various benzamidine derivatives demonstrates that synthetic inhibitors are capable of inhibiting relatively selectively one of the related enzymes.

DETERMINATION OF INHIBITORY ACTIVITY

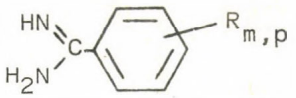
All derivatives presented are competitive inhibitors. The inhibitory activity was determined from the influence of the compounds on the thrombin- or trypsin-catalysed hydrolysis of Nα-benzoyl-D,L-arginine-p-nitroanilide at pH 8.0 and 25 °C (for details see Markwardt et al. 1973). When the K_i values for inhibition of thrombin decreased below 2 µmol/l the compounds were tight binding inhibitors and the more sensitive substrate H-D-Phe-Pip-Arg-pNA had to be used (Stürzebecher et al. 1983). In the case of trypsin, K_i values up to 0.2 µmol/l can be determined with Nα-benzoyl-D,L-arginine-p-nitroanilide as substrate. Data on the synthesis of the derivatives and their inhibitor constants have been reported in previous papers (Stürzebecher et al. 1976, 1981a, b, 1982, Walsmann et al. 1981).

BENZAMIDINE DERIVATIVES WITH NON-SPECIFIC INHIBITORY ACTIVITY

Studies of the inhibitory effect of benzamidine derivatives were started with the 3- and 4-substituted compounds listed in Fig. 1/Structure I (Markwardt et al. 1968, Walsmann et al. 1975).

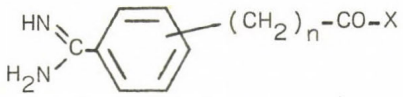
The inhibitory effect of some compounds is shown in Table 2. In most cases, the affinity of these compounds was higher for trypsin than for thrombin, the K_i values differed by about factor 10. Substituents with electron-donating and hydrophobic properties caused an increase in the inhibitory effect of benzamidine. This could be demonstrated also by Hansch analysis (Stürzebecher and Walsmann 1978, Labes and Hagen 1979).

Structure I



$R_{m,p}$ = Alkyl, Alkoxy, Aralkoxy, Halogen, NH_2

Structure II

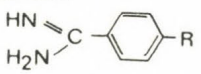


X = COOH, $COOC_2H_5$, Alkyl, Aryl; n = 1, 2

Fig. 1. Structure of benzamidine derivatives

From the structure-activity relationships the following binding sites can be postulated. First, the so-called specificity pocket to which the protonated side chain of arginine of the substrates is attracted is occupied by the benzamidine ring. Substituents with electron-donating properties influence the amidinophenyl moiety so that the affinity for the specificity pocket increases. Then, hydrophobic interactions with secondary binding sites occur.
Comparatively potent inhibitors were found among the 4-amidinophenyl compounds with ß-carbonyl function in the side chain

Table 2. Inhibition of thrombin and trypsin by benzamidine derivatives

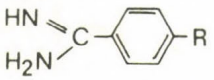


K_i . µmol / l

R	Thrombin	Trypsin
NO_2	>1000	330
COOH	>1000	200
OCH_3	720	70
Cl	380	40
H	220	35
NH_2	80	12
OC_8H_{17} (n)	24	6.0
CH_2O- 	58	9.0
CH_2O- 	6.6	2.6

(Fig. 1/Structure II). The antithrombin and antitrypsin activities of some representative compounds are listed in Table 3. In comparison with the isosteric compounds it is shown that the carbonyl function enhances the affinity of the inhibitors for the enzymes. In this group, no specific thrombin inhibitors were found. However, the ratio of the K_i values for inhibition of thrombin and trypsin is less than ten.
By quantitative structure-activity relationships detailed information was obtained about the mode of interaction between the carbonyl function and the enzyme. In order to describe the inhibitory effect, an electronic parameter of the inductive influence of the substituent X (Fig. 1/Structure II) localised at the carbonyl function was necessary besides parameters of the electronic and hydrophobic properties of the whole side chain. Both terms describe the binding of the benzamidine ring in the specificity pocket and hydrophobic interactions as in compounds of Structure I (Stürzebecher et al. 1976). This additional electronic parameter represents the interaction of the carbonyl function with the enzyme. That means, electron-accepting substituents that enhance the positive charge of the carbonyl function increase the inhibitory strength of the compound. Thus, the carbonyl function is believed to interact with a nucleophilic group of the enzyme, possibly with the active site serine alkoxide, by formation of a tetrahedral arrangement.

Table 3. Inhibition of thrombin and trypsin by benzamidine derivatives with a keto group



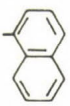
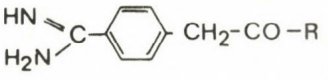
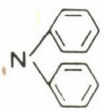
R	K_i . µmol/l Thrombin	Trypsin
CH_2-CH_2-CH_3	320	38
CH_2-CO-CH_3	68	40
CH_2-CH_2-COOH	>1000	500
CH_2-CO-COOH	6.5	1.6
CH_2-O- 	58	9.0
CH_2-CO- 	7.5	12
CH_2-CO-CH_2OCO- 	1.4	1.2

Table 4. Inhibition of thrombin and trypsin by amides of 4-amidinophenylacetic acid



K_i, µmol/l

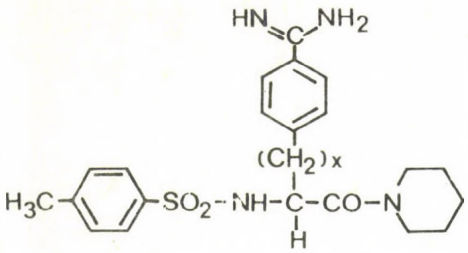
R	Thrombin	Trypsin
NH-CH_3	340	18
NH-C_3H_7	89	16
NH-C_4H_9	100	10
	28	43
	44	25
	20	62

BENZAMIDINE DERIVATIVES WITH SPECIFIC INHIBITORY ACTIVITY

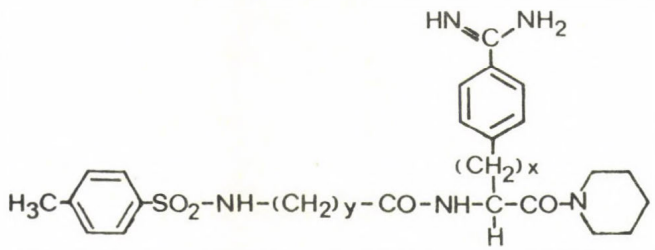
A number of compounds with certain thrombin specificity were found with secondary amides of 4-amidinophenylacetic acid (Walsmann et al. 1981) (Table 4). The primary and secondary amides possess the same antitrypsin activity, the secondary amides, however, are more potent inhibitors of thrombin. Since primary and secondary amide groups exert different inductive effects (Hansch et al. 1973, 1977), the carbonyl function seems to play a role, particularly in the binding of these inhibitors to thrombin.

This finding prompted us to examine further amides of amidinophenylalkylcarboxylic acids, particularly those whose side chain is branched via a sulfonamide group. The basic structure of the first type of inhibitors designed - the Nα-arylsulfonylated ω-amidinophenyl-α-aminoalkylcarboxylic acids - is shown in Fig. 2/Structure I. Variation of the position of the amidino group and chain length (x) and modification of the Nα- and the carboxyl substituent led to interesting structure-activity relationships which will be illustrated by some examples (Stürzebecher et al. 1981a, b, 1982).

An essential prerequisite for the specific inhibition of thrombin is a secondary amide structure, as shown in Table 5 for several amides of Nα-tosyl-(4-amidinophenyl)-alanine. Only the cyclic amides possess high affinity for thrombin This is also true for cyclic amides of various chain length (Table 6). The number of methylene groups is of great importance for the inhibitory activity. Only derivatives of 4-amidinophenylalanine (x = 1) and 2-amino-5-(4-amidinophenyl) valeric acid (x = 3) are able to inhibit thrombin effectively.



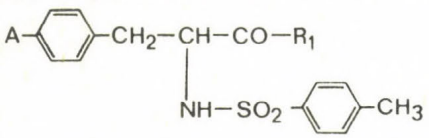
Structure I



Structure II

Fig. 2. Basic structure of Nα-arylsulfonylated (Structure I) and Nα-arylsulfonylaminoacylated (Structure II) ω-amidinophenyl-α-aminoalkylcarboxylic acid amides. The formulae show the piperidides with tosyl as arylsulfonyl residue.

Table 5. Inhibition of thrombin and trypsin by amides of Nα-tosyl-4-amidinophenylalanine [A = $C(NH)NH_2$]



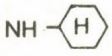
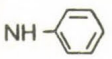
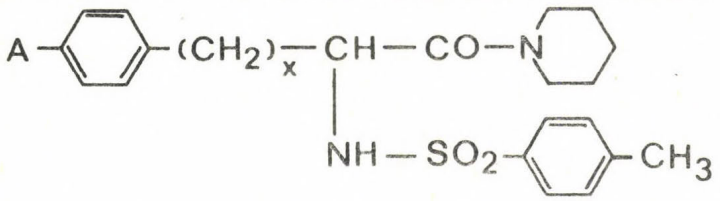
	K_i, µmol/l	
R_1	Thrombin	Trypsin
$NH-C_2H_5$	120	160
$NH-C_4H_9$	23	150
$NH-C_6H_{13}$	60	48
	340	130
	100	31
	5.9	58
	2.3	64
	5.4	80

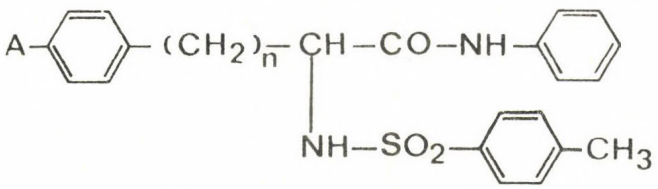
Table 6. Inhibition of thrombin and trypsin by piperidides of Nα-tosyl-(4-amidinophenyl)-α-aminocarboxylic acids



K_i, µmol/l

x	Thrombin	Trypsin
0	61	550
1	2.3	64
2	80	37
3	0.36	7.0

Table 7. Inhibition of thrombin and trypsin by anilides of Nα-tosyl-(4-amidinophenyl)-α-aminocarboxylic acids

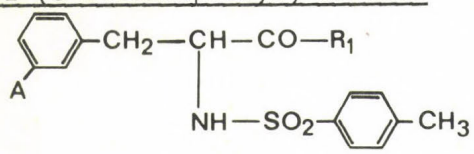


K_i, µmol/l

n	Thrombin	Trypsin
1	100	31
2	40	0.34
3	16	4.1

In contrast, the affinity for trypsin is enhanced with increasing chain length. The corresponding primary amides show another specificity of inhibition. As shown in Table 7 for the anilides, the antithrombin activity is enhanced with increasing chain length, however, it does not reach the inhibitor strength of the cyclic amides. All compounds of this type inhibit trypsin more potently than thrombin. Primary amides of 2-amino-4-(4-amidinophenyl)-butyric acid (x = 2) exhibit

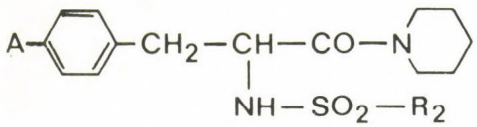
Table 8. Inhibition of thrombin and trypsin by amides of Nα-tosyl-(3-amidinophenyl)-alanine



K_i, μmol/l

R_1	Thrombin	Trypsin
$NH-C_4H_9$	0.36	3.9
	0.35	2.2
	0.34	1.2
	0.17	0.7

Table 9. Inhibition of thrombin and trypsin by piperidides of Nα-substituted 4-amidinophenylalanine



K_i, μmol/l

R_2	Thrombin	Trypsin
Tos	2.3	64
αNas	2.8	110
βNas	0.42	23
Tos – Gly	0.048	1.7
αNas – Gly	0.014	1.6
βNas – Gly	0.006	0.69
Tos – βAla	0.17	13
Tos – εAcp	13	49

Tos = tolylsulfonyl, αNas = α-naphthylsulfonyl, βNas = β-naphthylsulfonyl, εAcp = ε-aminocaproyl

the most pronounced inhibitory effects specifically directed against trypsin.

All inhibitors presented are 4-amidinophenyl derivatives. As shown in Table 8, also the isomeric 3-amidinophenyl compounds are potent thrombin inhibitors. The variation of the position of the amidino moiety, however, leads to one order of magnitude lower K_i values for thrombin and trypsin. The affinity of the butyl amide is increased by more than one order of magnitude. Thus, primary and secondary amides of 3-amidinophenylalanine possess similar inhibitory potency.
The most striking increase in affinity of 4-amidinophenylalanine derivatives for thrombin was attained after variation of the Nα-substituent (Table 9). Since the aromatic nucleus in P_2 contributes only slightly to the binding of the inhibitors to the enzymes - replacement of the tosyl residue for a naphthylsulfonyl residue had no effect - no further variation of the aromatic ring structure was carried out. Instead of this, an ω-aminoalkylcarboxylic acid was interpositioned between the α-nitrogen of 4-amidinophenylalanine and the arylsulfonyl residue (Stürzebecher et al. 1983). The secondary amides of these pseudo-peptides, especially the pyrrolidide and piperidide, with glycine as spacer are highly potent inhibitors of thrombin. The K_i of the most potent compounds reach the nanomolar range. Although these compounds exert more pronounced effects on trypsin than the corresponding Nα-arylsulfonyl derivatives, the K_i values for thrombin inhibition decrease more largely than those for inhibition of trypsin. In the case of the primary amides introduction of an aminoalkylcarboxylic acid spacer has no influence on the inhibitory effect of the Nα-arylsulfonylated compounds both of thrombin and trypsin.
In contrast to glycine, ß-alanine and ε-aminocaproic acid as spacer did not enhance significantly the inhibitory effect of the Nα-arylsulfonylated 4-amidinophenylalanine amides.

DISCUSSION

The question arises which structural elements of the amidinophenylamino acid derivatives account for the specific inhibitory activity and which interactions occur. As mentioned before, the extent and specificity of inhibition caused by these derivatives depend on the type of the amide component, the Nα-substituent and the chain length. Obviously, the carbon amide structure plays an important role in the enzyme-inhibitor interaction besides the benzamidine moiety. The carbon amide moiety of the inhibitor molecule might contribute to the interaction with the enzyme either by the hydrophobic part of the amine component or by the carbonyl group. Differences in hydrophobic interactions do not account for the specific affinity for both thrombin and trypsin, since the hydrophobic parameters of the various amines lie in the same order of magnitude. The electronic parameters of the amine groups, however, differ greatly. Secondary cyclic amine groups possess electron-accepting properties,primary amines are electron-donators (Hansch et al. 1973, 1977). That means, the carbonyl group of the two amide types is differently polarized. In the cyclic amides the electron density of the carbonyl carbon is reduced. In the primary amides, however, the electron density of the carbonyl oxygen is enhanced. Thus,

different interactions with the enzyme may occur. In the case of binding of secondary amides to thrombin an interaction between a nucleophilic enzyme moiety and the carbonyl carbon is believed. The nucleophile could be the Ser-195 alkoxide. In this case, a tetrahedral arrangement may occur without covalent binding. Similar enzyme-inhibitor interactions have been postulated for benzamidines with keto structure (Stürzebecher et al. 1976).

Since the electron density is enhanced in the carbonyl oxygen, the primary amides are believed to form preferentially hydrogen bonds. Such bridges could be formed with the amide nitrogens of Gly-193 and Ser-195 which form the so-called oxyanion hole. Interactions of this type have been demonstrated in the inhibition of trypsin by aprotinin (Bode 1979).

In the association reaction of inhibitors of the benzamidine type with thrombin or trypsin first the amidinophenyl moiety occupies the specificity pocket and next, secondary binding sites are involved. Topographical differences in the structure of the active sites of either enzyme might be the cause that the inhibitors with a carbonamide group are bound to thrombin preferentially via a nucleophilic mechanism, while they are bound to trypsin via an electrophilic mechanism. Optimum binding occurs when the carbonyl function is in a favourable position. Thus, only secondary amides of ω-amidinophenyl-α-aminoalkylcarboxylic acids with a certain length of the alkyl chain possess high and specific affinity for thrombin. In contrast, potent and specific inhibitors of trypsin were found among primary amides of 4-amidinophenyl-α-aminobutyric acid. In the case of Nα-arylsulfonylglycyl derivatives of 4-amidinophenylalanine, optimum binding of the Nα-substituent seems to occur which is not achieved with the Nα-arylsulfonyl derivatives. There are many hints that hydrophobic side chains of amino acids bound to the α-nitrogen of arginine play an essential role in the binding of peptide substrates and inhibitors to thrombin (Blombäck et al. 1969, Bajusz et al. 1978). Nevertheless, it does not appear reasonable to assume that the considerable increase in inhibitory activity by two orders of magnitude caused by introduction of a glycine spacer is solely due to hydrophobic binding. We would rather say that the interaction between the inhibitor carbonamide moiety and the active site of the enzyme is promoted as a consequence of the ideal fixation of the Nα-substituent on secondary binding sites. Summarising, Fig. 3 shows the enzyme binding sites occupied by a tight binding inhibitor.

The structure-activity relationships show some differences in the binding sites of thrombin and trypsin. Similar differences were observed also with other serine proteinases. Future development of even more active inhibitors could lead to still more detailed knowledge of the topography of the active site of serine proteinases and could lead to the development of highly specific inhibitors.

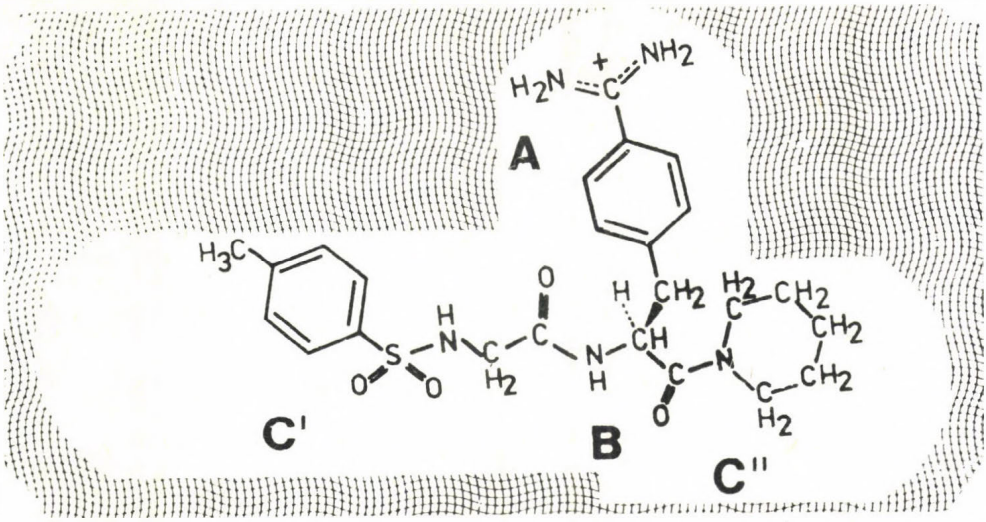


Fig. 3. Binding of Nα-tosyl-glycyl-(4-amidinophenyl)-alanine piperidide to the active centre

A = specificity pocket, B = catalytic mechanism, C', C'' = secondary binding sites

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DISCUSSION

JOCHUM:

You have shown a tremendous number of good thrombin inhibitors. Have you ever used one in animal models or clinically?

STÜRZEBECHER:

Synthetic low molecular weight inhibitors of thrombin are potential drugs for controlling blood coagulation. In our laboratory all potent inhibitor-candidates for thrombin have been examined for their effect on coagulation both in vitro and in vivo. They are able to prevent thrombus formation in animals. Some compounds have already been introduced in preclinical studies.

KORANT:

Have you seen any changes in the X-ray structure of trypsin bound to benzamidine? Since the compounds bind so tightly, there must be several points of attachment between the enzyme and the inhibitor.

STÜRZEBECHER:

Up to now, X-ray data have been obtained only for the trypsin-benzamidine-complex and not for complexes formed between trypsin and our new compounds of higher affinity.

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INTERACTION OF TRYPSIN-LIKE ENZYMES WITH SMALL INHIBITORS

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PEPTIDE INHIBITORS OF TRYPSIN-LIKE ENZYMES

The structure of peptide inhibitors and substrates of highly specific trypsin-like enzymes can be derived from the sequence at the cleavage site of physiological protein substrates. As the first example, peptide esters with anticoagulant activity were derived from the thrombin sensitive region of fibrinogen by Blombäck et al. /1969//Table 1/. Each peptide of this series contained Arg at the C-terminus and, the active members, like H-Phe-Val-Arg-OMe, Val as the penultimate residue which is identical with that residing at position P_2 of the fibrinogen Aα-chain. The N-terminal Phe was taken from position P_9 of the native substrate.

The peptide esters thus obtained are actually thrombin substrates rather than inhibitors. However, as such they can compete with the native substrate for the enzyme, thereby delaying coagulation in the thrombin-fibrinogen system. To double the clotting time of fibrinogen the ester substrates have to be present in about a 40-fold molecular excess over the native substrate. In addition, the inhibitory action of peptide esters disappeares progressively in some 10 min as the hydrolysis of the ester bond proceeds since the free peptides, which cannot combine with thrombin, are not inhibitory.

Abbreviations: -pNA: p-nitroanilide; -Boc: t-butyloxycarbonyl-;
-Bz-Arg-OEt: benzoyl-arginine-ethylester;
-Agm: agmatine

Table 1.

Sequence at substrate cleavage site of trypsin-like enzymes and peptide inhibitors derived therefrom

Enzyme	Physiological substrates	Sequence P_3—P_2—P_1-↓-P_1'	Inhibitors*
Thrombin	Fibrinogen A α-chain	Gly-Val-Arg-↓-Gly	H-Phe-Val-Arg-OMe
	Prothrombin	Ile-Pro-Arg--Ser	H-D-Phe-Pro-Arg-H**
	Factor XIII	Val-Pro-Arg--Gly	Boc-D-Phe-Pro-Arg-H
Plasmin	Fibrin/ogen/	Asn-Phe-Lys--Ser	Boc-Gln-Phe-Lys-H
		Glu-Trp-Lys--Ala	
		Gly-Tyr-Arg--Ala	
Kallikrein	Kininogen	Pro-Phe-Arg--?	H-D-Pro-Phe-Arg-H***
			H-D-Glp-Phe-Arg-H

*H-Phe-Val-Arg-OMe was prepared by Blombäck et al. /1969/, the others, Bajusz et al. /1975, 1978, 1982/

, ***Chloromethyl ketone derivatives were prepared by Kettner and Shaw /1978*, 1979**/

To overcome these shortcomings, the terminal ester function has to be replaced by a non-hydrolyzable group, like the aldehyde, chloromethyl ketone or, perhaps, by a secondary amide group. Of these, the aldehyde function was chosen in our studies /Bajusz et al., 1975/. The first representatives of peptide aldehyde inhibitors of serine proteases, mainly tri- and tetrapeptides, such as leupeptin and antipain, were natural products, isolated from culture filtrates of Streptomyces species by Umezawa and his colleagues /cf. Umezawa, 1972/.

Accordingly, in our work, the findings of Blombäck and Umezawa were combined. The C-terminal and the middle residues were taken from the physiological substrates, those found at positions P_1 and P_2, respectively, while the N-terminal moieties were selected experimentally.

In the case of thrombin inhibitors Pro at middle position, corresponding to the sequence of native substrates prothrombin and factor XIII, proved to be more favourable than Val residing at P_2 of the fibrinogen Aα-chain. Both Boc- and H-D-Phe-Pro-Arg-H showed high anticoagulant activity with an I_{50} value* of 1.4×10^{-7} M.

In contrast to thrombin, which can only split two Arg-Gly bonds of fibrinogen, plasmin has much less specificity since it cleaves - at different rates - about 20 of the 362 Arg-X and Lys-X bonds of fibrin. Differences in the susceptibility of peptide bonds to plasmin may mainly be due to differences in accessibility, but some correlation can be observed between the hydrolysis rate of peptide bonds P_1-P_1' and the subsites P_2 and P_1. Lys at P_1 seems to be more susceptible than Arg since most of the bonds cleaved during 10-min digestion are Lys-X, and most of the fragments attacked by plasmin first contain an aromatic residue at P_2. Three of such sequence portions are shown in Table 1. From these structural elements, Boc-Gln-Phe-Lys-H was developed as a rather good inhibitor of the plasmin-fibrin reaction with an I_{50} value* of 1.1×10^{-7} M

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*Molecular concentration required for doubling the reaction time.

/Bajusz et al., 1981/. H-D-Pro-Phe-Arg-H also showed the expected inhibitory action on plasma kallikrein both *in vitro* and *in vivo*. Namely, the esterolytic activity of the enzyme, measured with Bz-Arg-OEt, could completely be inhibited at a peptide concentration of about 1 x 10 M, and a dose of 5 mg/kg could completely block the paw-oedema-producing effect of kallikrein in the rat /Tolnay, P., Janáky, I., unpublished results/.

Except Boc-D-Phe-Pro-Arg-H, the tripeptide aldehydes proved to be specific inhibitors. For instance, H-D-Phe-Pro-Arg-H, the highly potent inhibitor of the thrombin-fibrinogen reaction, has practically no effect on the plasmin degradation of fibrin gel and on the hydrolytic actions of kallikrein. Its Boc derivative, the other powerful thrombin inhibitor, neither has any effect on kallikrein but, additionally, it can inhibit fibrinolysis by plasmin as well as plasminogen activation by urokinase and the proteolytic action of trypsin. Thus one may take Boc-D-Phe-Pro-Arg-H as a "general inhibitor" of a group of trypsin-like enzymes - at least in systems involving protein substrates /Bajusz et al., 1978/.

The chloromethyl ketone analogue of the two tripeptide aldehydes, H-D-Pro-Phe-Arg-H and H-D-Phe-Pro-Arg-H, have been prepared by Kettner and Shaw /1978; 1979/ and found to be highly active and specific irreversible inhibitors of kallikrein and thrombin, respectively.

BINDING OF INHIBITORS TO TRYPSIN-LIKE ENZYMES

Peptide aldehydes and peptidyl chloromethyl ketones

The findings presented above may indicate a certain distribution of specificity-determining sites of these enzymes /Fig. 1/.

Common specificity of trypsin-like enzymes is obviously determined by their Asp-189 residue /when using chymotrypsin numbering/ which can recognize and bind the basic side chain of Arg or Lys at position P_1 of substrates and inhibitors.

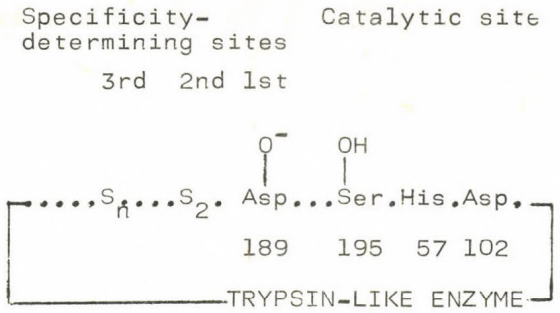

Fig. 1. Specificity determining- and catalytic sites of trypsin-like enzymes. S_2 and S_n denote subsites being responsible for binding residues P_2 and P_n, respectively, of substrates/inhibitors. Chymotrypsin numbering is used.

Thus, Asp-189 corresponds to the subsite residing at position S_1 of these type of enzymes. Subsite at S_2 which is to recognize and bind the amino acid residue at P_2 of substrates and inhibitors seems to be responsible for the individual specificity of enzymes. This specificity can be increased further with the assistance of subsites at positions S_3, S_4, etc.

There can be no doubt that peptide inhibitors and substrates being flexible molecules bind to the enzymes according to the "zipper" model /Burgen et al., 1975/ which term covers a kind of stepwise binding.

Schematic representation of the zipper model for binding of fibrinogen and inhibitors to thrombin is given in Fig. 2. The initial, "nucleation" complex can be formed by the ionic interaction discussed above. This is followed by a series of conformational rearrangements of the pertly bound substrates and inhibitors leading to binding of Val and Pro at P_2 of fibrinogen and the inhibitor, respectively, to the corresponding subsite at S_2, then the remaining segments at P_n positions combine with their appropriate subsites at S_n positions. Finally, the CO group of the Arg residue at P_1 reacts with the active OH group of thrombin. In the case of the

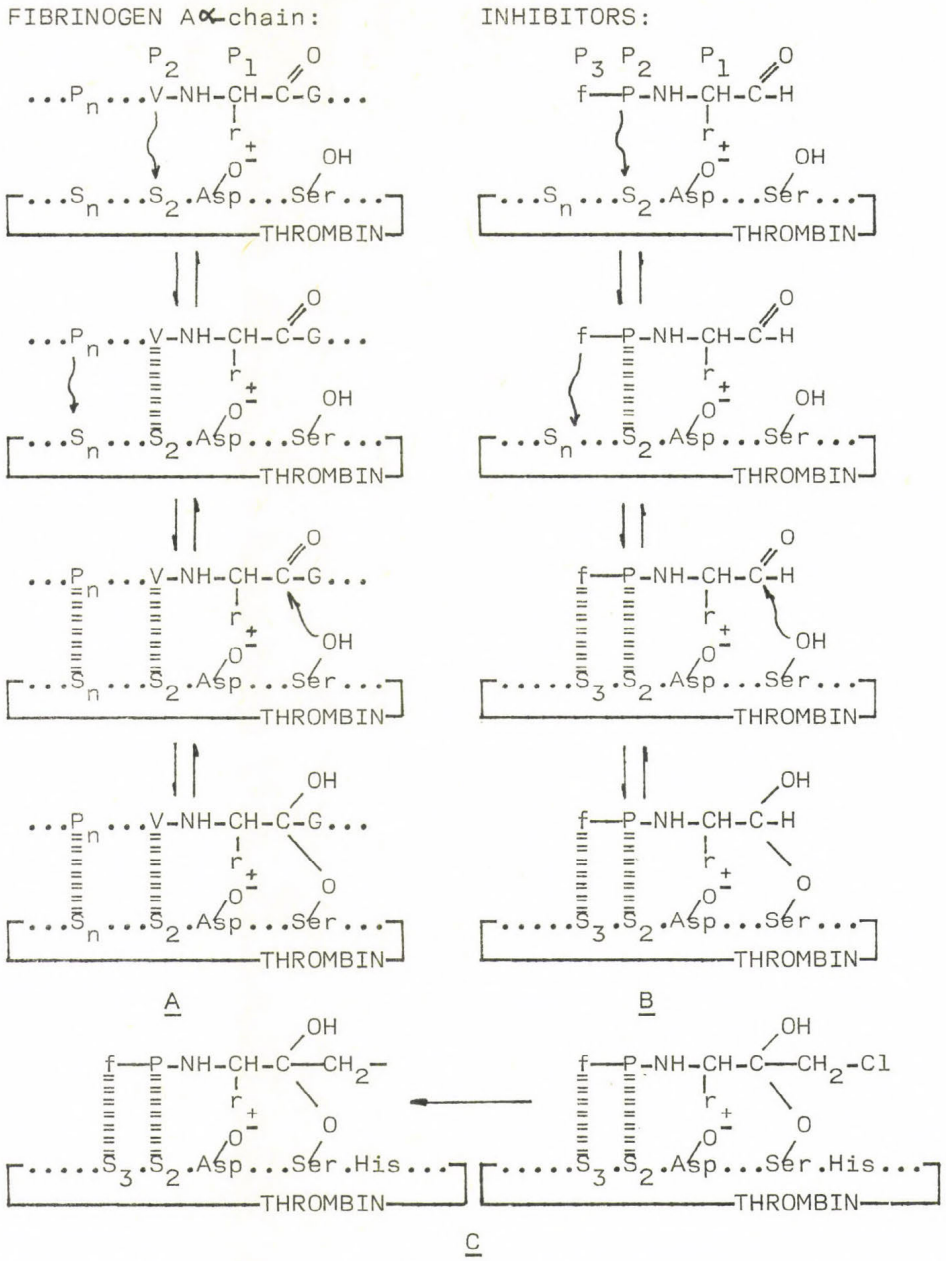

Fig. 2. Interaction of thrombin with fibrinogen (A) and inhibitors: H-D-Phe-Pro-Arg-H(B) and H-D-Phe-Pro-Arg-CH_2Cl(C). G, P, V and f are one-letter symbols for Gly, Pro, Val and D-Phe, resp., r_+ stands for the side chain of Arg.

chloromethyl ketone, a second covalent bond can also be formed by which this inhibitor will be irreversibly fixed in the active center of thrombin.

The high anticlotting activity of these tripeptide inhibitors may indicate that the enzyme-inhibitor complex is formed readily even in the presence of the native substrate fibrinogen. It seems likely that the thrombin subsite at S_2 shows higher affinity to Pro than Val at P_2 of fibrinogen. In addition, the N-terminal D-Phe, having an "unnaturally" oriented side chain, may bind to a subsite at an S_3 which is not involved in complexing fibrinogen.

According to this model, if no favourable interactions between subsites at P and S positions, in particular between those at P_2 and S_2, can be formed, the "nucleation" complex, being stabilized by a single salt bridge only, will dissociate, and so no hydrolysis or inhibition can occur.

Peptide inhibitors lacking terminal reactive groups

The primary importance of side chains in binding of peptide inhibitors to thrombin could be demonstrated by the fact that H-D-Phe-Pro-Arg-H and H-D-Phe-Pro-Arg-CH_2Cl were by 3-4 orders of magnitude more inhibitory than H-Gly-Val-Arg-H and H-Gly-Val-Arg-CH_2Cl, respectively. In this context we may also examine the significance of the C-terminal reactive group when it is linked to a suitably assembled peptidyl portion. For this purpose we prepared /Bajusz et al., 1983/ H-D-Phe-Pro-Agm, an analogue of the two highly potent inhibitors, lacking only the -CHO and -$COCH_2Cl$ functions, respectively, /Fig. 3/ and compared its anticlotting activity with that of the corresponding tripeptide aldehyde /Table 2/.

The high potency of H-D-Phe-Pro-Agm, about 1/4 of that showed by H-D-Phe-Pro-Arg-H, supports the view that peptide portions /and thereby the so-called secondary forces, i.e. ionic and hydrophobic bonds/ can be determinant in the formation of enzyme-inhibitor complexes. The aldehyde group, or rather the covalent bond formed by this group, could increase

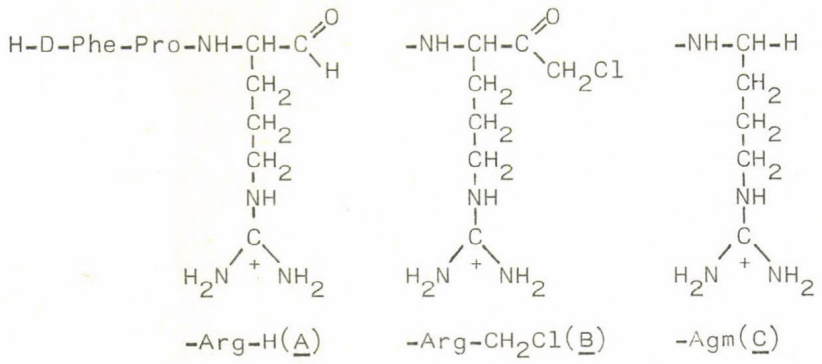

Fig. 3. Structure of H-D-Phe-Pro-Arg-H /A/, its chloromethyl ketone analogue /B/ and the corresponding agmatine peptide /C/.

the stability of the complex, which, in this particular case, resulted in a four-five-fold enhancement of the anticlotting activity. A much higher effect of the aldehyde group could be observed when activities of the former tripeptides /and their Boc derivatives/ were compared in systems comprising peptide substrates, e.g. Z-Phe-Val-Arg-pNA. In these experiments the antitrypsin activities were also examined.

Table 2

Antithrombin activity of tripeptides with fibrinogen substrate

Peptide	I^{*}_{50}	K_i
H-D-Phe-Pro-Arg-H	1.4×10^{-7} M	5×10^{-8} M
H-D-Phe-Pro-Agm	6.6×10^{-7} M	2×10^{-7} M

*Molecular concentration required for doubling the clotting time of fibrinogen.

K_i values presented in Table 3 indicated that both thrombin and trypsin were inhibited 20-30 times more ef-

fectively also by the free and the Boc tripeptide aldehydes than the corresponding Agm peptides. Moreover, inhibitory action of the Agm peptides on trypsin could only be observed after 10-min incubation. Accordingly, the three amino acid side chains of these inhibitors can play a more important role in binding of the highly specific thrombin than in that of the less specific trypsin - at least in the presence of the tripeptide substrate Z-Phe-Val-Arg-pNA.

Table 3

Antithrombin and antitrypsin activity of tripeptides with Z-Phe-Val-Arg-pNA substrate

Peptide	K_i /μM/ Thrombin	Trypsin
H-D-Phe-Pro-Arg-H	0.08	0.17
H-D-Phe-Pro-Agm	2.00	6.00*
Boc-D-Phe-Pro-Arg-H	0.20	0.08
Boc-D-Phe-Pro-Agm	4.60	2.00*

*Determined after 10-min incubation.

Since heparin has long been known to enhance the interaction of thrombin with its physiological protein inhibitor antithrombin-III, and, according to a recent note /Griffith et al., 1979/, it can also increase the binding affinity of peptidyl arginine chloromethyl ketones, the question may arise whether thrombin inhibition by our tripeptides, in particular by the Agm peptides, can be catalyzed by heparin /Bajusz et al., 1982; 1983/.

K_i values for the four tripeptides /Table 4/ were determined at heparin/thrombin molecular ratios* of 0.1 and 1 or 2, respectively, with fibrinogen and Z-Phe-Val-Arg-pNA, respectively, as substrates.

No substantial effect of heparin could be observed. Activities of the two aldehydes were not influenced at all. Using the synthetic substrate, a slight effect could only be found at a heparin/thrombin ratio of 2:1 when heparin can be inhibitory in its own right, too. As the best result K_i's for the two Agm peptides in the thrombin-fibrinogen system were lowered by a factor of about 3 at a 1:1 molecular ratio of heparin to thrombin. In this case the existance of a thrombin-heparin-peptide inhibitor ternary complex cannot be excluded either. In any case, the antithrombin activity of the free Agm peptide is more similar to that of the free tripeptide aldehyde, and so H-D-Phe-Pro-Agm could show significant anticlotting activity in vivo, too /Bajusz et al., 1982/.

Inhibitors with rigid conformation

According to some data, certain enzyme inhibitors having molecules with limited degrees of conformational freedom could be more effective than their flexible congeners. For instance, ornithine decarboxylase could be inhibited by

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* Calculation of the molecular activity of heparin was based on the findings of Rosenberg et al. /1978; 1979/ and Jordan et al. /1979/. Fractionation of commercial heparin resulted in polysaccharide chains with different activities and molecular weights. Some of the highly potent fractions with molecular weights of 6-7000 daltons had specific activities of 350-363 USP U/mg. Another species with approximate molecular weight of 20 000 daltons contained two binding sites for antithrombin-III and showed an activity of 738 USP U/mg. Based on the big difference between the binding constants obtained for the highly active and relatively inactive heparin, /i.e. $1x10^{-7}$ M and $1x10^{-4}$ M, respectively/ the effect of the less potent /4-12 USP U/mg/ and inactive components of commercial heparin was neglected. Thus an average molecular weight of 8000 daltons and a specific activity of 362 USP U/mg might be calculated. Accordingly, 2.9 USP U of heparin may be taken as 1 nmole.

Table 4

Thrombin inhibition by tripeptides /H/Boc-D-Phe-Pro-Agm/Arg-H/ and the catalytic effect of heparin thereon* / determined in the absence /A/ and presence /B/ of heparin; substrate: fibrinogen and Z-Phe-Val-Arg-pNA/

Peptide	K_i values /μM/ Fibrinogen A	Fibrinogen B[a]	Fibrinogen B[b]	Z-Phe-Val-Arg-pNA A	Z-Phe-Val-Arg-pNA B[b]	Z-Phe-Val-Arg-pNA B[c]
H-D-Phe-Pro-Agm	0.2	0.1	0.07	2.65	2.65	1.25
H-D-Phe-Pro-Arg-H	0.05	0.05	0.05	0.08	0.08	0.08
Boc-D-Phe-Pro-Agm	1.75	1.75	0.60	4.6	4.6	2.4
Boc-D-Phe-Pro-Arg-H	0.1	0.1	0.1	0.2	0.2	0.2

*Heparin/thrombin molecular ratios were 0.1[a], 1[b] and 2[c], respectively. 2.9 USP U of heparin and 113 NIH U of thrombin were taken as 1 nmole.

1,4-diaminobut-2-ene more efficiently than by the saturated analogue, 1,4-diaminobutane, and the unsaturated analogue of substrate ornithine, i.e. 3-dehydro-ornithine, could behave as inhibitor /Relyea and Rando, 1975/. Presumably, when the latter binds to the enzyme, and loses its carboxyl group, the unsaturated product, 1,4-diaminobut-2-ene, remained bound to the enzyme for a longer time than its saturated analogue. The double bond can stabilize the transform of the carbon chain, thereby giving a fixed orientation to both amino groups. By analogy, in our case, dehydro-agmatine might be profitable at the terminus of H-D-Phe-Pro. In this context it may be noted that highly potent reactants of trypsin-like enzymes, such as p-nitrophenyl-p-guanidinobenzoate /Chase and Shaw, 1967/, esters of p-guanidinophenylacetic acid and p-amidino-benzoic acid /Mares-Guia et al., 1967/, and substituted benzamidines /cf. Mares-Guia et al., 1977/ all have molecules with more or less rigid conformation. Prompted by these findings, we have prepared some dipeptide amides in which the above-mentioned rigid moieties were linked to the dipeptidyl portion H-D-Phe-Pro. These compounds together with their inhibitory action on the thrombin-fibrinogen reaction are given in Table 5 /Juhász, A., Bajusz, S., Barabás, É. and Bagdy, D.; unpublished results/.

Contrary to all expectations, replacement of the Agm residue by its unsaturated analogue led to a less potent compound. When compared at a higher level /indicated by values I_{80}/ a 3-fold decrease of activity could be observed.

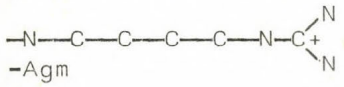
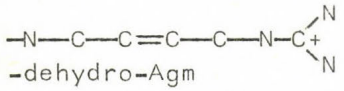
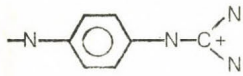
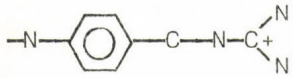
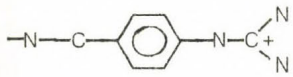
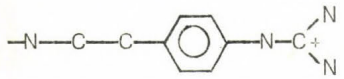
The other dipeptide amides were practically inactive in spite of comprising the favourable dipeptidyl portion H-D-Phe-Pro and the p-guanidinophenyl /or benzyl/ moiety of the highly potent aromatic reactants.

This failure may be interpreted as follows. The esters of p-guanidinobenzoic acid and p-guanidinophenyl-acetic acid being rigid small molecules can react according to the "lock-and-key" model. In this one-step binding, when the guanidino group forms a salt bridge with Asp-189, the ester function is simultaneously delivered to the catalytic site to

form an acyl-enzyme. In our compounds the p-guanidinophenyl moiety is carried by a bulky dipeptidyl portion being apt to binding to the enzyme rather than to be split off. In addition, unlike to the corresponding Agm peptide rearrangement of the side chains and thereby accomodation of the dipeptide portion to subsites at positions S_2 and S_3, could not take place because of the rigidity of the amide moiety.

Table 5

Antithrombin activity of H-D-Phe-Pro-Agm and analogues of type H-D-Phe-Pro-NH-X-NH-$\overset{+}{C}(NH_2)_2$ with fibrinogen substrate

Sceleton of amide portions -NH-X-NH-$\overset{+}{C}(NH_2)_2$	I^*_{50}	I^*_{80}
 -Agm	4×10^{-7}M	1×10^{-6}M
 -dehydro-Agm	5×10^{-7}M	3×10^{-6}M
	»1×10^{-3}M	-
	»1×10^{-3}M	-
	»1×10^{-3}M	-
	»1×10^{-3}M	-

*Molecular concentration required for prolonging the clotting time of fibrinogen by factors of 2 (I_{50}) and 5 (I_{80}), resp.

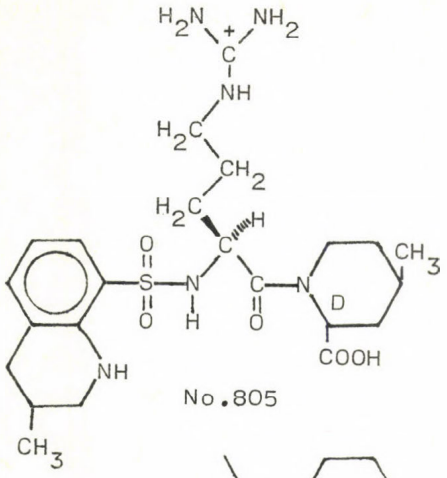
As it has been discussed above, inhibition of trypsin-like enzymes by peptide aldehydes involves - besides ionic and hydrophobic bonds - the formation of tetrahedral semi-

acetals /Westerik and Wolfenden, 1972/. p-Nitrophenyl p-guanidino-benzoate and related esters form acyl-enzymes which intermediates have been shown to deacylate unusually slowly, permitting their use as inhibitors /cf. Chase and Shaw, 1969/. H-D-Phe-Pro-Agm /Bajusz et al., 1983/ and derivatives of benzamidine /cf. Mares-Guia et al., 1977/ can combine with trypsin-like enzymes by ionic and hydrophobic bonds merely.

Acylamino acids

A further kind of complexes can be formed by acylamino acids, like arylsulfonyl arginine amides /Okamoto et al., 1975/ and the corresponding p-amidinophenylalanine /Wagner et al., 1981; Stürzebecher, 1982/ or p-guanidinophenylalanine /Claeson et al., 1983/ derivatives synthesized as thrombin inhibitors. A detailed analysis of structure-activity relationships among the Arg compounds /Okamoto et al., 1980; Kikumoto et al., 1980a, 1980b; Okamoto and Hijikata, 1981/ revealed the primary importance of an arylsulfonyl group as an N-substituent and an imino group, preferentially a cyclic imino acid residue, as the carboxamide portion. As it appears from the structure of the most potent compound of the Japanese group, No. 805 in Fig. 4, the imino acid portion, 4-methyl-2-piperidinecarboxylic acid, has D configuration. Four orders of magnitude less potency was reported for the L isomer. Accordingly, apart from its side chain, this highly active inhibitor of thrombin has nothing in common with the native substrates of this enzyme. Namely, the arylsulfonyl group residing in place of an amino acid residue at P_2 is unusual both in size and chemical character, i.e. while the peptide bond /CONH/ is practically neutral, the sulfonamide /SO_2NH/ is acidic. Moreover, like other trypsin-like enzymes, thrombin does not cleave peptide bonds involving a D-amino acid residue, and particularly not those formed by an imino acid, i.e. dialkyl amine, like the Arg-Pro bond.

It can be supposed that inhibitors of unusual structure may bind to enzymes through unusual interactions. Binding sites for the arylsulfonyl and carboximide portions of



Antithrombin activity

I_{50}	K_i
3.2×10^{-8}M	1.9×10^{-8}M
1.5×10^{-4}M	2.8×10^{-4}M

Fig. 4. Structure and antithrombin activity of No. 805 and one of its isomers /Okamoto and Hijikata, 1981/.

No. 805 and its congeners, must be other than those for the corresponding amino acid residues at positions P_2 and P_1' of the native substrates. It is also an open question whether the active OH group of thrombin can react with the dialkylamido group of the inhibitor to form a sort of tetrahedral adduct.

In my opinion the high antithrombin activity of No. 805 may just be due to the formation of such an adduct stabilizing the enzyme-inhibitor complex. In the case of its L-imino acid-containing analogue, however, the attack of the active OH group may not take place because of a steric hindrance caused by the carboxyl group of the L-imino acid portion. It is, of course, also possible that the COOH group of No. 805 can stabilize the enzyme-inhibitor complex by forming a salt bridge with an otherwise insignificant basic side chain of thrombin but this or similar ionic bond cannot be formed between the L-imino acid portion and the enzyme.

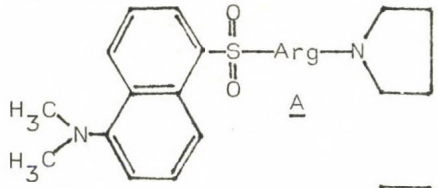
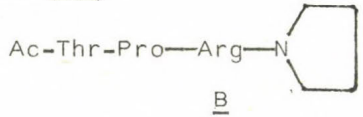
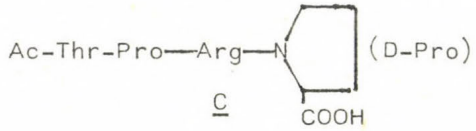
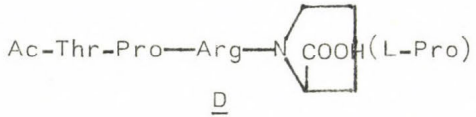
Pertaining to the structural requirements for the acyl and carboxamide portions of this type of inhibitors, Table 6 presents the anticlotting activity of dansyl-L-arginyl-pirrolidine of the series of Okamoto's group /Kikumoto et al., 1980a/ and some peptides prepared in our laboratory /Fauszt et al., 1983/. Comparing the potencies of compounds A/B and B/C/D, respectively, two conclusions may be drawn. First, as an acyl moiety, dansyl is much more favourable than the dipeptidyl portion Ac-Thr-Pro - in spite of endowing Pro at P_2. This finding in concert with those presented in Table 5 give evidence that combination of molecular portions with different binding patterns may not be fruitful. Secondly, if there is a substituent at the position of the carboxamide portion, it must have the D configuration - as it has been found with the highly potent inhibitor No. 805 /Fig. 4/.

The peptides presented in Table 6 were the Arg analogues of hirudine fragment Gly-Thr-Pro-Lys-Pro, which portion together with the preceeding segment Val-Thr-Gly-Glu, is the putative inhibitor site of this highly active and selective thrombin inhibitor of the leech /Magnusson et al., 1976/. As shown in Table 7, the D-Pro-containing one of the two Lys-peptides /Fauszt, I., Bajusz, S., Barabás, É., Diószegi, M. and Bagdy, D.; unpublished results/ possessed the higher activity, and both were more inhibitory than the corresponding Arg analogue, in spite of the fact that thrombin preferentially cleaves Arg-X bonds, when X is an amino acid residue other than Pro.

In the molecule of hirudin, the Pro-Gln bond in the segment Pro-Lys-Pro-Gln must unusually be distorted to make an interaction possible between the Lys-Pro bond and the active Oh of thrombin. Still, in my opinion formation of the thrombin-hirudin complex could be started by interactions between the clustered acidic residues of the C-terminal fragment of hirudin /Glu-Glu-Ile-Pro-Glu-Glu-Tyr[SO_3^-]-Leu-Gln/ and some of the five pairs of basic residues found in thrombin /i.e. Lys-Lys, Arg-Lys and Lys-Arg/ rather than by saltbridge for-

Table 6

Antithrombin activity of dansyl-L-arginyl-pirrolidine /A/ and relating Arg peptides /B-D/ with fibrinogen substrate

Inhibitor	I^{*}_{50}	I^{*}_{80}
 A	1×10^{-6} M**	-
 B	4×10^{-4} M	2.9×10^{-3} M
 C	9×10^{-4} M	4.3×10^{-3} M
 D	16×10^{-4} M	17.6×10^{-3} M

*See Table 5. **Kikumoto et al., 1980a.

mation between Asp-189 and the Lys residue of hirudin[×].

Table 7

Antithrombin activity of hirudin peptide Ac-Thr-Pro-Lys-Pro-NH_2 and analogues with fibrinogen substrate

Peptide	$I^{×}_{50}$, mM	$I^{×}_{80}$, mM
Ac-Thr-Pro-Lys-L-Pro-NH_2	0.78 /1.6/	3.0 /17.6/
Ac-Thr-Pro-Lys-D-Pro-NH_2	0.62 /0.9/	1.6 /4.3/

[×]Defined as in Table 5; values for the corresponding Arg peptides are given in parentheses.

With the findings and speculations concerning hirudin and its fragment-analogues I only wanted to exemplify that sometimes a peculiar enzyme-inhibitor interaction may be found in nature, too.

SUMMARY

According to the above, three main functional portions could be distinguished in the inhibitors of trypsin-like enzymes. The first one is a basic residue relating to the side chain of Arg or Lys, that forms a salt bridge, the first bond between the enzyme and the inhibitor. The second portion, usually comprising more than one grouping, endows the inhibitor with an ability to be accomodated to the given enzyme much better than to any other of the same type, thereby increasing specificity. The third portion, a reactive group, stabilizes the enzyme-inhibitor complex. Apart from the basic residue and the reactive group mentioned first and last, we hardly know anything about the structural requirements for

[×]Unsuitability of Lys at P_1 could be demonstrated by comparing the I_{80} values of 4×10^{-4} M and 7×10^{-7} M obtained for Boc-D-Phe-Pro-Lys-H and Boc-D-Phe-Pro-Arg-H, respectively.

the others comprised by the three main functional portions. As a corollary, construction of an inhibitor is nothing else but effort to find the most favourable counter-sites of certain completely unknown subsites of given enzymes in order to obtain a highly stable enzyme-inhibitor complex. That is the very reason why peptide inhibitors should be preferred. It has been examplified that binding pattern of peptides to enzymes has much in common with enzyme-substrate interactions, and sequences around the cleavage sites of physiological substrates are frequently known. With this knowledge, the two amino acid residues being favourable at positions P_2 and P_1 can easily be selected. Since a molecular size of a tripeptide or an acyl tripeptide is generally satisfactory, there is only one amino acid and possibly an acyl group left to be selected experimentally.

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DISCUSSION

STEPANOV:

You certainly mean geometric isomers, i.e. cis/trans isomers. In the case of dehydro-agmatine the trans-form can only be obtained.

BAJUSZ:

I know neither about the dehydro derivative nor about the activity of the cis isomer of D-Phe-Pro-ΔAgm.

JOCHUM:

Have you ever used your inhibitors in animal or clinical experiments?

BAJUSZ:

Preclinical testing of D-Phe-Pro-Arg-H has already been carried out. This reversible inhibitor has favourable properties both in vivo and in vitro, e.g. it does not bind irreversibly to the protein components of blood /red cells or other components of large surfaces and good binding properties/. I think this compound will soon find its way to clinical application.

Symposia Biologica Hungarica 25 (1984)

COMPARISON OF THE EFFECTS OF LYSOSOMAL PROTEOLYTIC INHIBITORS IN VIVO, ON THE PERFUSED LIVER AND ON ISOLATED LYSOSOMES

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SUMMARY

The effects of various proteolytic inhibitors - and leupeptin in particular - were tested in different experimental designs, namely in vivo, during liver perfusion and in vitro. Injection of leupeptin to rats caused a prompt increase in autophagic vacuoles by 0.5-1 hour. The proteolytic capacity of the isolated autophagic vacuoles was assessed as a function of treatment time. Generation of TCA-soluble radioactivity in isolated autophagic vacuoles from rats prelabelled with ^{14}C-L-leucine for 16 hours before sacrifice displayed a minimum /50% inhibition/ 0.5-1 hour following leupeptin injection. Conversely, after longer leupeptin exposure times /up to 16 hours/ the rate of proteolysis increased over the control value /catch up proteolysis/ with a maximum by 4 to 6 hours.

The effect of leupeptin on degradation of long and short lived proteins was studied also in the liver perfusion model. Proteins were labelled for 30 minutes or 16 hours by an in vivo injection of ^{14}C-L-leucine. Leupeptin was injected into rats 15 minutes before sacrifice and was also included in the perfusate. Leupeptin decreased the proteolytic rate assayed as release of TCA-soluble label to the perfusate by 30 and 50% for short and long labelling periods, respectively. The per-

Abbreviations: AVs: autophagic vacuoles; TCA: trichloroacetic acid.

fused livers were also subfractionated. The autophagic vacuoles were isolated and their proteolytic activity monitored. In this system leupeptin was found to cause 70% /short lived proteins/ and 80% /long lived proteins/ inhibition of breakdown. Extrapolating the inhibition of lysosomal proteolysis to 100% allows one to conclude that the nonlysosomal pathway/s/ would account for roughly 40% /long labelled/ and 55% /short labelled/ of total proteolytic activity in the perfused rat liver.

Isolated liver lysosomes from rats prelabelled for 30 minutes and 16 hours as above were also incubated in the absence and presence of either propylamine, leupeptin or chloroquine. The highest proteolytic rate was obtained following short labelling times /15 minutes and 2 hours/. Accordingly, we conclude that lysosomes are active in the degradation of both long and short lived proteins. Irrespective of labelling period and tested agent 100% inhibition was never recorded. As far as can be judged from experiments with isolated lysosomes the lysosomal inhibitors may thus not be used under the assumption that they cause a complete shut down of lysosomal proteolytic activity.

INTRODUCTION

The lysosomes have long been regarded as the only well documented and well characterized intracellular locus of protein degradation. On the basis of electron microscopic evidence it has generally been assumed that organelles such as e.g. mitochondria are degraded within lysosomes /Glaumann et al., 1981/. An important role for lysosomes in organelle degradation also appears likely since they contain all enzymes required to degrade proteins, lipids and carbohydrates /Barrett and Heath, 1977/. Experiments performed with bacteria /Goldberg and St. John, 1976/ and cell free reticulocyte extracts /Etlinger and Goldberg, 1977/ show that abnormal proteins have a higher rate of turnover than normal protein. Moreover, studies on cultured cells /Knowles and Ballard,

1976/ suggest a nonlysosomal pathway of degradation for abnormal and other short lived proteins. Recently, rat liver mitochondria have been shown to contain a proteolytic system that can hydrolyse proteins to constituent amino acids /Desautels and Goldberg, 1982/. The presence of one or several extra lysosomal pathways is appealing since the lysosomal sequestration mechanism is believed to be indiscriminate and is therefore difficult to reconcile with the finding that the half-lives of mitochondrial and cytosolic proteins differ considerably.

There are at least three different ways for endogenous proteins to gain access to the lysosomal apparatus, namely by means of autophagy, microautophagy and crinophagy /Marzella et al., 1981/. The process by which cells sequester and subsequently degrade their organelles has been designated autophagy. This mechanism operates in cells under normal conditions /De Duve, 1969; Glaumann et al., 1981/. However, the number of morphologically detectable autophagic vacuoles /AVs/ is usually small during basal steady state. This fact may in part be a result of the rapid transition of newly formed autophagic vacuoles into more mature lysosomes /Mortimore and Schworer, 1977; Pfeifer et al., 1978/. Another contributing factor could be, as suggested by Ballard /1977/, a relatively minor importance of the lysosomal pathway in basal proteolysis as compared to the extralysosomal pathway.

Autophagy appears to be especially activated in states of induced degradation such as starvation, cell injury and remodelling of organs. Its level may also be manipulated by the administration of hormones such as insulin /Pfeifer et al., 1978/ and glucagon /Deter and De Duve, 1967; Schworer and Mortimore, 1979/; microtubular inhibitors such as vinblastine /Marzella and Glaumann, 1980a, b/ and amino acids /Grinde and Seglen, 1981; Schworer and Mortimore, 1979; Kovacs et al., 1981/. Gray et al. /1981/ administered chloroquine /a weak base and inhibitor of cathepsin B_1/ and Furuno et al. /1982a/ leupeptin /an inhibitor of the lysosomal thiol proteases cathepsin B, H and L/ to rats. Both groups found an increase in the number of AVs in the liver tissue. It seems likely

that the marked increase in AVs is due to an impeded turnover of these organelles secondary to a drug inhibited intralysosomal degradation.

Most of the evidence supporting the hypothesis of a dual pathway for proteolysis comes from experiments with inhibitors of lysosomal enzymes, showing that degradation of long-lived proteins is generally more sensitive to inhibition than degradation of short-lived proteins. The sensitive pathway has tentatively been equated with the lysosomally mediated proteolysis. The different proteolytic inhibitors are useful tools when trying to identify the different intracellular ways of degradation, although the interpretation of the results is not clearcut. For example, in the majority of instances the inhibition of lysosomal enzymes has not been complete and for many inhibitors of proteolysis nonspecific effects such as decreased protein synthesis may well influence the results.

The aim of the present study was to compare the effects of certain proteolytic inhibitors in different experimental designs. These included 1/ administration of the inhibitor to rats in vivo followed by measurements of proteolysis in isolated lysosomes and autophagic vacuoles; 2/ comparison of the effects of leupeptin on protein degradation in the perfused liver - as measured in the perfusate - with the effects of the inhibitor on lysosomes isolated from the same liver; and 3/ evaluation of the effects of different lysosomal inhibitors following their addition to isolated lysosomes.

MATERIAL AND METHODS

Male Sprague-Dawley rats /150-200 g/ were used. They were maintained on standard laboratory chow and water ad libitum in an environmentally regulated room with automatic light control /dark between 6 p.m. and 6 a.m./.

Isolation of autophagic vacuoles and lysosomes

Leupeptin was dissolved in Ringer's solution /2 mg/ml/

and injected intraperitoneally /2 mg/100 g body weight/. The procedure for isolating autophagic vacuoles was based on the method by Wattiaux et al. /1978/ with several modifications according to Marzella et al. /1982/. Lysosomes were isolated according to Wattiaux et al. /1978/. Purity was checked by ultrastructural analysis and measurement of marker enzymes /Marzella et al., 1982/.

Measurement of proteolysis

The animals were isotopically labelled with ^{14}C-L-leucine at different time points before sacrifice. Lysosomes or autophagic vacuoles from 1 gram of liver suspended in 1 ml of isotonic sucrose were incubated with appropriate additions and adjusted pH at 37^{o}C in a gently shaking water-bath. TCA was added to stop the reaction. The test tubes were centrifuged /37 000 g for 15 minutes/ and the radioactivities in the supernatants were measured. Generation of TCA-soluble radioactivity during incubation was linear with time and protein and used as a measurement of protein degradation after substraction of blank values incubated in ice water-bath.

Chemicals

^{14}C-L-leucine, specific activity 54 mCi/mmol, Code nr CFA. 273, was purchased from the Radiochemical Centre /Amersham, U.K./. Other chemicals were of highest available purity and purchased from Sigma Chemicals Co. /St. Louis, Mo, U.S.A./.

Electron microscopy

Livers were perfusion-fixed with glutaraldehyde as described before /Glaumann et al., 1975/. The subcellular fractions /autophagic vacuoles or lysosomes/ were fixed overnight with glutaraldehyde and subsequently placed on the bottom of a 10 ml centrifuge tube and pelleted at 35 000 g for 60 minutes.

After an overnight rinse in 0.1 M cacodylate buffer /pH 7.4/ containing 0.1 M sucrose, the pellets were cut through the entire depth. The resulting strips were either embedded as such or cut in the transverse plane into two or three pieces.

Liver perfusion

Male rats /250-280 g/ were labelled with ^{14}C-L-leucine 0.5 or 16 hours before sacrifice. The animals were anaestetized and the bile canaliculi and portal vein rapidly cannulated. The liver was removed and connected to the perfusion apparatus /Beije et al., 1979/. The perfusion medium consisted of a 70 ml Krebs-Ringer bicarbonate buffer, containing 3% bovine albumin /Fraction V, Sigma/, 10 mM glucose and a suspension of fresh human red blood cells. The pH of the perfusate was adjusted to 7.2 and oxygenated with carbogen gas. During perfusion samples from the perfusate were taken for isotopical assay of protein degradation /TCA soluble radioactivity/. After 1 hour the perfusion was stopped and the livers processed for subcellular fractionation and measurement of proteolysis in the AVs.

RESULTS

Effect of leupeptin in vivo

Electron microscopy. Rats were given leupeptin and the livers were analyzed from 30 minutes to 16 hours after injection. For each time point autophagic vacuoles were isolated and characterized /Henell and Glaumann, 1984/. Only a few points pertinent to this study are presented here. Hepatocytes of rats treated for 30 minutes displayed a modest increase in autophagic vacuoles containing mitochondria, peroxisomes and portions of endoplasmic reticulum as well as glycogen. The sequestered organelles were well preserved /Fig. 1A/. The corresponding isolated autophagic vacuolar fraction was pure and consisted mainly of secondary lysosomes and some auto-

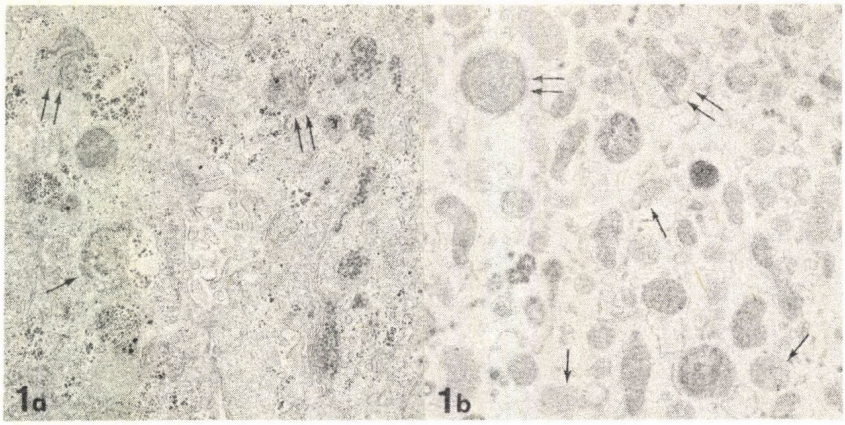

Fig. 1. Leupeptin treatment time thirty minutes.

A. Liver tissue. Nascent /arrows/ and more mature /double arrows/ autophagic vacuoles are seen. Most autophagic vacuoles contain particulate sequestered glycogen. X 14 500.

B. Fraction of autophagic vacuoles. Lysosomes /arrows/ are seen intermingled with autophagic vacuoles /double arrows/ containing material in early stages of degradation. X 13 200.

phagic vacuoles typically containing identifiable material /Fig. 1B/.

After one and especially two hours of leupeptin treatment the liver displayed a conspicuous increase in autophagic vacuoles. The identity of the sequestered material could no longer be determined. The one exception was glycogen particles which appeared relatively resistant to degradation. The relative number of autophagic vacuoles vis-a-vis other types of lysosomes had increased as compared to the fraction from animals treated for 30 minutes.

By four hours the autophagic vacuoles had become even more enlarged /Fig. 2A/. The nature of sequestered organelles could only rarely be determined since the degradation was far advanced. The isolated autophagic vacuoles contained mostly amorphous material /Fig. 2B/ as an indication of progressive degradation.

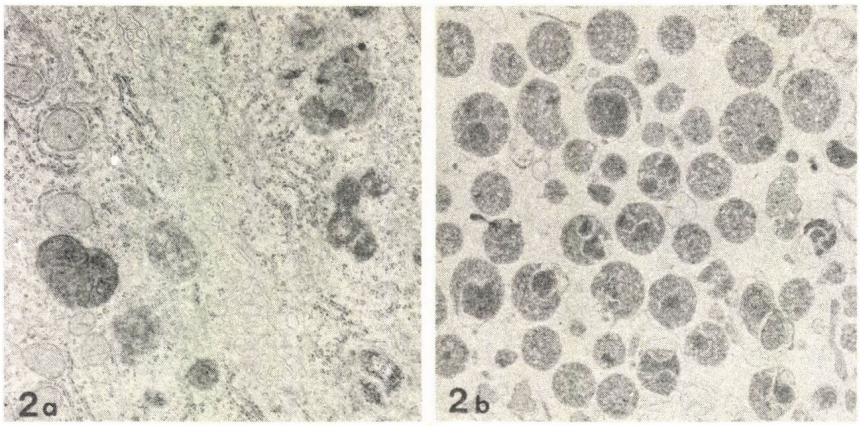

Fig. 2. Leupeptin treatment time four hours.

A. Liver tissue. The autophagic vacuoles have become larger than by the earlier time points. The sequestered organelles have been degraded to fine-granular material. X 13 200.

B. Fraction of autophagic vacuoles. The isolated organelles contained amorphous material. Only occasionally could more well preserved sequestered organelles be observed. X 10 000.

Eight hours following the injection of leupeptin abundant large autophagic vacuoles were still present in the hepatocytes. Particulate glycogen was detectable in some of the autophagic vacuoles, which is evidence that glycogen is only slowly degraded by lysosomes /Glaumann et al., 1979/.

By sixteen hours following the injection of leupeptin the ultrastructure of the hepatocytes was restored to normal. The huge autophagic vacuoles seen by eight hours had disappeared. Possibly a small increase in residual bodies of ordinary size and form could be detected.

Marker enzymes. We have characterized the autophagic vacuolar fraction obtained after 30 minutes of leupeptin treatment by means of marker enzyme analysis /Henell and Glaumann, 1984/. Cathepsin D /chosen because it is unaffected by leupeptin/ was enriched approximately twenty-fold as compared to the homogenate. As judged by negligible activities of marker enzymes for endoplasmic reticulum and mitochondria contamination of the autophagic vacuolar fraction was small confirming the morphological results.

Proteolysis in isolated autophagic vacuoles. Having ascertained that the autophagic vacuolar fractions are pure with satisfactory enzyme latencies /not shown/ we assessed their proteolytic capacities. In Fig. 3 the rates of proteolysis in the autophagic vacuolar fractions are shown as a function of the time interval following the injection of leupeptin.

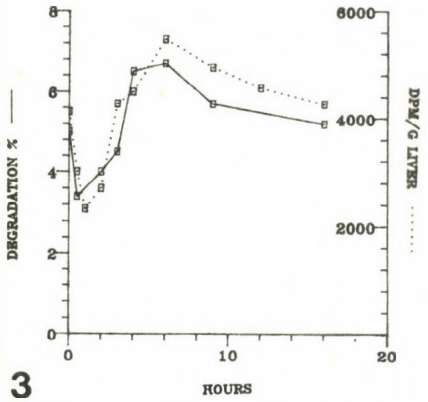

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Fig. 3. Proteolysis in the isolated autophagic vacuolar fraction as a function of leupeptin exposure time. Rats were given an injection of ^{14}C-L-leucine 16 hours before sacrifice. At the indicated time points following the injection of leupeptin /2 mg/100 g body weight/ the autophagic vacuoles were isolated. Proteolysis is given both on a per cent basis /per cent of total activity in the fraction/ and as the generation of TCA soluble DPM in the isolated vacuolar fraction from one gram of liver during incubation.

Maximal inhibition occurred after 30 minutes of treatment. If the animals were subjected to longer leupeptin exposures /up to 16 hours/ before fractionation the rate of proteolysis increased with a peak occurring after 4-6 hours exceeding the control value by 50%, a phenomenon called "catch up" proteolysis /Amenta and Brocher, 1980/. Hereafter peoteolysis decreased reaching control values by 16 hours.

Liver perfusion

The effect of leupeptin on proteolysis was also studied by means of the rat liver perfusion technique. The perfusion model has proven extremely valuable for studies on protein breakdown, since indirect effects often inherent in in vivo studies can be avoided to a large extent /Mortimore and Schworer, 1977/.

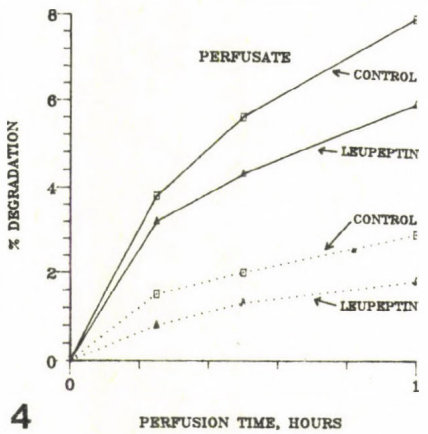

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Fig. 4. Effect of leupeptin on the release of degradation products to the perfusate during liver perfusion. Proteins were labelled by an injection of ^{14}C-L-leucine 0.5 /——/ hours /for labelling of proteins with short half-lives/ and 16 /..../ hours /for labelling of proteins with long half-lives/ before sacrifice. Leupeptin was injected 15 minutes before sacrifice and was also included in the perfusion medium. Aliquots were taken from the perfusion medium at indicated time points and analysed for acid soluble radioactivity.

We tried to evaluate whether or not there exists a correlation between total "net" proteolysis as measured in the perfusate on the one hand and in the isolated lysosomes on the other hand at two levels of protein breakdown, namely with and without the addition of leupeptin. This correlation was tested for both short time labelled /short-lived/ and long time labelled /long-lived/ proteins since it is generally believed that the regulation of the breakdown of these classes of proteins is different.

To compare the effects of leupeptin on the degradation of long lived and short lived proteins we injected rats with ^{14}C-L-leucine 30 minutes and 16 hours before sacrifice and removal of the livers for perfusion. Leupeptin /2 mg/100 g/ was injected to rats 15 minutes before sacrifice and was also included in the perfusion medium /2 mg/100 ml/. The time course of release of TCA-soluble radioactivity to the perfusate is shown in Figure 4. As is seen the rate of protein breakdown is two to three times higher following short time prelabelling in comparison with long prelabelling with ^{14}C-L-leucine. Leupeptin decreased the release of TCA-soluble radioactivity by 55% as measured during the last 30 min of perfusion /period 30-60 min/ following 16 hours of ^{14}C-L-leucine prelabelling. The corresponding value for short labelled proteins was 30% inhibition. The perfused livers were also used for the isolation of autophagic vacuoles /Marzella et al., 1982/. Generation of TCA-soluble radioactivity from isolated autophagic vacuoles was linear during the in vitro incubation for 30 minutes. Leupeptin treatment resulted in 80% inhibition of the degradation of both long and short labelled /-lived/ proteins in isolated autophagic vacuoles /Fig. 5/.

Electron microscopy. Perfusion of liver for 1 hour with a medium lacking amino acid supplementation causes some increase in the number of autophagic vacuoles /Fig. 6A/. The lysosomal fraction from such a liver contains autophagic vacuoles /Fig. 6B/ in addition to the secondary lysosomes characterizing the corresponding fraction from the nonperfused liver. Leupeptin increases the number of autophagic vacuoles even more in liver /Fig. 7A/. The lysosomal fraction from corresponding animals consisted mainly of autophagic vacuoles /Fig. 7B/.

In summary our data from liver perfusion demonstrate that leupeptin decreased the breakdown of both long and short lived proteins. This inhibitory effect was monitored in the perfusate and also in the autophagic vacuolar fraction. These results indicate - in some contradiction to the general notion - that lysosomes are engaged in the degradation of all classes of proteins, be they short or long lived. The next

series of experiments were devoted to a more thorough study of this question.

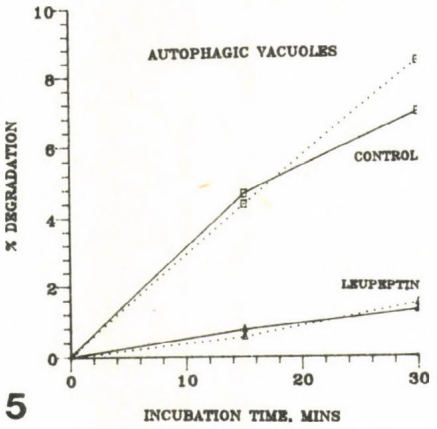

Fig. 5. Effect of leupeptin on proteolysis in autophagic vacuoles isolated from perfused liver. Rats were given an injection of ^{14}C-L-leucine 0.5 /——/ and 16 /..../ hours before sacrifice. When used, leupeptin was injected 15 minutes before sacrifice and also included in the perfusion medium. The liver was perfused and subsequently subfractionated /Marzella et al., 1982/. After 15 minutes of incubation the AVs were assayed for released radioactivity. Degradation is given in per cent /per cent of total radioactivity in the fraction/.

Characterization of lysosomal proteolysis in vitro

Experiments were first performed to evaluate the effect of pH variation of the incubation medium on lysosomal protein degradation. As could be expected maximal proteolytic rate was seen in the acidic range at pH 4-5 /not shown/, since most of lysosomal enzymes have an acidic pH optimum.

Proteolytic rates after different labelling times in vivo. As was discussed above the general opinion is that lysosomes are preferentially engaged in the degradation of long lived proteins as opposed to proteins which turn over rapidly. To elucidate this specificity further, rats were prelabelled with ^{14}C-L-leucine for different time-intervals ranging from 15 mi-

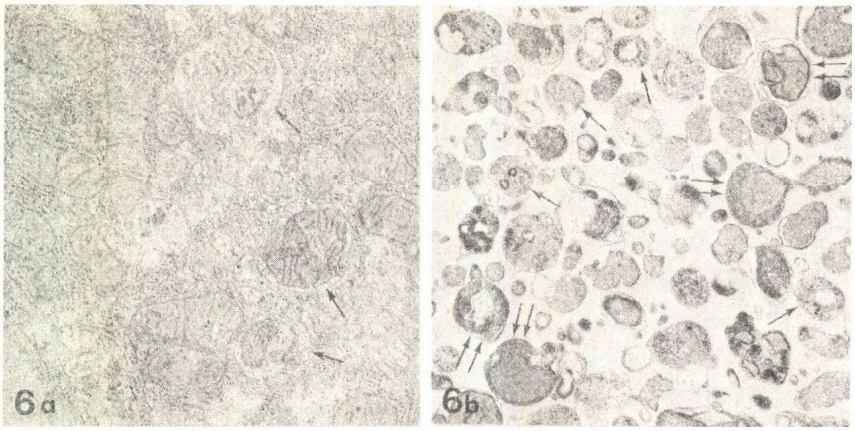

Fig. 6. Control liver. Perfusion time one hour.

A. Liver tissue. Perfusion causes some increase in the number of autophagic vacuoles /arrow/. The sequestered material consisted of mitochondria, glycogen and endoplasmic reticulum. X 15 000.

B. Fraction of autophagic vacuoles. Fraction consists of mainly secondary lysosomes /arrow/ but several nascent autophagic vacuoles /double arrows/ are also seen. X 13 200.

nutes to 16 hours, followed by subfractionation of the livers. Figure 8 summarizes the results from measurements of proteolysis in isolated lysosomes. A higher rate /about two to three fold/ of proteolysis was noted at pH 5 in comparison with pH 7 irrespective of labelling time. The highest fractional rates were obtained by shorter labelling times /15 minutes and 2 hours/.

Inhibition of proteolysis. The effect of different inhibitors of proteolysis was also tested on isolated lysosomes from rats prelabelled with ^{14}C-L-leucine for 15 minutes and for 16 hours. The lysosomes were incubated with the different inhibitors in increasing concentrations.

The following inhibitors were tested /Table 1/: Propylamine which impedes proteolysis by increasing the lysosomal pH /Seglen and Gordon, 1980/; leupeptin inhibiting lysosomal cathepsins B, H and L /Barrett and Heath, 1977/; chloroquine which impairs the action of proteinases by elevating intra-

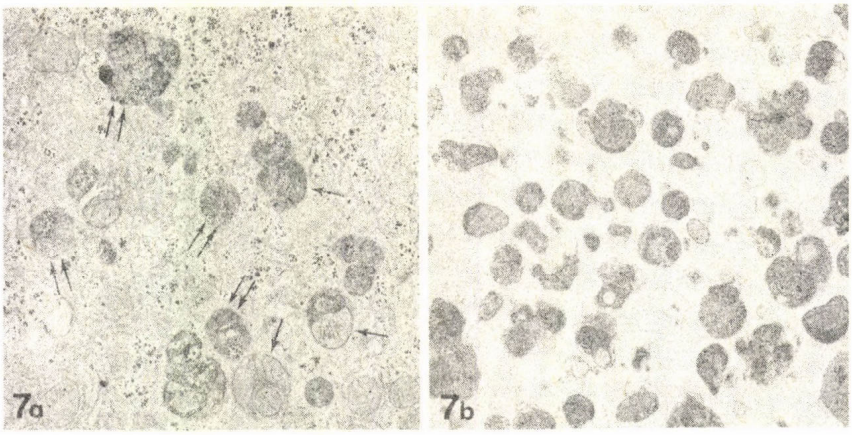

Fig. 7. Leupeptin treatment. Perfusion time one hour.

Leupeptin was injected into the rat /15 min before sacrifice/ and also included in the perfusion medium.

A. Liver tissue. Leupeptin causes a conspicuous increase in autophagic vacuoles /arrow/. Many of these contain glycogen particles /double arrows/ as evidence that cytosol is also taken up and degraded within lysosomes. X 9 500.

B. Fraction of autophagic vacuoles. This fraction consists of autophagic vacuoles in early stages of maturation as is evident from their identifiable contents. X 10 000.

lysosomal pH as well as by inhibiting cathepsin B_1 /Wibo et al., 1974/. As to propylamine no more than maximally 36 and 69% inhibition was obtained for long and short labelled proteins, respectively, even at high concentration /100 mM/. Leupeptin impeded proteolysis in lysosomes by 50% for both classes of proteins. Chloroquine was the most effective inhibitor tested. It resulted in 65 and 80% decrease of the degradation of slowly and rapidly turning over proteins, respectively. Neither addition of Triton nor sonication of the lysosomal suspension increased the inhibition of any agent.

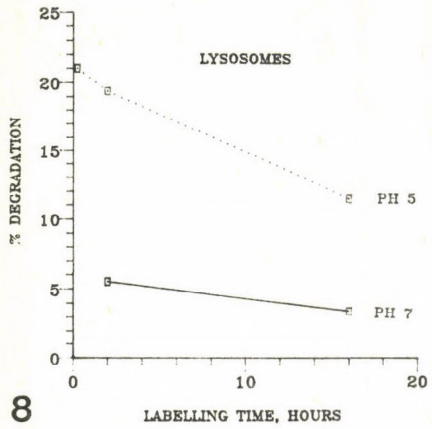

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Fig. 8. Correlation between degradation rate in isolated lysosomes and labelling times in vivo.
The rats were prelabelled with ^{14}C-L-leucine at different time points before sacrifice. Lysosomes were prepared as described in Material and Methods and were incubated for 30 minutes at pH 5 and 7. TCA-soluble activity was measured after centrifugation at 37 000 g for 15 minutes. Blanks were run at +4 °C. Degradation is expressed as TCA soluble DPM divided by total DPM in the same fraction.

DISCUSSION

As is apparent from the present study leupeptin increases the occurrence of autophagic vacuoles in rat liver considerably. This is due to the retarded degradation of sequestered organelles and thus prolonged lifespan of the autophagic vacuoles. The lower degradation rate of the sequestered organelles is in turn due to the inhibition of cathepsins B, H and L caused by leupeptin. The increase in number of autophagic vacuoles following leupeptin administration seems to be a good approach for their subsequent isolation into a pure fraction as is evident from the marker enzyme and electron microscopic analyses /Furuno et al., 1982b; Henell and Glaumann, 1984/. This conclusion is valid also for the perfused liver.

During basal proteolytic conditions obtained when cells are grown in, incubated in, or when an organ is perfused with a fully supplemented medium, autophagic vacuoles are compara-

Table 1

Effect of different inhibitors on proteolysis in isolated lysosomes

Inhibitor	Short labelling time		Long labelling time	
	degradation %	inhibition %	degradation %	inhibition %
None	8.0	-	4.5	-
Propylamine 50 mM	3.0	63	3.0	33
Propylamine 100 mM	2.5	69	2.9	36
Leupeptin 20 µg	4.8	40	3.0	33
Leupeptin 100 µg	3.6	55	2.5	45
Leupeptin 200 µg	3.5	56	2.2	52
Chloroquine 25 mM	0.8	78	1.75	62
Chloroquine 100 mM	0.6	81	1.55	66

Rats were prelabelled with ^{14}C-L-leucine for 15 minutes /short labelling time/ or 16 hours /long labelling time/ before sacrifice. Lysosomes /Wattiaux et al., 1978/ from 1 g of liver suspended in isotonic sucrose were incubated with the inhibitor at pH 5.0 for 15 minutes at 37°C. The reaction was stopped by adding an equal amount of cold 24% TCA. Radioactivity was measured in the supernatant after centrifuging the suspension 37 000 x g for 10 minutes. Blanks were run at 4°C. Mean of four experiments.

tively few. Experiments along these lines indicate that the lysosomal pathway of protein degradation is of less importance during basal conditions. However, it should be taken into consideration that the half-life of the autophagic vacuoles is short or approximately 8-9 minutes /Pfeifer et al., 1978/. This means that a substantial portion of cytoplasm can be taken up and degraded by the lysosomal apparatus in spite of the fact that this organelle only constitutes a small frac-

tional volume of the cell. Our experiments with leupeptin illustrate this. As soon as the transition of AVs into secondary lysosomes is blocked a dramatic increase in AVs occurs. This phenomenon is seen within one hour following leupeptin treatment. Accordingly, leupeptin treatment illuminates the importance of AVs in organelle degradation and unveils these organelles, which at least during basal conditions easily escape detection in the electron microscope.

We found no specificity as to the sequestration or the degradation of various organelles in autophagic vacuoles in accordance with earlier results from heterophagocytosis of cell organelles demonstrating that lysosomes have the ability to digest all types of biological membranes including lysosomes /Glaumann et al., 1975; Henell et al., 1983/. The only exception was glycogen, which was degraded comparatively slowly inside lysosomes.

The perfusion model has the advantage that total liver proteolysis can be estimated by monitoring the release of degradation products to the perfusate; such measurements are difficult to perform in vivo. Recent development in subcellular fractionation techniques has made possible the isolation of pure lysosomes /Wattiaux et al., 1978/ and autophagic vacuoles /Marzella et al., 1982/. Accordingly, by first perfusing a liver and then subfractionating the same liver it is possible to estimate the lysosomal contribution to total endogenous protein breakdown.

The present results clearly demonstrate that leupeptin inhibits protein breakdown both in vivo and in the perfused liver. As to the in vivo model, maximal inhibition of proteolysis in isolated autophagic vacuoles occurred after 30 minutes of treatment. At later intervals /4-6 hours/ the proteolytic rate increased now exceeding the initial basal rate. Amenta and coworkers /1980/ have designated this phenomenon "catch up" proteolysis, apparently a result of the elimination of the leupeptin block. By setting up an isotopic labelling protocol in which rats were given ^{14}C-L-leucine and sacrificed at 30 minutes and 16 hours later it was possible to label

preferentially short lived /30 minutes incorporation time/ and long lived proteins /16 hours of incorporation/. As to the degradation of these two classes of proteins, leupeptin inhibits the release of degradation products to the perfusate from short- and long labelled proteins by 30% and 50%, respectively.

Regarding the cellular site of this inhibition, we tried to disaggregate total protein breakdown by dissecting out one of its components, namely the lysosomes, in an effort to localize the effect of leupeptin more precisely. By this approach it was possible to demonstrate that leupeptin inhibits lysosomally mediated protein breakdown by approximately 70% /short lived proteins/ and 80% /long lived proteins/. /Corresponding values for the degree of inhibition of the release of degradation products to the perfusate were 30 and 50% for short and long lived proteins, respectively./ Extrapolating the leupeptin mediated inhibition of lysosomal proteolysis to 100% would allow one to conclude that the nonlysosomal pathway would account for roughly 40% /long lived/ and 55% /short lived/ of the total proteolytic activity in the perfused liver.

To characterize further the lysosomal route of degradation, we tested some additional lysosomal inhibitors often used as tools in assessing the relative importance of lysosomes in protein breakdown /Table 1/. The compounds were added in vitro to lysosomes isolated from ^{14}C-L-leucine labelled rats. Chloroquine was the most effective inhibitor, followed by leupeptin and propylamine. However, none of the agents produced total inhibition. The lack of 100% inhibition was not due to insufficient intralysosomal concentration of the inhibitor since rupture of the lysosomal membrane did not enhance the effect of the inhibitors /not shown/. The extent of inhibition was highest for short lived proteins. This finding is contradictory to the general notion that the turnover of short lived proteins is less affected by lysosomotropic agents than long lived proteins. However, this discrepancy might be more apparent than real, since when using inhibitors in intact cells one is studying not only the degradative phase but also the supply

of protein substrates, i.e. the input of proteins into lysosomes. With isolated lysosomes only the degradative phase is monitored. Accordingly, comparison between earlier results on intact cells with the present data on isolated lysosomes is not necessarily valid.

As was discussed above, the general notion is that amino acid analogue containing and other short lived proteins are degraded by a nonlysosomal ATP-dependent cytosolic process. However, our results from measurements of degradation in isolated lysosomes, demonstrate that already following 15 minutes and 2 hours of isotopic labelling time, proteolytic activity was recorded in lysosomes. Moreover, the fractional proteolytic rate was in fact somewhat higher for the short labelling time intervals than for the 16 hour interval. Accordingly, we conclude that lysosomes are active in the degradation of all types of proteins, irrespective of their half lives.

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BAJUSZ:

Leupeptin seems to be a rather weak inhibitor of Cathepsin B. Don't you think of testing a better one?

GLAUMANN:

Yes, we have tried various inhibitors. You can combine them, too, and get better and higher inhibition. One of the main point of this lecture is that if you use these inhibitors and you use them as a weapon to distinguish between two types of the lysosomal pathway of degradation and non-lysosomal pathway of degradation, this is not a good tool, because they don't inhibit completely.

BAJUSZ:

I see, thank you. But if you want to try a better one, I'd suggest you such a tripeptide aldehyde, namely Boc-DPhe-Pro-Arg-H.

BOHLEY:

The values of residual activity you found in lysosomes after inhibition with leupeptin are exactly in agreement with ours. What kind of proteinases might be responsible for this residual activity in your opinion?

GLAUMANN:

In the case of leupeptin, several proteinases such as aminopeptidases, cathepsin D are not affected by the inhibitor. As far as lysosomotropic bases are concerned, the increased pH might not completely inhibit the activity of all cathepsins.

SOHÁR:

It is known from several publications that pepstatin + leupeptin can reduce the increased proteinase activities in dystrophic skeletal muscles. Have you measured the

effect of pepstatin or pepstatin together with leupeptin on lysosomal protein degradation?

GLAUMANN:

Yes, we have tested pepstatin on proteolysis in isolated lysosomes. Pepstatin is a weak inhibitor decreasing protein breakdown by 20%. Since pepstatin does not freely permeate membranes these experiments were performed with ruptured lysosomes. Pepstatin has some /10%/ additional inhibitory effect to leupeptin.
Concerning the second part of your question. I think both leupeptin and pepstatin inhibit protein breakdown as long as it is mediated by lysosomes, be it induced or basal state.

BARRETT:

Perhaps one reason for the incomplete inhibition of lysosomal enzymes in these systems may be the very high /1-2 mM/ concentration of the cathepsins in the lysosomes?

GLAUMANN:

The same result was found even after lysis of the lysosomes.

BAICI:

Is it possible to speculate on the concentration of leupeptin in lysosomes after administration in vivo?

GLAUMANN:

I don't really know. It has been measured, I guess, because it has been labelled.

JOCHUM:

Is there anything known whether neutral proteinases e.g. elastase-like or chymotrypsin-like /cathepsin G/ are also present in liver lysosomes as it is the case for polymorpho-nuclear granulocytes? Or are there only cathepsins there?

GLAUMANN:

Well, there are a number of cathepsins there.

JOCHUM:

Yes, but not chymotrypsin-like cathepsin G, so if there is a small amount of these neutral proteinases, this might be the reason why you cannot completely block the proteolysis.

KORANT:

What is the principal effect of reduced pH on increased rate of protein degradation: is it to activate the lysosomal cathepsins or to denature the intralysosomal proteins and make them better as substrates?

GLAUMANN:

To activate the enzymes.

KORANT:

Doesn't it also denature the substrate?

GLAUMANN:

Well, could well be, yes. Make it a better substrate.

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STUDIES ON THE INTERACTION OF SERINE (PRO)ENZYMES WITH PROTEIC INHIBITORS

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INTRODUCTION

Serine proteinases participate in several physiological and pathological processes which are regulated by protein proteinase inhibitors, either circulating or specifically acting on selected organs. These inhibitors can be divided, on the basis of their sequences and catalytic properties, into at least ten homologous families (Laskowski and Kato, 1980).

The bovine basic pancreatic trypsin inhibitor (BPTI), which is the prototype molecule of the Kunitz family, has been object of extensive investigations both from a structural and dynamic point of view (see: Laskowski and Kato, 1980 and references cited therein). However, to date, its physiological role is uncertain, and its distribution in the animal kingdom is very limited and puzzling. Conversely, the porcine (p.) and bovine (b.) pancreatic secretory trypsin inhibitor (PSTI), which belongs to the Kazal family and which is widely distributed among mammals, has been subject only of preliminary examinations. PSTI is secreted in the pancreatic juice with the zymogens, and its physiological role seems to prevent the premature activation of proteinase(s) within the pancreas itself (Greene et al., 1976).

In the present paper, we analyze in parallel thermodynamics, kinetics and stereochemistry of binding of both native and chemically modified PSTI and BPTI to bovine β-trypsin (trypsin), bovine trypsinogen (trypsinogen; both in the presence and absence of the effector dipeptide H-Ile-Val-OH), human urinary kallikrein (h.u. kallikrein), porcine pancreatic kallikrein (p.p. kallikrein)

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and bovine α-chymotrypsin (chymotrypsin) describing the common structural elements which are believed to be responsible for the enzyme inhibition.

THERMODYNAMICS OF BPTI AND PSTI BINDING TO SERINE (PRO)ENZYMES

Equilibrium dissociation constants (K_d) for the binding of the native and the chemically modified PSTI and BPTI to serine (pro)enzymes have been determined by measuring: (i) the loss of the enzymatic activity upon inhibitor binding (see: Antonini et al., 1983a and references cited therein); (ii) the spectral changes in the ultraviolet region accompanying the formation of the PSTI: and BPTI:(pro)enzymes adducts (see: Antonini et al., 1983a and references cited therein); (iii) the spectral changes accompanying proflavine displacement upon inhibitors binding (see: Quast et al., 1978 and references cited therein); and (iv) by competition experiments involving two (pro)enzymes with different affinities for the inhibitor (Vincent and Lazdunski, 1976; Antonini et al., 1983a).

Values of K_d obtained by the different methods agree very well one another and are independent on (pro)enzymes concentration. Moreover, under all the experimental conditions the inhibitor:(pro)enzyme complexes formation conforms to a simple equilibrium.

Table 1 shows the values of K_d, at pH 8.0, for the binding of the native and the chemically modified p. and b. PSTI and BPTI to several serine (pro)enzymes.

In agreement with the exact predetermined complementarity of the (re)active sites of trypsin and BPTI, as shown by the comparative X-ray analysis on the free enzyme, free inhibitor and enzyme:inhibitor adduct (see: Huber and Bode, 1978 and references cited therein), trypsin binds BPTI with the lowest K_d value known (0.5 to 1.5×10^{-13}M) (Vincent and Lazdunski, 1972; Finkenstadt et al., 1974). In accordance with the specificity properties of trypsin, which catalyses undistinguishably the hydrolysis of both arginine and lysine residues (Ascenzi et al., 1982), the chemically modified Arg15-BPTI binds to trypsin with the same K_d value shown by the native BPTI, which has a lysine at site P_1 (position 15) (Jering and Tschesche, 1976). Furthermore, both p. and b. PSTI, which show, respectively, a lysine and an arginine at site P_1 (position 18) bind to trypsin with the same K_d value (Schweitz et al., 1973; Menegatti et al., 1983).

It is of interest to remark that in spite of inherent differences at the (re)active sites of the serine (pro)enzymes and inhibitors examined, the interaction between Asp189, present in the specificity pocket of serine (pro)enzymes acting on cationic substrates, and Lys15 or Lys18 (or Arg18), at the reactive site loops of the native BPTI and p. or b. PSTI, respectively,

TABLE 1

Dissociation equilibrium constants (K_d) for the binding of native and chemically modified p. or b. PSTI and BPTI to various serine (pro)enzymes (pH 8.0, I=0.1; T=21 to 25°C).

Inhibitor	(Pro)enzyme	K_d(M)
p. PSTI	trypsin	9.6×10^{-11}
	trypsinogen	2.8×10^{-5}
	trypsinogen (+0.02 M H-Ile-Val-OH)	$<6.0\times10^{-7}$ ≠
	chymotrypsin	7.1×10^{-7}
b. PSTI	trypsin	9.0×10^{-11}
BPTI	trypsin	0.5 to 1.5×10^{-13}
	trypsinogen	2.0×10^{-6}
	trypsinogen (+0.02 M H-Ile-Val-OH)	$<6.0\times10^{-7}$ ≠
	h.u. kallikrein	9.0×10^{-11}
	p.p. kallikrein	9.0×10^{-10}
	chymotrypsin	9.5×10^{-9}
Arg15-BPTI	trypsin	6.0×10^{-14}
	p.p. kallikrein	$\sim4.5\times10^{-10}$
	chymotrypsin	$\sim1.4\times10^{-8}$
Phe15-BPTI	trypsin	1.5×10^{-6}
	chymotrypsin	2.8×10^{-9}
Trp15-BPTI	trypsin	2.8×10^{-6}
	chymotrypsin	2.8×10^{-9}

≠Maximum K_d value that may be determined by the method used here (see: Antonini et al., 1983a).

is strictly conserved (Huber and Bode, 1978; Bolognesi et al., 1982; Chen and Bode, 1983).

In the case of p. PSTI and BPTI binding to trypsinogen, rather low affinity values have been observed (Vincent and Lazdunski, 1976; Antonini et al., 1983a). In fact the specificity pocket and the activation domains of the free proenzyme are not structured and the productive interaction Asp189-LysP_1 (18 or 15 respectively) can occur only in parallel to partial activation of trypsinogen to a trypsin-like structure (Huber and Bode, 1978; Bolognesi et al., 1982). The observed increased affinity of both p. PSTI and BPTI for trypsinogen by the effector dipeptide H-Ile-Val-OH, is in accordance with the proposed structural mechanism for the activation of this proenzyme (Huber and Bode, 1978; Bode, 1979). Thus, BPTI binding to the trypsinogen:H-Ile-Val-OH complex is undistinguishable, on structural grounds, from that of the inhibitor to trypsin (Huber and Bode, 1978). The same relation, conceivably, holds for p. PSTI binding to the trypsinogen:H-Ile-Val-OH adduct (Antonini et al., 1983a).

It is well known that both p.p. kallikrein and h.u. kallikrein preferably catalyse the hydrolysis of arginine rather than lysine residues (Antonini et al., 1982; Ascenzi et al., 1982). This peculiar catalytic behaviour has been related to the presence at position 226 of a serine residue which seems to play a dominant role in the binding of the arginine side chain (Bode et al., 1983; Chen and Bode, 1983) and is replaced by a glycine in trypsin (Bode and Schwager, 1975). Thus, Arg15-BPTI binds to p.p. kallikrein with a higher affinity than that of the native Lys15-BPTI (Jering and Tschesche, 1976). The lower affinity of BPTI for both kallikreins as compared to that for trypsin has been related to the different geometries that the so called "kallikrein polypeptide loops" assume in these enzymes. In fact, these domains limit the accessibility of macromolecular inhibitors and substrates to the active site of both kallikreins (Bode et al., 1983; Chen and Bode, 1983).

The relatively low affinities of BPTI and p. PSTI for chymotrypsin (Vincent and Lazdunski, 1973; Antonini et al., 1983a) may be essentially related to the absence of the salt linkage between Lys15 or Lys18, present at the P_1 site of BPTI and p. PSTI respectively, and the Asp189, since this residue is replaced by a neutral serine (Birktoft and Blow, 1972). In this respect, chemically modified BPTI inhibitors, in which Lys15 has been replaced by a tryptophan (Trp15-BPTI) or by a phenylalanine (Phe15--BPTI) show higher affinities for chymotrypsin (Jering and Tschesche, 1976). Conversely, Phe15-BPTI and Trp15-BPTI show lower affinities for trypsin if compared to the native BPTI (Jering and Tschesche, 1976).

As shown in Table 1, the affinity of p. and b. PSTI for trypsin, trypsinogen (in the presence and absence of H-Ile-Val-OH)

and chymotrypsin is systematically lower as compared to that of BPTI, moreover p. PSTI does not inhibit both kallikreins up to an inhibitor concentration of 3 mM (Antonini et al., 1983a). The low affinity of p. and b. PSTI for the (pro)enzymes examined has been related to the wider radius of the reactive site loop of these inhibitors as compared to that of BPTI (see Fig. 1). In fact, while upon BPTI binding trypsinogen adopts a structure which is coincident with that of activated trypsin (Huber and Bode, 1978), p. PSTI induces in the zymogen a novel conformation of the activation domain which differs from that of trypsin (Bolognesi et al., 1982), and may thus be energetically less favourable. On the same basis, lack of p. PSTI binding to both kallikreins may be explained (Antonini et al., 1983a).

The pH dependence of K_d for p. PSTI and BPTI binding to trypsin, trypsinogen and chymotrypsin is roughly the same between pH 5 and 9.5 for both the inhibitors (see Fig. 2). In spite of difficulties in the determination of the acidic asymptotes of log K_d, the data may be fitted with a simple pH transition with an average pK_a of 6.2. This finding implies an acid-pK_a shift of a group upon inhibitor(s) binding from $pK \simeq 7.2$, in the free (pro)-enzymes, to $\simeq 5.2$, in the (pro)enzymes:inhibitors adducts.

Inspection of the different active site residues capable of affecting p. PSTI and BPTI binding, suggests to assign the observed acid-pK_a shift upon (pro)enzymes-inhibitors complexes formation to His57. Inhibitor binding and the related acid pK_a-shift are associated with considerable strengthening of the Ser195OG--His57NE2 hydrogen bond, which is observed in all the trypsin-(ogen)-inhibitors adducts and is very weak in the free (pro)-enzymes (Huber and Bode, 1978; Bolognesi et al., 1982; Chen and Bode, 1983).

At pH values below 5, BPTI binding to trypsin is affected by a cooperative three proton transition (Finkenstadt et al., 1974). On the other hand, p. PSTI and BPTI binding to chymotrypsin is unaffected by pH (Vincent and Lazdunski, 1973; Antonini et al., 1983a). Probably one of the three residues affecting BPTI binding to trypsin, below pH 5, is Asp189 (Finkenstadt et al., 1974), which plays a dominant role in the catalytic pathway, in spectral and ligand binding properties of serine proteinases acting on cationic substrates (Ascenzi et al., 1982), and is replaced by a neutral serine in chymotrypsin (Birktoft and Blow, 1972).

KINETICS OF BPTI AND PSTI BINDING TO SERINE PROTEINASES

The kinetic data available up to now on the reactions of trypsin and chymotrypsin with native and modified (P_1-P_1' reaction site bond split) BPTI and b. or p. PSTI are consistent with the generally accepted reaction scheme:

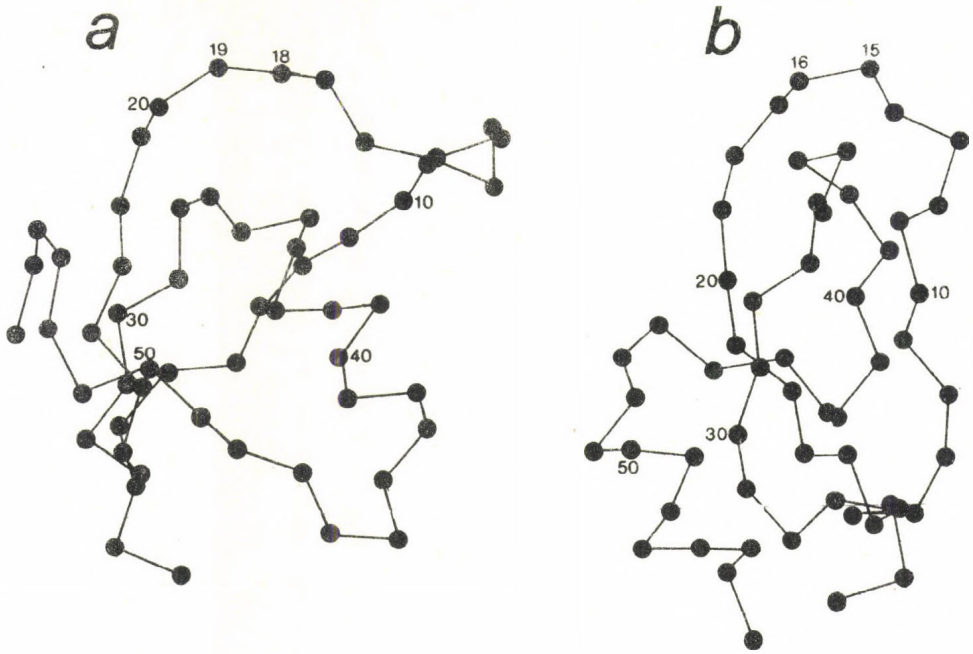

FIG. 1: C-alpha skeletal models of p. PSTI (a) and BPTI (b). The reactive site bonds of inhibitors (18-19 in p. PSTI and 15-16 in BPTI) have been positioned, for ease of comparison, in approximately the same orientation. (Modified from Antonini et al., 1983a).

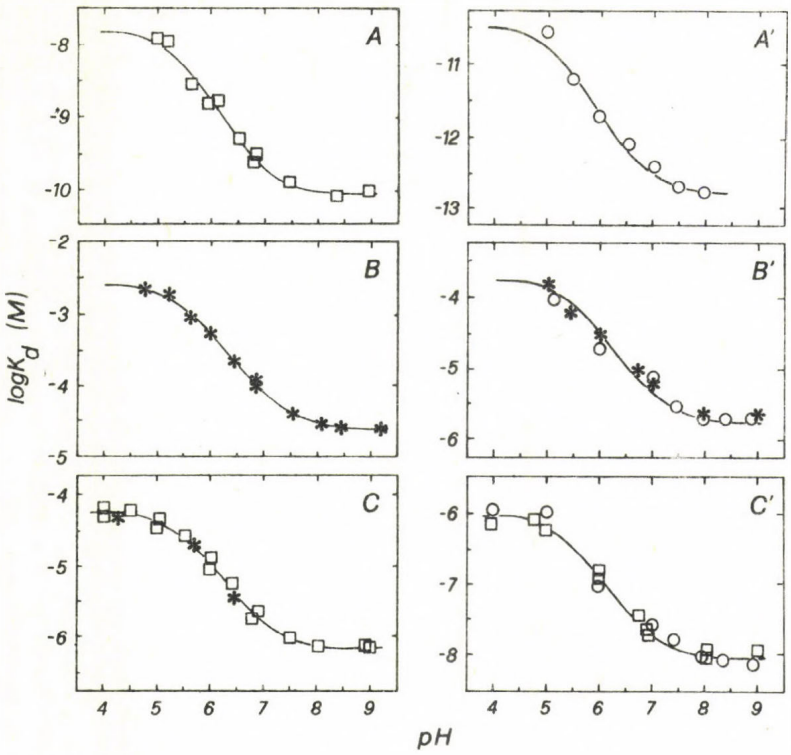

FIG. 2: pH dependence of the dissociation equilibrium constant (K_d) for: (i) p. PSTI (A) and BPTI (A') binding to trypsin; (ii) p. PSTI (B) and BPTI (B') binding to trypsinogen; and (iii) p. PSTI (C) and BPTI (C') binding to chymotrypsin at 21°C. Squares indicate values of K_d obtained by the evaluation of the inhibitory effect of p. PSTI and BPTI on the enzymes catalyzed hydrolysis of synthetic substrates. Asterisks indicate values of K_d obtained by spectrophotometric titrations. Values of K_d (O) for BPTI binding to trypsin (A': from Vincent and Lazdunski, 1972; Finkenstadt et al., 1974), trypsinogen (B': from Vincent and Lazdunski, 1976) and chymotrypsin (C': from Vincent and Lazdunski, 1973) are reported for comparison. Continuous lines are the simple theoretical titration curves with the following pK_a values: A: 6.15; B: 6.38; C: 6.25; A': 6.0; B': 6.30; C': 6.26. (Modified from Antonini et al., 1983a).

$$E + I \rightleftharpoons L \rightleftharpoons X \rightleftharpoons C \rightleftharpoons X^{+} \rightleftharpoons L^{\neq} \rightleftharpoons I^{x} + E \qquad (1)$$

where E is the enzyme; I and I^{x} are the virgin and the modified inhibitors, respectively; L and $L^{\neq}$ are loose complexes of E with I and I^{x}, respectively; X and X^{+} are relative long lived intermediates observed in the reaction of trypsin with I and I^{x}, respectively (Quast et al., 1978; Antonini et al., 1983b) and C is the stable inactive enzyme-inhibitor adduct.

With inhibitors in excess over the enzymes ($[I] \geq 5x\,[E]$), the time course of the reaction of trypsin and chymotrypsin with BPTI and p. PSTI corresponds to a pseudo-first order process (see: Antonini et al., 1983b and references cited therein). Under all the experimental conditions, the concentration dependence of the rate is second order at low inhibitors concentrations, reflecting a relatively rapid pre-equilibrium followed by a limiting first order process.

The kinetics of the formation and dissociation of the complexes of native and reactive site bond split BPTI and p. or b. PSTI with trypsin and chymotrypsin have been investigated using four different signals: (i) the spectral changes accompanying proflavine binding or displacement as a consequence of macromolecular inhibitors competition; (ii) the intrinsic optical density changes in the ultraviolet region accompanying the formation or the dissociation of the enzyme-inhibitor complexes; (iii) the spectral changes accompanying benzamidine binding or displacement as a consequence of macromolecular inhibitors competition; (iv) the loss and recovery of the enzymatic activity, upon binding or dissociation of inhibitors (see: Antonini et al., 1983b and references cited therein).

The values of the second order rate constants for the trypsin- and chymotrypsin:BPTI and - p. PSTI complexes formation, determined around neutrality and at low pH (3.5 to 4), are reported in Table 2. Moreover, Fig. 3 shows the dependence of the apparent pseudo-first order rate constant for the trypsin:BPTI complex formation on the inhibitor concentration for the displacement of proflavine, the intrinsic spectral changes in the ultraviolet region, the displacement of benzamidine and the loss of the enzymatic activity, at pH 6.8 and 3.5. The values of the kinetic parameters for the enzymes-inhibitors adducts formation, determined by the four different methods, agree one another, around neutrality. At pH 3.5, the kinetic parameters for the reaction of chymotrypsin with BPTI and p. PSTI are independent on the different signals. On the other hand, the kinetic parameters for the trypsin-BPTI and -p. PSTI complexes formation differ widely for the processes followed with the different signals.

At pH 3.5, proflavine displacement from trypsin, upon BPTI binding, preceeds the intrinsic optical density changes in the ultraviolet, and the latter preceeds the benzamidine displacement

TABLE 2

Values of the second order rate constant ($k_{(on)i}$) for the binding of BPTI and p. PSTI to trypsin and chymotrypsin (I=0.1; T=21 to 25°C).

Inhibitor	Enzyme	pH	$k_{(on)i}$ ($M^{-1}s^{-1}$)		
			Proflavine displacement	The uv optical density changes	Loss of the enzymatic activity
BPTI	trypsin	6.8	2.5×10^5	2.5×10^5	2.5×10^5
		3.5	2.5×10^4	3.2×10^2	8.0×10^1
p. PSTI	trypsin	6.8	-----	-----	1.1×10^6
		3.5	8.0×10^3	1.1×10^3	1.5×10^2
BPTI	chymotrypsin	8.0	6.7×10^5	7.0×10^5	1.1 to 5.3×10^5
		4.0	0.7 to 2.0×10^3	2.0×10^3	2.0×10^3
p. PSTI	chymotrypsin	6.8	4.0×10^3	4.0×10^3	4.0×10^3
		3.5	4.0×10^2	4.0×10^2	4.0×10^2

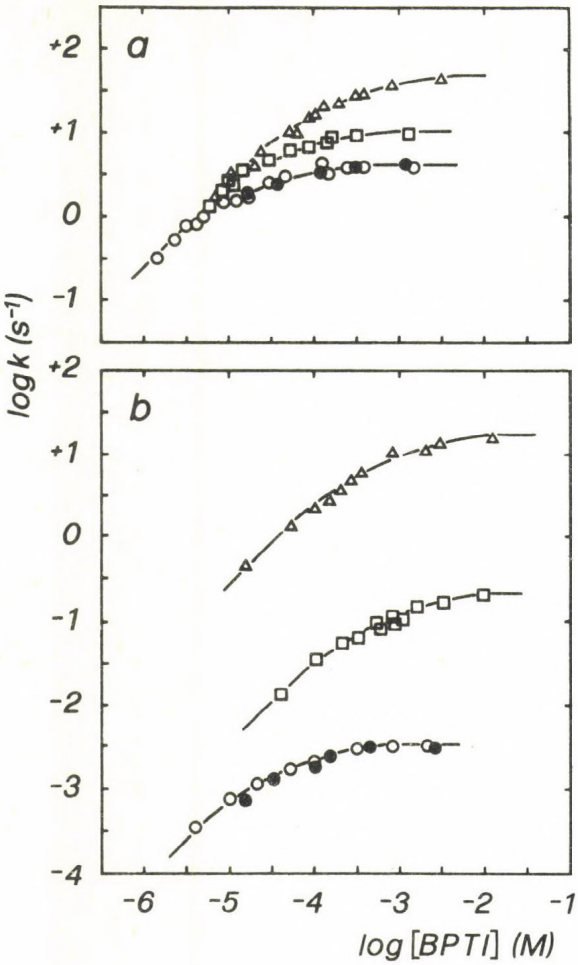

FIG. 3: Dependence of the apparent pseudo-first order rate constant (k) for trypsin-BPTI complex formation on BPTI concentration for (i) the displacement of proflavine (Δ); (ii) the spectral changes in the ultraviolet region (□); (iii) the displacement of benzamidine (●); and (iv) the loss of the enzymatic activity (○) at pH 6.8 (a) and pH 3.5 (b) ($T=21\pm0.5^{\circ}C$; $[BPTI] \geq 5 \times [E]$). The continuous lines were calculated from the expression :

$$k = k_{+i}/(1 + K_i / [BPTI])$$

where k_{+i} is the limiting pseudo-first order rate constant and K_i the pre-equilibrium dissociation constant. (Modified from Antonini et al., 1983c).

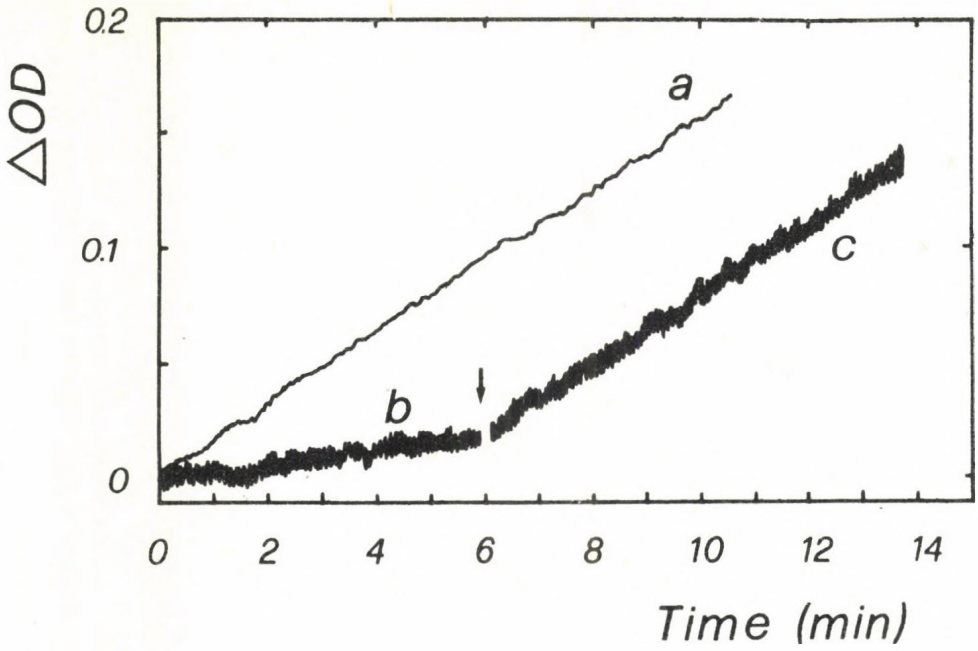

FIG. 4: Time course of the trypsin-catalysed hydrolysis of N-α-carbobenzoxy-L-lysine p-nitrophenyl ester (ZLysONp), at pH 3.5 and 21±0.5°C. Trace a in the absence of both BPTI and proflavine; trace b in the presence of 1 mM proflavine; trace c same as b but after the addition of BPTI, indicated by the arrow. Trypsin concentration: 0.1 μM; ZLysONp concentration: 85 μM; BPTI concentration: 20 mM; λ = 360 nm. (Modified from Antonini et al., 1983c).

and loss of the enzyme activity (which procede at the same time) by several orders of magnitude (see Fig. 3). These data led to the prediction that, at pH 3.5, BPTI addition to the trypsin--proflavine complex would initially remove proflavine inhibition and, since the acridine dye is a competitive inhibitor for trypsin, the enzyme would recover transiently its catalytic activity, before being irreversibly inhibited by completion of BPTI binding. The kinetic evidences shown in Fig. 4 verified this prediction, indicating that during the trypsin-BPTI complex formation one proflavine-displaced-(inhibitor-bound) intermediate occurs, which is not able to bind proflavine but may bind and hydrolyse the substrate. The catalytic properties of this transient are indistinguishable from those of free trypsin both in terms of k_{cat} and K_m (Antonini et al., 1983c).

It may be remarked that between pH 3.5 and 8.0 the values of the dissociation constants for trypsin- and chymotrypsin-BPTI and -p. PSTI complexes are independent of the different signals.

Finally, it is of interest to observe that also the formation of the stable inactive BPTI-trypsin complex from trypsin and the reactive site bond split BPTI is a multistage process, characterized by the presence of the relatively long-lived intermediate X^+ (see scheme 1). On the other hand in the case of chymotrypsin, the reaction with the reactive site bond split inhibitor, may be regarded as an all-or-none process. The dissociation of the modified BPTI from trypsin- and chymotrypsin--inhibitor complexes may be regarded as a simple process (see: Quast et al., 1978 and references cited therein).

The kinetic observations here reported indicate that, in contrast with chymotrypsin, the reactions of trypsin with the native and modified BPTI and p. PSTI involve several distinct intermediates, rather than being an all-or-none process, this being expecially evident at low pH.

Although it is obviously impossible to explain these kinetic data in terms of static three-dimensional structures, the multistage processes observed in the BPTI: and p. PSTI-trypsin complexes formation is in keeping with the proposed reaction mechanism which takes into account a zipper-like sequential formation of hydrogen bonds, polar and apolar interactions at the contact area (Huber and Bode, 1978).

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SUBSTRATE SPECIFICITIES AND CATALYTIC MECHANISMS OF SERINE PROTEASES ON THE FACET OF THEIR THREE-DIMENSIONAL STRUCTURE

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INTRODUCTION

The aim of the present study is to compare side by side the catalytic properties, the chemical and the three-dimensional structures of some serine (pro)enzymes, namely bovine β-trypsin (trypsin), bovine trypsinogen (trypsinogen):H-Ile-Val-OH adduct, bovine thrombin (thrombin), porcine pancreatic kallikrein (p.p. kallikrein), human urinary kallikrein (h.u. kallikrein), human urokinase (urokinase), the thrombin-like proteinase from the Ancistrodon rhodostoma venom (Ancrhod), all acting on cationic substrates, as well as bovine α-chymotrypsyn (chymotrypsin), porcine pancreatic elastase (elastase) and free trypsinogen, acting on noncationic substrates.

The characterization of kinetic and thermodynamic properties of these (pro)enzymes has been conducted along the following lines of investigation: (i) steady-state and pre-steady-state kinetics of the catalyzed hydrolysis of a number of p-nitrophenyl esters of (L-)amino acids having cationic and noncationic side chains. Particularly, such studies have been performed with N-α-carbobenzoxy-L-lysine p-nitrophenyl ester (ZLysONp) which results to be one of the most favorable substrate for (pro)enzymes acting on cationic substrates; (ii) "promoter" effect of ethylamine on the catalyzed hydrolysis of the noncationic substrate N-α-carbobenzoxy-L-alanine p-nitrophenyl ester (ZAlaONp); (iii) inhibition by ethylamine, benzamidine and polybenzamidine derivatives of the catalyzed hydrolysis of ZLysONp; (iv) spectral properties of free (pro)enzymes.

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The values of the individual equilibrium and kinetic constants for the catalyzed hydrolysis of p-nitrophenyl esters examined have been analyzed (Gutfreund, 1972; Antonini and Ascenzi, 1981) in the framework of the minimum three-step catalytic mechanism (scheme 1):

$$(P)E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (P)E\cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} (P)E\cdot P + P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} (P)E + P_2 \qquad (1)$$

where (P)E is the (pro)enzyme, S is the substrate (i.e. the p-nitrophenyl ester of the N-α-carbobenzoxy-(L-)amino acid), K_s ($=k_{-1}/k_{+1}$) is the pre-equilibrium dissociation constant for the formation of the (pro)enzyme·substrate complex (P)E·S, (P)E·P is the acyl intermediate and P_1 and P_2 are the hydrolysis products (i.e. p-nitrophenol and N-α-carbobenzoxy-(L-)amino acid, respectively).

For all the (pro)enzymes examined the formation of the (P)E·S complex may be regarded as a pseudo first order process with a minimum value of the second order rate constant of $\simeq 1\times10^{6}M^{-1}s^{-1}$. Moreover, values of k_{-2} and k_{-3} are always closed to zero.

The dependence of the initial velocity on substrate and (pro)enzyme concentration and the time course of the reaction follow simple Michaelis-Menten kinetics, under all the experimental conditions.

It will be shown that serine (pro)enzymes acting on cationic substrates notably, trypsin, trypsinogen:H-Ile-Val-OH, thrombin, p.p. and h.u. kallikrein, urokinase and Ancrhod, but not chymotrypsin, elastase and free trypsinogen, acting on noncationic substrates, contain a similarly located ionizable group, Asp189, the ionization of which affects the binding of cationic substrates, inhibitors and ligands, the rate of the acylation step and the spectral properties. Subtle changes in the specificity of (pro)enzymes examined are also reported and their structural grounds discussed.

THE (PRO)ENZYMES CATALYZED HYDROLYSIS OF ZLysONp

Fig. 1 shows the pH dependence of the values of steady--state and pre-steady-state parameters for trypsin and chymotrypsin catalyzed hydrolysis of ZLysONp (Antonini and Ascenzi, 1981; Ascenzi et al., 1982).

Values of log k_{cat} and log k_{cat}/K_m for all (pro)enzymes acting on cationic substrates may be fitted with two pH transitions ($\simeq$4.5 and $\simeq$7). On the other hand, values of log k_{cat} and log k_{cat}/K_m for chymotrypsin, elastase and free trypsinogen may be fitted with a single pK_a value ($\simeq$7) (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983). Values of K_m

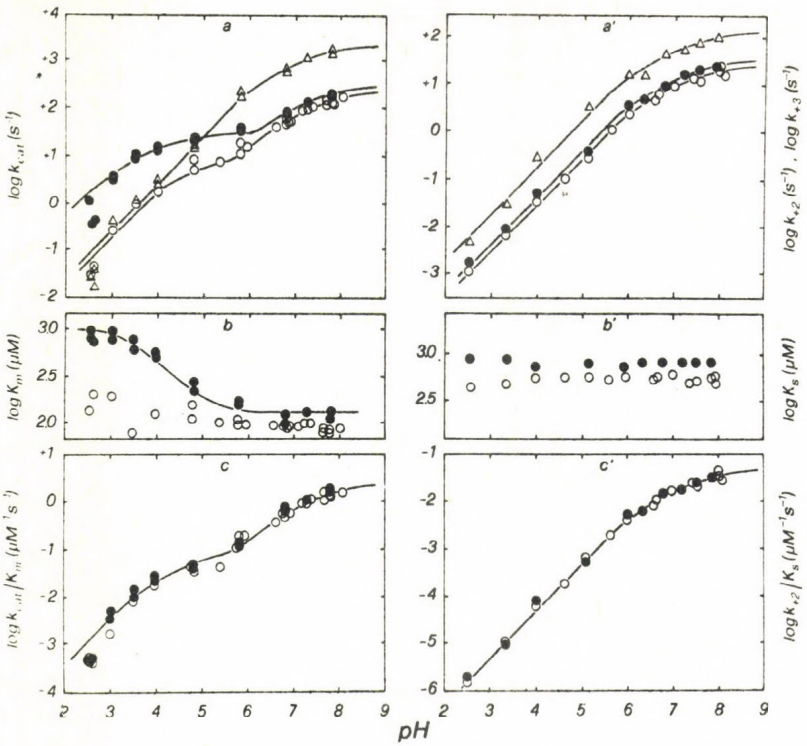

FIG. 1: pH dependence of log k_{cat} (○), log K_m (○), log k_{+2} (●), log k_{+3} (△), log K_s (●), log k_{cat}/K_m (○) and log k_{+2}/K_s (●) for the trypsin (a,b,c) and chymotrypsin (a',b',c') catalyzed hydrolysis of ZLysONp at 21±0.5°C and I=0.1M. Data for the trypsin catalyzed hydrolysis of ZLysONp may be fitted with the following pK_a values: k_{cat} -4.6±0.1, 7.1±0.1; k_{+2} -3.73±0.10, 7.05±0.10; k_{+3} -6.95±0.10; K_s -4.2±0.1; k_{cat}/K_m ($=k_{+2}/K_s$) -4.5±0.1, 7.1±0.1. Values of Km (○) for the trypsin catalyzed hydrolysis of ZLysONp are pH independent with an average value of 124±25 μM. Data for the chymotrypsin catalyzed hydrolysis of ZLysONp may be fitted with the following pK_a values: k_{cat} -7.05±0.10; k_{+2} -6.93±0.10; k_{+3} -6.93±0.10; k_{cat}/K_m ($=k_{+2}/K_s$) -7.0±0.1. Values of K_s (●) and K_m (○) for the chymotrypsin catalyzed hydrolysis of ZLysONp are pH independent with average values of 722±64 μM and 568±62 μM, respectively.
(Modified from Antonini and Ascenzi, 1981; Ascenzi et al., 1982).

are essentially independent of pH, between pH 2 and 8.5. (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983) except for Ancrhod (pK_a=4.3±0.1) (unpublished results).

The k_{+2} versus pH profile for (pro)enzymes acting on cationic substrates implicates two ionizing groups ($pK_a \simeq 3.6$ and $\simeq 7$). On the other hand for chymotrypsin, elastase and free trypsinogen, values of k_{+2} depend on a single ionizing group ($pK_a \simeq 7$), over the whole pH range explored (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983).

The value of K_s for the catalyzed hydrolysis of ZLysONp by (pro)enzymes acting on cationic substrates has been found to increase of about five folds going from pH $\simeq$ 6 to pH $\simeq$ 3. The data correspond to a simple titration curve with an average pK_a value of 4.2. The value of K_s is pH independent, over the same pH range for chymotrypsin, elastase and free trypsinogen (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983).

For all (pro)enzymes examined, values of k_{+2}/K_s, determined at different pH values, from experiments where $[E_o] \geq 5 \times [S_o]$ are in excellent agreement with those of k_{cat}/K_m determined from steady-state experiments. This finding provides direct evidence for the conformity of the systems to the minimum three-step mechanism (scheme 1).

For all (pro)enzymes examined the pH dependence of k_{+3} conforms to a single ionizing group ($pK_a \simeq 7$) (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983), except for p.p. kallikrein in which k_{+3} depends on two pK_a's (4.6±0.15 and 7.0±0.1) (Ascenzi et al., 1983b).

From the inspection of Fig. 1, it may be observed that in the catalyzed hydrolysis of ZLysONp by trypsin, as well as by other serine (pro)enzymes acting on cationic substrates, k_{+3} is rate limiting in catalysis at pH $\lesssim$ 4, whereas at pH $\gtrsim$ 6, k_{+2} becomes rate limiting. On the other hand, the rate limiting step (k_{+2}) for the chymotrypsin, elastase and free trypsinogen catalyzed hydrolysis of ZLysONp is pH independent (Ascenzi et al., 1982, 1983a; 1983b; Antonini et al., 1982; 1983). As expected (Gutfreund, 1972) when $k_{+2} > k_{+3}$ and $[S_o] \geq [E_o]$, only at pH values where the deacylation step is rate limiting in catalysis, a burst of p-nitrophenol release (of amplitude $A = \alpha \cdot [E_o]$, with a first order rate constant k) is detectable.

THE (PRO)ENZYMES CATALYZED HYDROLYSIS OF ZAlaONp

Fig. 2 shows the pH dependence of steady-state and pre-steady-state parameters for the trypsin and chymotrypsin catalyzed hydrolysis of ZAlaONp (Kassera and Laidler, 1970; Antonini and Ascenzi, 1981; Ascenzi et al. 1982).

Values of k_{cat}, k_{+2}, k_{+3} and k_{cat}/K_m ($=k_{+2}/K_s$) for all the (pro)enzymes examined, may be fitted with a single pH transition with an average pK_a of 7. Moreover, values of K_m and K_s are es-

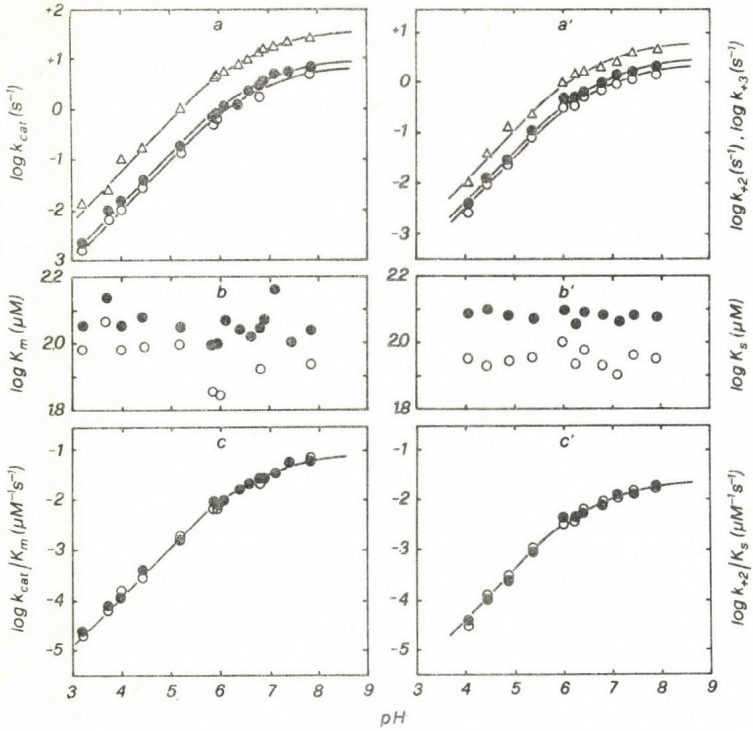

FIG. 2: pH dependence of log k_{cat} (○), log K_m (○), log k_{+2} (●), log k_{+3} (△), log K_s (●), log k_{cat}/K_m (○) and log k_{+2}/K_s (●) for the trypsin (a,b,c) and chymotrypsin (a',b',c') catalyzed hydrolysis of ZAlaoNp at 21±0.5 °C and I=0.1M. Data for the trypsin catalyzed hydrolysis of ZAlaONp may be fitted with the following pK$_a$ values: k_{cat} -6.83±0.10; k_{+2} -6.83±0.10; k_{+3} -6.75±±0.10; k_{cat}/K_m (=k_{+2}/K_s) -6.83±0.10. Values of K_m (○) and K_s (●) for the trypsin catalyzed hydrolysis of ZAlaONp are pH independent with average values of 88±18 μM and 117±22 μM, respectively. Data for the chymotrypsin catalyzed hydrolysis of ZAlaONp may be fitted with the following pK$_a$ values: k_{cat} -6.8±0.1; k_{+2} -6.9±0.1; k_{+3} -6.75±0.10; k_{cat}/K_m (=k_{+2}/K_s) -6.72±0.10. Values of K_m (○) and K_s (●) for the chymotrypsin catalyzed hydrolysis of ZAlaONp are pH independent with average values of 98±12 μM and 118±8 μM, respectively.
(Modified from Antonini and Ascenzi, 1981; Ascenzi et al., 1982).

sentially pH independent between pH 2 and 8.5. At all pH's, values of k_{cat}/K_m, determined from experiments where $[E_o] \geq 5x [S_o]$ are in agreement with those of k_{cat}/K_m determined where $[S_o] \geq 5x [E_o]$ (Ascenzi et al., 1982; Antonini et al., 1983). Since, under all the experimental conditions, k_{+2} is rate limiting in catalysis for all the (pro)enzymes examined, no burst phase is detectable.

THE EFFECT OF ETHYLAMINE ON THE (PRO)ENZYMES CATALYZED HYDROLYSIS OF ZAlaONp

The effect of adding ethylamine on the catalyzed hydrolysis of ZAlaONp by (pro)enzymes acting on cationic substrates is reflected in the increase (from 4 to 10 folds) of the rate of hydrolysis. Moreover, the pH dependence of the pre-steady-state parameters for the hydrolysis of ZAlaONp by (pro)enzymes acting on cationicsubstrates, in the presence of saturating levels of ethylamine, show that at pH ≤ 4, the k_{+3} step is rate limiting in catalysis, whereas at pH ≥ 6, the k_{+2} step becomes rate limiting. On the other hand, in the absence of ethylamine, k_{+2} is rate limiting in the (pro)enzymatic hydrolysis of ZAlaONp, over the whole pH range explored. In contrast, chymotrypsin, elastase and free trypsinogen do not bind ethylamine and the catalyzed hydrolysis of ZAlaONp is unaffected by the presence of this alkylamino compound. Furthermore, at all pH values, both in the presence and absence of ethylamine, k_{+2} remains the rate limiting step in catalysis and the values of K_s, in all cases, are pH independent (Antonini and Ascenzi, 1981; Antonini et al., 1982; 1983; Ascenzi et al., 1982).

The dissociation constant for ethylamine binding to trypsin varies with pH with a pK_a of 4.1 ± 0.1 (see Fig. 3) (Antonini and Ascenzi, 1981; Ascenzi et al., 1982).

COMPARISON OF STEADY-STATE PARAMETERS OF THE (PRO)ENZYMES CATALYZED HYDROLYSIS OF N-α-CARBOBENZOXYGLYCINE p-NITROPHENYL ESTER (ZGlyONp), ZAlaONp, N-α-CARBOBENZOXY-L-LEUCINE p-NITROPHENYL ESTER (ZLeuONp), N-α-CARBOBENZOXY-L-ARGININE p-NITROPHENYL ESTER (ZArgONp), ZLysONp AND N-α-CARBOBENZOXY-L-TYROSINE p-NITROPHENYL ESTER (ZTyrONp)

The values of k_{cat} and k_{cat}/K_m for the catalyzed hydrolysis of ZArgONp by (pro)enzymes acting on cationic substrates change with pH and may be fitted with the acidic and neutral transitions ($pK_a \simeq 4.5$ and $pK_a \simeq 7$) observed for the hydrolysis of ZLysONp; those for the hydrolysis of neutral substrates show only the neutral transition ($pK_a \simeq 7$). On the other hand, the values of k_{cat} and k_{cat}/K_m for the catalyzed hydrolysis of all the p-nitrophenyl esters by chymotrypsin, elastase and free trypsinogen have the same pH dependence corresponding to a single pH transition of $pK_a \simeq 7$. Moreover, the values of K_m have

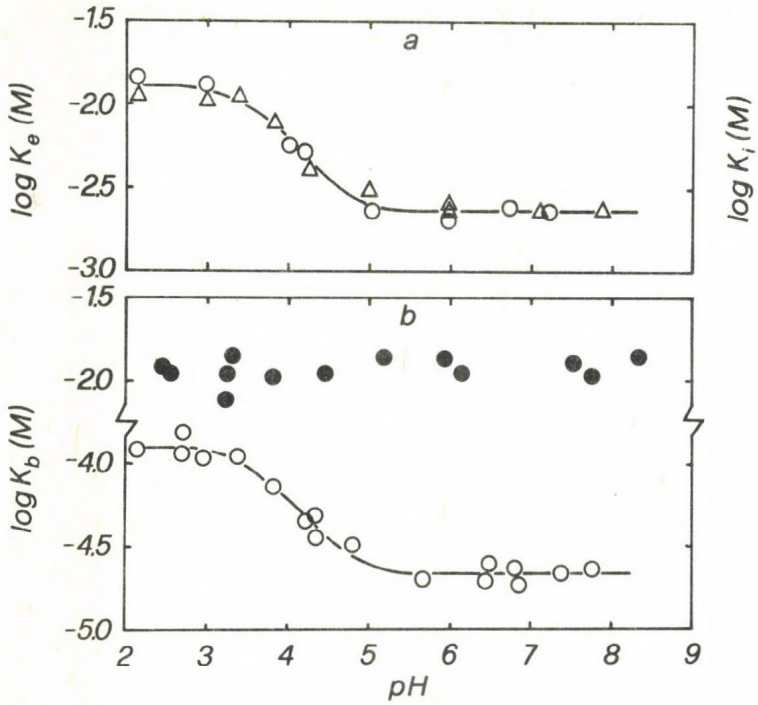

FIG. 3: (a) pH dependence of the dissociation constant for ethylamine binding (K_e, K_i) to trypsin at 21±0.5°C and I=0.1M. Triangles indicate values of K_e obtained from the promoter effect of ethylamine on the trypsin catalyzed hydrolysis of ZAlaONp. Circles indicate values of K_i obtained from the inhibition of the trypsin catalyzed hydrolysis of ZLysONp by ethylamine. The data may be fitted with a single pH transition with a pK_a value of 4.1±0.1. (b) pH dependence of the dissociation constant for benzamidine binding (K_b) to chymotrypsin (●) and trypsin (○) at 21±0.5°C and I=0.1M. Values of K_b have been obtained from the inhibition of the chymotrypsin and trypsin catalyzed hydrolysis of ZLysONp by benzamidine. Values of K_b for benzamidine binding to chymotrypsin are pH independent with an average value of 0.011±0.002 M. Values of K_b for benzamidine binding to trypsin may be fitted with a simple pH transition with a pK_a value of 4.15±0.10.
(Modified from Antonini and Ascenzi, 1981; Ascenzi et al., 1982; Menegatti et al., 1982).

been found to be essentially pH independent in the pH range explored (2-8.5) for all substrates (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1983) except for the Ancrhod catalyzed hydrolysis of ZArgONp (pK_a=4.80±0.15) (unpublished results).

Inspection of Table 1 shows that the catalytic parameters for the same substrates vary largely, reflecting the different specificity profiles of the various (pro)enzymes.

THE BINDING OF BENZAMIDINE AND POLYBENZAMIDINE DERIVATIVES TO SERINE (PRO)ENZYMES

Fig. 3 shows the effect of pH on the inhibition constant of benzamidine on the trypsin and chymotrypsin catalyzed hydrolysis of ZLysONp (Ascenzi et al., 1982; Menegatti et al., 1982). The pH dependencies of the inhibition constants for the binding of benzamidine and polybenzamidine derivatives to serine (pro)enzymes acting on cationic substrates may be generally fitted with a simple pH transition with an average pK_a value of 4.1 (see: Menegatti et al., 1982 and references cited therein).

Additional ionizing groups, well outside the recognition site, have been found to affect the binding of polybenzamidine derivatives (i.e. tetra-p-amidinophenoxy-propane; TAPP) to some serine (pro)enzymes acting on cationic substrates (see: Menegatti et al., 1982 and references cited therein).

No pH effects on the value of the inhibition constant for the binding of benzamidine and its derivatives to chymotrypsin and free trypsinogen has been observed (Ascenzi et al., 1982; Menegatti et al., 1982; Antonini et al., 1983). Moreover, elastase does not bind at all benzamidine and polybenzamidine derivatives (Menegatti et al., 1982).

Finally, in agreement with the specificity towards positively charged substrates, (pro)enzymes acting on cationic substrates show generally an higher affinity for benzamidine and its derivatives than chymotrypsin and free trypsinogen.

SPECTRAL PROPERTIES OF SERINE (PRO)ENZYMES

Fig. 4 shows the effect of pH on the spectral properties of trypsin, between 230 and 330 nm. The pH dependent spectral changes of free (pro)enzymes acting on cationic substrates reflect the ionization of a single group with an average pK_a value of 4.5 (see Fig. 4). On the other hand the absorption spectra of chymotrypsin, elastase and free trypsinogen are unaffected by pH in the same wavelength range (Ascenzi et al., 1982; 1983a; 1983b; Antonini et al., 1982; 1983).

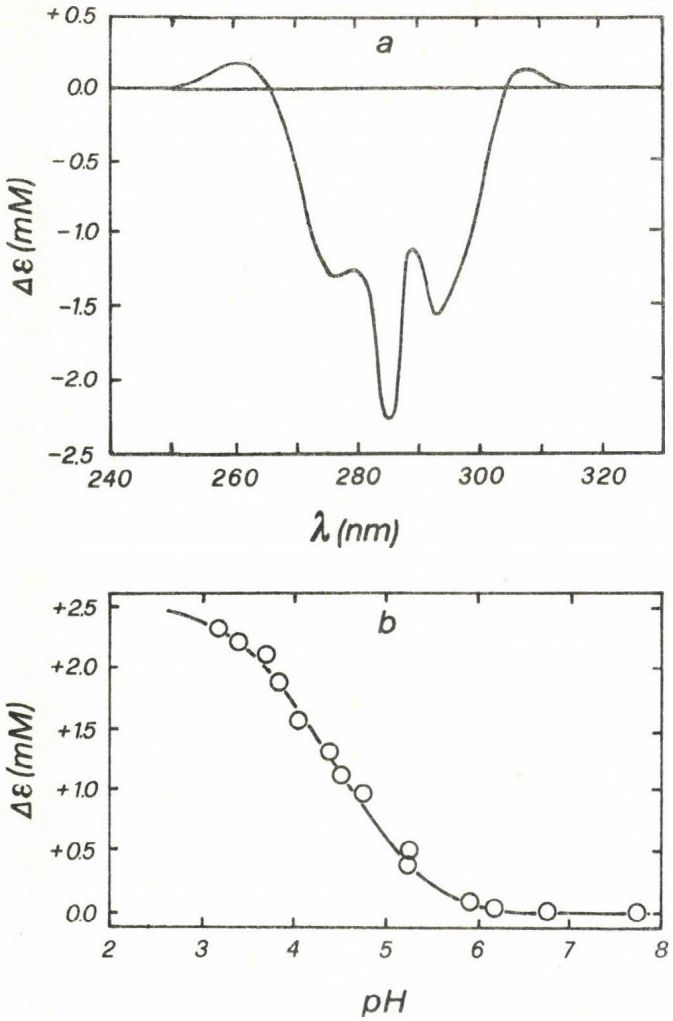

FIG. 4: (a) pH difference spectrum of trypsin at 21±0.5°C and I=0.1M. The reference solution was at pH 7.5 and sample solution was at pH 2.7. (b) pH titration of trypsin at 21±0.5°C and I=0.1M. The fit of data with a simple pH transition yields a pK_a value of 4.5±0.1.
(Modified from Ascenzi et al., 1982).

CONSIDERATIONS ON STRUCTURE-FUNCTION RELATIONSHIPS

From the inspection of Table 1, it may be observed that, in regard to substrate specificity, arginine derivatives are more sensitive substrates for p.p. and h.u. kallikrein, thrombin and Ancrhod than the corresponding ones of lysine. The greater rate of catalysis is reflected in higher values of k_{cat}/K_m. Moreover, in the case of Ancrhod, is also probably reflected in the different effect of pH on values of K_m for the catalyzed hydrolysis of ZArgONp and ZLysONp. The catalytic parameters for the trypsin catalyzed hydrolysis of substrates containing arginine and lysine, are undistinguishable. On the other hand, urokinase shows more favourable steady-state parameters in the hydrolysis of lysine rather than arginine derivatives. Chymotrypsin, elastase and free trypsinogen show no preference for cationic substrates and their catalysis is unaffected by ethylamine.

The rate limiting step in the catalyzed hydrolysis of ZLysONp, ZArgONp, or ZAlaONp in the presence of ethylamine, by serine (pro)enzymes acting on cationic substrates changes with pH. For all these (pro)enzymes, k_{+3} is rate limiting in catalysis at pH $\lesssim 4$, whereas at pH $\gtrsim 6$, k_{+2} becomes rate limiting. On the other hand, the rate limiting step (k_{+2}), for the chymotrypsin, elastase and free trypsinogen catalyzed hydrolysis of both cationic and neutral substrates does not change with pH.

The pH dependence of the ratio k_{cat}/K_m ($=k_{+2}/K_s$) for the catalyzed hydrolysis of ZLysONp by (pro)enzymes acting on cationic substrates indicates (according to Peller and Alberty, 1959) that the free form of the (pro)enzymes undergoes two pH transitions with pK_a values of about 4.5 and 7. On the other hand, chymotrypsin, elastase and free trypsinogen undergo only one acid-alkaline transition with a pK_a value of about 7.

The increase of the value of K_s for the catalyzed hydrolysis of ZLysONp by serine (pro)enzymes acting on cationic substrates (as well as of K_m for the Ancrhod catalyzed hydrolysis of ZArgONp and ZLysONp) from pH 6 to 4, reflects, according to linkage relations (Wyman, 1964), an acid-shift in the pK_a value of the acidic group ($pK_a \simeq 4.5$ in free (pro)enzymes) on binding ZLysONp amounting to 0.5-1 pH units (as shown from the pH dependences of k_{+2}).

In addition, the effect of pH on the dissociation constant for the binding of ethylamine and benzamidine to serine (pro)enzymes acting on cationic substrates, implies a pK_a-shift from $\simeq 4.5$ in free (pro)enzymes, to $\simeq 3.7$ in the complexes.

The absorption spectra of the free (pro)enzymes acting on cationic substrates change with pH according to a simple titration curve with an average pK_a value of 4.5. The pH difference absorption spectra of free (pro)enzymes acting on cationic

TABLE 1

Values of k_{cat}/K_m ($\mu M^{-1} s^{-1}$) for the (pro)enzymatic hydrolysis of cationic and neutral substrates at pH 8 (phosphate buffer, I=0.1M; T=21±0.5 °C).

(Pro)enzymes	Substrates					
	ZGlyONp	ZAlaONp	ZLeuONp	ZArgONp	ZLysONp	ZTyrONp
Elastase	8.3×10^{-3}	1.5×10^{-1}	2.4×10^{-3}	1.6×10^{-4}	1.5×10^{-4}	1.2×10^{-5}
P.p. kallikrein	9.6×10^{-3}	7.3×10^{-3}	4.6×10^{-3}	2.5	1.1×10^{-1}	5.4×10^{-5}
H.u. kallikrein	4.3×10^{-3}	6.7×10^{-3}	---	2.7	7.1×10^{-1}	5.0×10^{-4}
Thrombin	6.1×10^{-3}	8.6×10^{-3}	3.1×10^{-2}	2.3	5.9×10^{-1}	2.8×10^{-1}
Ancrhod	4.2×10^{-3}	3.3×10^{-3}	---	1.7	1.8×10^{-1}	1.3×10^{-2}
Trypsin	4.1×10^{-2}	5.9×10^{-2}	6.4×10^{-2}	1.4	1.4	3.6×10^{-1}
Trypsinogen: H-Ile-Val-OH	1.2×10^{-4}	1.4×10^{-4}	3.2×10^{-3}	2.3×10^{-3}	3.0×10^{-3}	1.2×10^{-3}
Urokinase	2.1×10^{-3}	2.4×10^{-3}	4.2×10^{-4}	2.8×10^{-3}	1.5×10^{-1}	2.5×10^{-4}
Trypsinogen	2.2×10^{-5}	1.8×10^{-5}	5.2×10^{-4}	2.8×10^{-5}	3.6×10^{-5}	4.2×10^{-4}
Chymotrypsin	1.6×10^{-2}	2.0×10^{-2}	6.4×10^{-1}	5.3×10^{-2}	5.4×10^{-2}	2.3

substrates are also similar (Antonini et al., 1982; Ascenzi et al., 1982).

The data here reported indicate that steady-state and pre-steady-state parameters for the catalyzed hydrolysis of p-nitrophenyl esters examined by all serine (pro)enzymes are affected by a common neutral ionization which probably reflects the acid-base equilibrium of His57, involved in the catalytic triad (Kraut, 1977). Moreover, as postulated from Kassera and Laidler (1969), only serine (pro)enzymes acting on cationic substrates, Ancrhod and H-Ile-Val-OH:trypsinogen adduct, contain a similarly located ionizable residue, of $pK_a \simeq 4.5$, in free (pro)enzymes, and $\simeq 3.7$ in their complexes, which affects the binding of cationic substrates, ligands or inhibitors, the spectral properties and the rate limiting step in catalysis. Such residue should be absent in chymotrypsin and elastase and differently located in free trypsinogen. From the inspection of the amino acid sequences and of the three-dimensional structures of the serine (pro)enzymes examined (except Ancrhod) (Magnusson, 1971; Birktoft and Blow, 1972; Huber and Bode, 1978; Sawyer et al., 1978; Bode, 1979; Lottspeich et al., 1979; Schaller et al., 1982; Bode et al., 1983; Chen and Bode, 1983), the residue may be identified with Asp189 which is present in serine (pro)enzymes acting on cationic substrates and is replaced by a neutral serine in chymotrypsin and elastase.

The role of Asp189 on the catalytic, ligand binding and spectral properties of serine (pro)enzymes acting on cationic substrates is outlined from the effect of the dipeptide H-Ile--Val-OH on the physico-chemical properties of trypsinogen. It is well known that in free trypsinogen, Asp189 is pointed out to the solvent and the specificity site disordered (Huber and Bode, 1978). The interaction of H-Ile-Val-OH, which mimics the two N-terminal residues of the activated trypsin, with Asp194 of the (p-guanidinobenzoate:)trypsinogen(:bovine basic pancreatic trypsin inhibitor [(pGB:)trypsinogen(:BPTI)] complex(es) is accompanied by a series of conformational changes in the (pGB:)--zymogen(:BPTI) complexes, which assume trypsin-like properties. In this respect in the H-Ile-Val-OH:trypsinogen:BPTI complex, Asp189 assumes a geometry closed to that of β-trypsin and the specificity pocket becomes ordered (Huber and Bode, 1978; Bode, 1979).

The high specificity of p.p. kallikrein for the δ-guanidino group rather than the ε-amino one and the peculiar pH dependence of k_{+3} in the hydrolysis of ZLysONp have been related to the presence at the specificity site of this proteinase of a serine residue at position 226. In fact this residue cannot only make a hydrogen bond with Asp189, but also can partially shield this residue from the bulk water. Ser226 is replaced by a glycine in trypsin (Bode et al., 1983; Chen and Bode, 1983).

The peculiar catalytic and ligand binding properties of elastase as compared to that of chymotrypsin, have been related to the presence at the recognition site, of two residues showing an high steric hindrance, notably Val216 and Thr226. These residues are replaced by two glycine side chains in chymotrypsin and trypsin (Sawyer et al., 1978).

Finally, a tentative assignment can be given also for the additional ionizing groups affecting polybenzamidine binding to serine (pro)enzymes acting on cationic substrates. On the basis of simple stereochemical consideration, drawn on the analogous three-dimensional structures of β-trypsin and of sequence comparison (Huber and Bode, 1978; Magnusson, 1971), only Glu149 and Asp192 residues of thrombin seem to meet the requirement for productive binding of a second TAPP benzamidine substituent. These two residues are unique to thrombin, while no other negatively charged side chains appear to be accessible to TAPP once one of its benzamidine moiety is bound into the enzyme specificity pocket (Menegatti et al., 1982).

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PRE-STEADY-STATE KINETIC ANALYSIS OF THE INTERACTION OF PROTEINASES WITH SLOW-BINDING INHIBITORS

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This report reviews a method for determining the rate constants for the formation and dissociation of a reversible enzyme-inhibitor complex, based on the analysis of reaction progress curves. The concepts of slow-binding and tight-binding inhibition are discussed taking as example the cathepsin B-leupeptin system. Practical advices are given for a more general application of the method in the field of proteinases.

INTRODUCTION

The reaction of proteinases with naturally occurring protein inhibitors is usually characterized by a very high affinity between the reaction partners and the same is also valid for some low-molecular weight inhibitors.

Slow-binding and slow, tight-binding inhibiton processes represent probably a more generalized phenomenon than previously realized. For instance, the interaction of pepsin with pepstatin (Rich and Sun 1980) and of cathepsin B with leupeptin (Baici and Gyger-Marazzi 1982) are two examples of slow, tight-binding inhibition. These two cases fit very well into the concept of enzyme hysteresis (Frieden 1970). Recently, a slow, tight-binding mechanism was demonstrated for the inhibition of human leukocyte elastase by eglin, an inhibitor protein from the leech *Hirudo medicinalis* (Baici and Seemüller 1983).

Slow processes characterize the binding of oxidized human plasma α_1-proteinase inhibitor to human leukocyte elastase, of native human plasma α_1-proteinase inhibitor to human pancreatic cationic trypsin, plasmin and thrombin (Beatty *et al.* 1980), of human plasma inter-α-inhibitor to trypsin and chymotrypsin (Aubry and Bieth 1976).

The knowledge of the detailed inhibition mechanism is of paramount importance in the field of proteinase inhibitors since this is an elegant way to predict their physiological role. In particular, not only K_i, but also the individual rate constants k_{on} and k_{off} for formation and dissociation of the complex, respectively, should be taken into account (Bieth 1980).

An indirect method using competition kinetics has been employed for measuring k_{on} and k_{off} in the trypsin-pancreatic trypsin inhibitor system (Vincent and Lazdunski 1972). The same technique has been succesfully applied to characterize the association of human leukocyte elastase with α_1-proteinase inhibitor by allowing this enzyme and bovine chymotrypsin to compete for the binding with the inhibitor (Beatty *et al.* 1980). An extension of this method has been reported by Gauthier *et al.* (1982) for determining the rate of association of human leukocyte elastase with human bronchial inhibitor (leukocyte elastase was allowed to compete for the binding with α_1-proteinase inhibitor and bronchial inhibitor and the residual α_1-proteinase inhibitor was assayed with pancreatic elastase).

These methods are applicable as long as appropriate inhibitors and competition systems are available for the enzyme considered.

The present report reviews a relatively simple technique, which is independent on coupled reactions, and allows the direct determination of the association and dissociation rate constants for enzyme-inhibitor systems characterized by a slow-binding or a slow, tight-binding process.

DEFINITIONS AND NOMENCLATURE

The nomenclature of slow-inhibition and tight-binding inhibition is not yet universally accepted. A criterion has been proposed by Agarwal *et al.* (1977) for classifying reversible enzyme inhibitors in three categories depending on the shape of reaction progress curves. To determine the class to which an inhibitor belongs, the enzyme is assayed in two ways: 1) enzyme and inhibitor are preincubated and the reaction is started by adding the substrate; 2) substrate and inhibitor are preincubated and the reaction is started with the enzyme. When the time courses of the two assays are identical the inhibitor is classified as *readily reversible*, when they are different but attain the same steady-state velocity within a reasonable period of time the inhibitor is called *semi-tight-binding*, and when the reaction profiles are different and do not attain the same steady-state velocity over a long period of time the inhibitor is said to be *tight-binding*. These definitions are justified by the fact that with increasing strength of interaction between enzyme and inhibitor, a stage is reached in which the equilibrium of the reaction can not be established rapidly.

In a concise review, Morrison (1982) pointed out that the slow establishment of equilibrium for the formation of an enzyme-inhibitor complex is not an unique feature of tight-binding inhibitors. In fact, tight-binding inhibitors are known, for which the formation of the EI complex is a fast process and, conversely, slow-binding can occur without concomitant tight-binding (Williams *et al.* 1980, Williams and Morrison 1979). Therefore, other nomenclatures were proposed: *slow, tight-binding* as an equivalent of the above mentioned semi-tight-binding (Williams *et al.* 1979) and *very slow tight binding* as a synonim of

tight-binding (Williams and Morrison 1979). In any case, a sharp line of demarcation can not be drawn between various inhibitor classes because of the subjectivity in defining the time scale during which the inhibition process is observed and a critical K_i value which defines the limits of tight-binding and classical inhibition.
In this report the nomenclature proposed by Morrison (1982) is adopted and is schematically reproduced in Table 1.

TABLE 1

Classification of reversible enzyme inhibitors.

Class of inhibitor	Relative concentrations of enzyme and inhibitor $[E]_o$ and $[I]_o$	Rate of establishment of the equilibrium $E + I \rightleftharpoons EI$
Classical	$[I]_o >> [E]_o$	fast
Tight -binding	$[I]_o \cong [E]_o$	fast
Slow-binding	$[I]_o >> [E]_o$	slow
Slow, tight-binding	$[I]_o \cong [E]_o$	slow

THEORETICAL REMARKS

The treatment presented below applies to the model shown in Scheme 1, which represents a fully competitive, reversible inhibition mechanism. It is assumed that: a) Steady-state conditions are reached rapidly between enzyme (E), substrate (S) and the ES complex, whereas a slow process characterizes the binding of inhibitor (I) to the enzyme; b) The reverse reaction (catalytic step) is negligibly slow. Further necessary conditions, which can be realized experimentally, are: c) $[E]_o << [I]_o$, which means also $[I]_o \cong [I]$; d) $[I]_o >> K_i$; e) $[S]_o >> [E]_o$; f) The experiments are performed only while the conversion of substrate to product has no effect on the reaction velocity (substrate depletion and accumulation of product).

$$E + S \rightleftharpoons ES \longrightarrow \cdots\cdots \longrightarrow E + \text{Products}$$

$$+$$

$$I$$

$$K_{on} \downarrow \uparrow K_{off} \qquad K_i = k_{off}/k_{on}$$

$$EI$$

Scheme 1

Time-dependent equations for the mechanism in Scheme 1 and also for more complex systems have been reported (Cha 1975, 1976, 1980). In the general case (Cha 1980) the restrictions c) and d) above are not necessary, but in this case the graphical method described below is no longer applicable. This is due to the fact that for $[E]_o \cong [I]_o$ there will be a substantial proportion of I

bound in the EI complex and the condition $[I]_o \cong [I]$ will no longer be valid. In this case, a factor accounting for the depletion of free inhibitor can be introduced in the equations and calculations must be performed using computer iterative procedures (Cha 1980, Williams *et al.* 1979). For the derivation of the basic rate equations the reader is referred to the cited literature. Since the algebraic symbolism used there is somewhat cumbersome for the treatment of Scheme 1, the equations described by Baici and Gyger-Marazzi (1982) will be adopted here.

There are two fundamental experimental conditions which can influence the shape of the reaction progress curve: Enzyme preincubated or not preincubated with the inhibitor. The effect on the reaction profile will be macroscopic because the rate equation for the appearance of product contains also the $[EI]_o$ term, i.e. the concentration of the EI complex at the beginning of the reaction. Typical curves for reversible slow-binding inhibition are shown in Fig. 1, while the integrated rate equations are:

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on} [I]}{(1+\sigma)\lambda^2} (1-e^{-\lambda t}) \qquad (1)$$

$$[P]^* = \frac{v_o k_{off}}{\lambda} t - \frac{v_o k_{off}}{\lambda^2} (1-e^{-\lambda t}) \qquad (2)$$

The asterisk in Eq. (2) and in the following expressions denotes that the enzyme has been preincubated with inhibitor, whereas symbols without asterisk mean no preincubation. v_o is the initial velocity in the absence of inhibitor, $\sigma = [S]/K_m$ and λ is an apparent pseudo-first-order rate constant which describes the exponential approach to the steady-state:

$$\lambda = \frac{k_{on}}{1+\sigma} [I] + k_{off} \qquad (3)$$

After a given time the curvatures shown in Fig. 1B, C approach a linear steady-state phase and the exponential terms in Eqs. (1) and (2) become negligibly small. Therefore the two equations can be rewritten as follows:

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on} [I]}{(1+\sigma)\lambda^2} \qquad (4)$$

$$[P]^* = \frac{v_o k_{off}}{\lambda} t - \frac{v_o k_{off}}{\lambda^2} \qquad (5)$$

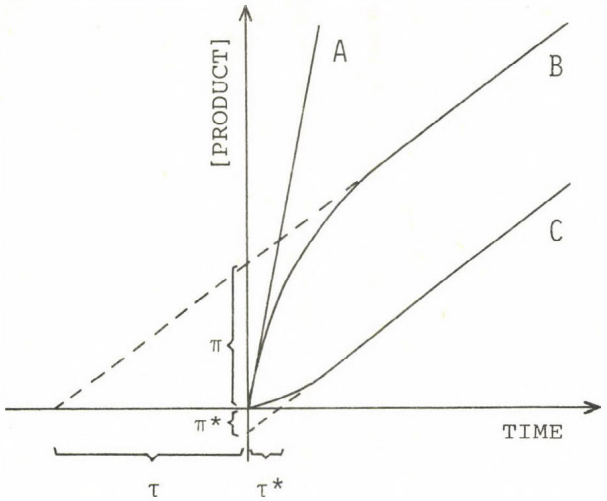

Fig. 1. Progress curves for slow-binding inhibition. (A) Control curve in the absence of inhibitor; (B) Inhibitor not preincubated with enzyme; (C) Enzyme and inhibitor preincubated. Curves (B) and (C) were generated using Eqs. (1) and (2), respectively.

Equations (4) and (5) describe straight lines with the same slope and different intercepts. The slopes are equal to v_i, the inhibited reaction velocity in the steady-state for fully competitive inhibition:

$$\text{Slope} = v_i = \frac{v_o k_{off}}{\lambda} = \frac{V[S]/K_m}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}}$$

Equations (4) and (5) are very useful for the graphical calculation of the rate constants. Extrapolating back the linear portions of curves B and C in Fig. 1 we obtain intercepts with the [P]-axis:

$$\pi = \frac{v_o k_{on}[I]}{(1+\sigma)\lambda^2} \tag{6}$$

$$\pi^* = -\frac{v_o k_{off}}{\lambda^2} \tag{7}$$

and intercepts with the time axis:

$$\tau = -\frac{k_{on}[I]}{k_{off}(1+\sigma)\lambda} \tag{8}$$

$$\tau^* = 1/\lambda \qquad (9)$$

Various combinations of Eqs. (6)-(9) allow the calculation of k_{on}, k_{off} and K_i. Of the several possible combinations, the following are very useful. From Eqs. (8) and (9), introducing the explicit formula for λ (Eq. 3):

$$k_{off} = \frac{1}{|\tau| + \tau^*} \qquad (10)$$

Combining Eqs. (6), (7) and (10):

$$k_{on} = \frac{\pi(1 + \sigma)}{|\pi^*|(\tau^* + |\tau|)[I]} \qquad (11)$$

Since τ and π^* are negative numbers, their absolute values $|\tau|$ and $|\pi^*|$ are considered. Combining Eqs. (4), (5), (8) and (9):

$$k_{off} = \frac{v_o - v_i}{v_o} \frac{1}{|\tau|} \qquad (12)$$

In some instances the following transformation of Eq. (6) can be used (when π and λ are known with precision):

$$k_{on} = \frac{\pi(1+\sigma)\lambda^2}{v_o[I]} \qquad (13)$$

Rearranging Eq. (8) into the reciprocal form we obtain:

$$\frac{1}{|\tau|} = K_i k_{off}(1+\sigma)\frac{1}{[I]} + k_{off} \qquad (14)$$

Thus, a plot of $1/|\tau|$ versus $1/[I]$ will give a straight line with an ordinate intercept corresponding to k_{off} and abscissa intercept equal to $-1/(1+\sigma)K_i$.

The values of π and λ to be used in Eq. (13) can be obtained with sufficient approximation from a semilogarithmic plot according to Guggenheim (1926) applied to a reaction progress curve started by addition of the enzyme (Fig. 1B). In order to be successful, this analysis must be performed at the very beginning of the progress curve. In other words, the semilogarithmic plot should be made at time points for which the first term on the right side of Eq. (1) is negligible. This method allows the calculation of π by means of the following relation:

$$\pi = \frac{e^{int}}{1 - e^{-\lambda\Delta t}} \qquad (15)$$

Where Δt is the time interval used for calculating the semilogarithmic plot and λ and *int* are the slope and the intercept of the plot with the ordinate axis, respectively.

ANALYSIS OF A REAL CASE

The slow, tight-binding inhibition of cathepsin B by leupeptin.

Full details on the purification of human spleen cathepsin B and on the inhibition mechanism of this enzyme by leupeptin were reported by Baici and Gyger-Marazzi (1982). This system will be briefly discussed here to illustrate the procedures for calculating k_{on} and k_{off} (Scheme 1) from progress curves. Fig. 2 shows some profiles of reactions started by adding the enzyme. The substrate used was N-carbobenzoxy-L-phenylalanyl-L-arginine-7-amino-4-methylcoumarylamide (Z-Phe-Arg-NMec) and the reaction conditions are given in the legend of the figure.

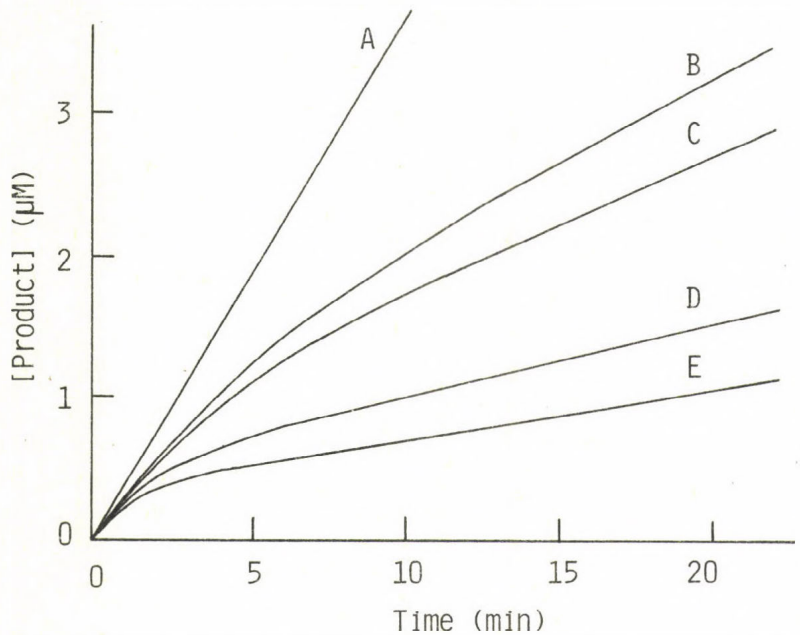

Fig. 2. Inhibition of human spleen cathepsin B by leupeptin. The curves represent continuous-recording traces for the release of 7-amino-4-methylcoumarin monitored fluorimetrically. The solution contained 2.0 mM EDTA, 2.0 mM dithiothreitol, 0.18 mM Z-Phe-Arg-NMec in 0.1 M phosphate buffer, pH 6.00. Leupeptin concentrations were (nM): 0 (A), 27 (B), 44 (C), 87 (D) and 148 (E). Reactions were started by the addition of cathepsin B that was preactivated with 2.0 mM dithiothreitol for 15 min. The final concentration of the enzyme was 0.22 nM, based on protein content and a molecular weight of 27,000.

Fig. 3 shows experiments in which the enzyme was preincubated (curve C) or not preincubated (curve B) with the inhibitor and illustrates practically what is shown in the simulated curves in Fig. 1. From Fig. 2 we obtain the following information: 1) The slope of curve A corresponds to v_0; 2) The slopes of the linear parts of curves B-E correspond to the inhibited steady-state velocities v_i; 3) Extrapolating back the linear parts of curves B-E we obtain four values for π and τ. The reciprocals of the τ values may be plotted versus the reciprocals of the corresponding leupeptin concentrations (not shown) giving a straight line with an ordinate intercept of $7.9 \times 10^{-4} s^{-1} = k_{off}$, and an abscissa intercept of $-1.3 \times 10^{8} M^{-1}$ corresponding to $-1/(1+\sigma)K_i$. Since $K_m = 0.23$ mM and $[S] = 0.18$ mM (Baici and Gyger-Marazzi 1982), the ratio $\sigma = [S]/K_m = 0.78$ and $K_i = 4.3$ nM. Alternatively, k_{off} can be calculated by means of Eq. (12). k_{on} can now be calculated from K_i and k_{off} or using Eq. (13) with the values of λ and π obtained from semilogarithmic plots.

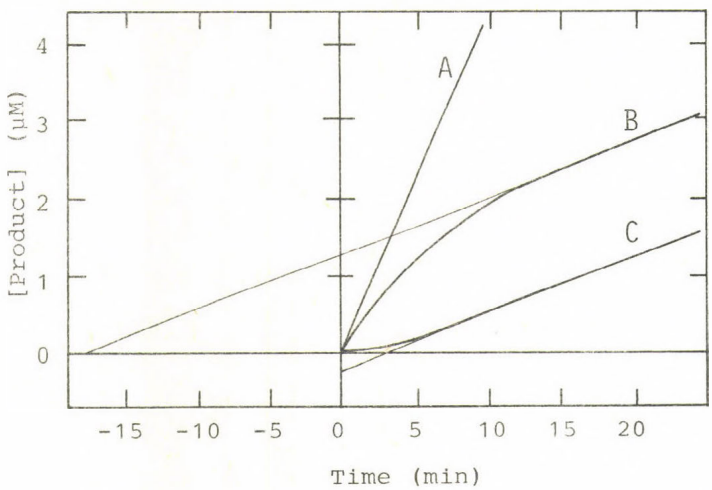

Fig. 3. Inhibition of human spleen cathepsin B by leupeptin. (A) No inhibitor added; (B) and (C) leupeptin 44 nM. In curve B the reaction was started by adding the enzyme and in curve C by adding the substrate (preincubation of enzyme and inhibitor for 20 min). All other conditions as in Fig. 2.

The information given by Fig. 3 is manyfold: $\pi = 1.26$ μM, $|\pi^*| = 0.22$ μM, $|\tau| + \tau^* = 1269$ s. From these values k_{on} and k_{off} can be calculated with the aid of Eqs. (11) and (10), respectively: $k_{on} = 1.82 \times 10^5 M^{-1}s^{-1}$; $k_{off} = 7.88 \times 10^{-4} s^{-1}$; $K_i = k_{off}/k_{on} = 4.3$ nM.

From the calculations above it is evident that the analysis

has a high degree of internal consistency. Several experiments as those shown in Figs. 2 and 3 afforded the following average values ± S.D.: k_{on} = 1.8 ± 0.1 x 10^5 $M^{-1}s^{-1}$; k_{off} = 7.9 ± 0.1 x 10^{-4} s^{-1}; K_i = 4.4 ± 0.3 nM.

For the cathepsin B-leupeptin system it has been also possible to measure K_i using a classical steady-state approach. Using the specific velocity plot (Baici 1981) a K_i value of 5.0 ± 0.4 nM could be calculated (Baici and Gyger-Marazzi 1982). This value is in reasonable agreement with the figure of 4.4 nM obtained by pre-steady-state analysis and suggests that k_{on} and k_{off} were determined correctly.

CRITICISM, COMMENTS AND PRACTICAL ADVICES

The success of the pre-steady-state kinetic analysis discussed above is subordinated to several factors. As explained in the theoretical part, no limitations are set as concerns the relative concentrations of enzyme and inhibitor if the complete equation is used (Cha 1980). This method implies the use of a statistical approach using computer techniques which may not be accessible to all investigators. The recent progress made in the synthesis of peptide substrates having very sensitive fluorogenic leaving groups (most notably peptide substrates containing 7-amino-4-methylcoumarin) offers powerful assay methods for the assay of several proteinases. Owing to the high sensitivity of the fluorimetric technique, the condition $[E]_o << [I]_o$ can probably be realized for several slow, tight-binding proteinase inhibitor systems. The lower limit of enzyme concentrations which can be used will depend on the stability of the enzymes in very diluted solution (denaturation, adsorption, etc.).

Whenever possible, experiments should be performed using a sensitive fluorogenic substrate and at inhibitor concentrations which are ten or more times that of the enzyme: In this way the tight-binding condition will be elegantly circumvented and the slow-binding process can be easily analysed. Of course, continuous monitoring of the reaction is essential.

It is very important that the slow kinetics are studied under conditions in which substrate depletion and product accumulation are negligible (in other words, the control curve A in Fig. 1 should be observed and must remain linear for a time corresponding to that of curves B and C). The occurrence of a linear steady-state after the lag phases (Fig. 1 B, C) is a demonstration that the slow-binding process is reversible.

By inspection of Fig. 1 and Eqs. (6)-(9) it is clear that π^* and τ^* can be determined with less precision than π and τ. In particular, for relatively high inhibitor concentrations, both π^* and τ^* become very small. On the contrary, experiments in which the reaction is started by adding the enzyme [Eqs. (1) and (4), Fig. 1B] give more accurate results, as it has been verified experimentally (Baici and Gyger-Marazzi 1982, Baici and Seemüller 1983, Hanozet *et al.* 1981). For this reason, the calculation of k_{on} and k_{off} by using equations requiring the concomitant presence of τ^* and π^* with τ and/or π [as Eq. (11)] should

only be performed if τ^* and π^* can be measured with high accuracy.

The use of Eq. (13) requires careful attention. In fact, it can be criticized that the values of π and λ are obtained using the semilogarithmic plot proposed by Guggenheim (1926) to analyse first-order reaction kinetics. Strictly speaking, this method is exactly applicable to a purely exponential reaction profile, whereas Eq. (1) (Fig. 1B) is the sum of an exponential term and of a linear term. However, if the analysis is made using the very beginning of the progress curve, the exponential term in Eq. (1) will be much greater than the linear one, thus justifying the approximation. The following are controls for internal consistency: 1) The semilogarithmic plot is a straight line only if the method is correctly applied or is curved upwards if the time interval used is too large; 2) The value of π can be compared with that obtained by extrapolation of the steady-state curve. Using simulated curves it could be verified that this approximation gives k_{on} values within 3% of the true value when [I] is at least $10K_i$, and the precision increases with increasing $[I]/K_i$ ratio. Thus, the uncertainty introduced in the determination of k_{on} by using Eq. (13) is certainly less than the overall experimental error.

Besides the relatively simple competitive mechanism shown in Scheme 1 other pathways implying slow-binding or slow, tight-binding inhibition could exist. General expressions for non-competitive and uncompetitive inhibition and an extension of the rate equation to two-substrate reactions have been analysed by Cha (1980). Also for these mechanisms the graphical method can be used if the conditions above apply.

CONCLUDING REMARKS

The pre-steady-state kinetic analysis of progress curves for reactions involving slow, (tight)-binding inhibitors offers a relatively simple tool for evaluating the individual rate constants of the inhibition process.

The importance of knowing k_{on} and k_{off} for EI formation and dissociation has been emphasized by Bieth (1980) and will be no longer discussed here. The only concept I would like to remember is that k_{on} is a direct measure of the time required for a proteinase to be inhibited *in vivo*. Thus, an inhibitor, although efficient *in vitro*, may be physiologically irrelevant if its concentration is insufficient to achieve a sufficiently rapid encounter with the enzyme. The cathepsin B-leupeptin system analysed above illustrates well this concept. From $K_i \cong 5$ nM we would suppose that a tenfold excess of leupeptin over this value could effectively inhibit cathepsin B. However, from $k_{on} \cong 2 \times 10^5$ $M^{-1}s^{-1}$ and a leupeptin concentration of 50 nM, we can calculate a delay time for inhibition of 500 s, which is about 8 minutes [delay time for inhibition = $5 \times t_{1/2} = 5/\{k_{on}[I]\}$ (Bieth 1980)]. In order for leupeptin to be a physiologically significant inhibitor of cathepsin B, its concentration at the target site must be at least 10 µM (binding faster than 2s), otherwise the extracellularly released enzyme will bind its natural substrates

faster than the inhibitors.

In the past history of enzymology, a relatively limited number of detailed studies have been made using the kinetic approach outlined in this paper. In the particular field of proteinase inhibitors the concepts of slow-binding, tight-binding and slow, tight-binding inhibition were discussed in only few cases. It is well possible that several pertinent examples have been overlooked or not sufficiently analysed in the literature.

The slow process accompanying tight-binding inhibition may in several instances be a manifestation of enzyme hysteresis (Frieden 1970) and, therefore, may be related to some important regulatory function of the enzyme-inhibitor system.

From the mechanistic point of view, slow, tight-binding proteinase inhibition may involve slow interactions of E and I or slow isomerizations of rapidly formed EI complexes. Furthermore, transition state analogues may give rise to kinetic patterns falling in the category of slow, tight-binding inhibition. An accurate analysis of the pre-steady-state kinetics will certainly help interpreting these features.

I suggest that the concepts of slow-binding inhibition, slow, tight-binding inhibition and enzyme hysteresis be reevaluated, correctly used and, in the pertinent cases, be introduced in the current terminology of proteinase inhibitors.

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DISCUSSION

TURK:

Did you compare your experimental results with those obtained by the classical method?

BAICI:

Yes, I did. In the case of cathepsin B-leupeptin system the agreement between K_i's from pre-steady-state and from steady-state analysis is satisfactory (4.4±0.3 nM compared with 5.0±0.4 nM). The agreement was also satisfactory for the elastase-eglin system (0.8 nM from pre-steady-state compared with a value of 0.5-1.5 nM from steady-state analysis).

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PROTEINASE-PROTEINASE INHIBITOR IMBALANCE IN INFLAMMATION AND MULTIPLE TRAUMA WITH SPECIAL EMPHASIS ON RELEASE OF GRANULOCYTIC LYSOSOMAL ELASTASE*

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INTRODUCTION

Severe injury or infection trigger the so-called inflammatory response of the organism. This response includes besides other reactions the activation of humoral systems such as clotting, fibrinolysis, complement and kallikrein-kinin cascades, and of various cellular systems, especially of phagocytes, mast cells and lymphocytes, but also of stress hormone producing cells.

Phagocytes such as polymorphonuclear granulocytes and macrophages contain high numbers of lysosomes equipped with a powerful hydrolytic or proteolytic potential (Dingle, 1977). Normally the cell uses this enzyme equipment, in addition to oxidizing agents, for mainly two purposes (Klebanoff and Clark, 1978):
(i) the maintenance of the intracellular protein catabolism including the degradation of wasted endogenous substances, and
(ii) the defense of invasive organisms by degradation after phagocytosis of viruses and bacteria.

If released extracellularly, they may enhance the inflammatory response via two major routes (Fig. 1):

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*An extented version of this article will be published in Clinical Chemistry (H. Fritz et al.: Granulocyte Proteinases as Mediators of Unspecific Proteolysis in Inflammation. A Review).

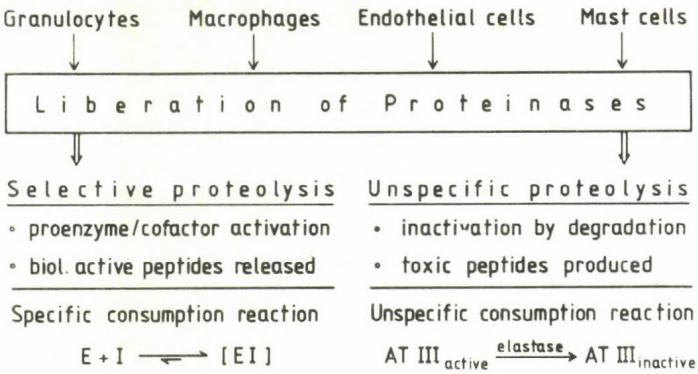

Fig. 1. Proteolytic processes due to liberation of lysosomal proteinases from various body cells.

Selective proteolysis leads to proenzyme and/or cofactor activation including the formation of biologically highly potent peptides, as for example kinins and anaphylatoxins. The proteinases formed are eliminated by specific interactions with their target inhibitor proteins. Hence, reaction mechanisms of high selectivity are responsible for the so-called specific consumption of factors of the clotting, fibrinolysis, complement and kallikrein-kinin cascades.

Unspecific proteolysis means inactivation of soluble factors or destruction of structural elements simply by proteolytic digestion. Such unspecific consumption reactions may be accompanied also by the production of toxic peptides like, for example, the clot formation inhibiting fibrin/fibrinogen degradation products.

LYSOSOMAL PROTEINASES

Of the lysosomal proteinases known so far, elastase and

cathepsin G, the neutral proteinases from polymorphonuclear granulocytes, deserve special interest. Like the acidic thiol and aspartate proteinases, the various cathepsins, they are stored in the lysosomes in fully active form. The most striking feature of both, elastase and the chymotrypsin-like cathepsin G, is an almost unlimited cleavage specifity (Fig. 2).

Lysosomal Proteinases (preformed)

neutral (pH 6-9)	acidic (pH 3-7)
• Elastases	• Cathepsin B
• Cathepsin G	• Cathepsins H, L
° Collagenases	• Cathepsins A, C
° Kininogenases (Kallikreins)	• Cathepsin D (Leukokininogenase)

cleavage specificity: • broad; ° high

Fig. 2. Lysosomal proteinases of neutrophils.

They are able to degrade numerous humoral factors including proteinase inhibitors as well as structural elements like elastin and collagen type III and IV at physiological pH. The potential lysosomal digestion capacity in humans is clearly demonstrated by the calculated synthesis rate of more than 1 g of neutral proteinases per day. However, inside the cells, the lysosomal proteinases are kept under control by either assemblage in membrane coated organelles or by proteinase inhibitors present in the cytosol.

PLASMA PROTEINASE INHIBITORS

Lysosomal proteinases escaping from their natural target cells are faced usually with potent antagonists, the wellknown proteinase inhibitor proteins (Travis and Salvesen, 1983).

α_2-Macroglobulin (α_2M) inhibits effectively serine proteinases as well as thiol, aspartate and metallo proteinases; due to its high molecular weight, its function is normally restricted to the vascular bed.

α_1-Proteinase inhibitor (α_1PI), the major antagonist of the lysosomal neutrophil elastase, is present in remarkable high concentration in blood, but occurs also in interstitial fluid and mucous secretions.

α_1-Antichymotrypsin (α_1AC), a rapidly responding acute phase reactant - it can reach up to six times its normal concentration during the inflammatory response - is a potent inhibitor of lysosomal neutrophil cathepsin G and mast cell chymase.

Compared to α_1PI and α_1AC, the plasma concentrations of the other given inhibitors are clearly lower. Nevertheless, the proteinase inhibitor proteins represent approximately 60 % of the residual plasma proteins after removal of albumin and the immunoglobulins. This is an indirect indication upon the significance of proteinase inhibitors as regulatory proteins of the organism.

The association between known plasma proteinase inhibitors and their target enzymes is schematically compiled in Fig. 3.

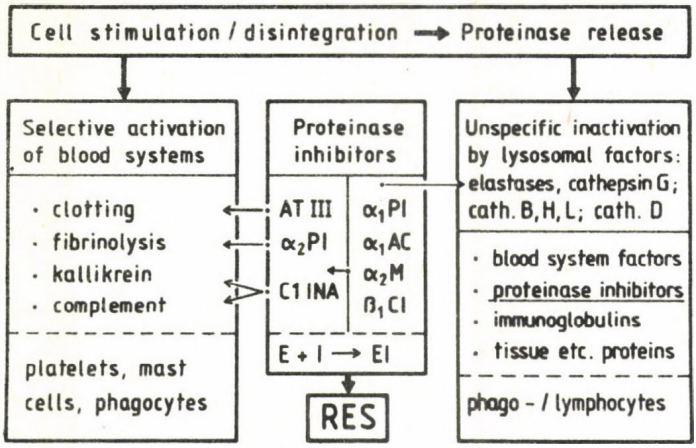

Fig. 3. Activation and consumption reactions: specific activation of blood systems by system-specific proteinases (left part); unspecific degradation of plasma factors by lysosomal proteinases (right part); complex formation with proteinase inhibitors and elimination of the enzyme-inhibitor complexes by phagocytes of the reticulo endothelial system (RES; central part).

Excessive activation of blood systems may be controlled primarily by three inhibitors: antithrombin III (AT III) regulates clotting, α_2-plasmin inhibitor (α_2PI) fibrinolysis, and C1-inactivator (C1-INA) both, the classical complement pathway and the intrinsic coagulation cascade, the latter by inhibition of plasma kallikrein and Hageman factor, respectively its low molecular weight fragment.

The occurrence of complexes between α_2-macroglobulin and plasma kallikrein or plasmin in plasma under certain pathological conditions indicates that this multifunctional glycoprotein is also involved in the regulation of the blood system cascades. However, present evidence suggests a predominant protective

role of α_2-macroglobulin in prevention of unspecific proteolysis by inhibition of all types of liberated lysosomal proteinases. Remarkable, in humans α_2M is, in contrast to α_1PI and α_1AC, not an acute phase reactant, although it is supposed to be the most important endogenous plasma proteinase inhibitor.

Inhibition of activated or liberated proteinases by the given inhibitors means that formation of vasoactive or toxic peptides such as kinins or anaphylatoxins as well as proteolysis-induced stimulation of cellular systems like, for example, thrombin-induced platelet aggregation or the anaphylatoxin-induced chemotactic response of granulocytes, is also depressed. Therefore, reduction of the proteinase inhibitory potential of the organism by unspecific proteolysis represents one of the most striking pathological effects caused by lysosomal proteinases. As we could show some years ago, AT III, for example, is rapidly inactivated in the presence of catalytic amounts of neutrophil elastase (Jochum et al., 1981 a).

The same holds true for α_2PI and C1-INA (Brower and Harpel, 1982). Even α_1PI, the major antagonist of neutrophil elastase, can be proteolytically inactivated by a lysosomal metallo enzyme from macrophages (Banda et al., 1980). Moreover, oxidation of the methionine residue in the enzyme-reactive site of α_1-proteinase inhibitor leads to a significant reduction of the affinity of this inhibitor to neutrophil elastase (Beatty et al., 1980). Such oxidizing agents, for example, superoxide, hydroxyl radicals and hydrogen peroxide, are produced in high amounts in the phagolysosomes to facilitate intracellular protein breakdown and may be released simultaneously with the lysosomal enzymes under pathological conditions (Klebanoff and Clark, 1978).

Hence, severe injury or infection can induce consumption of proteinase inhibitor proteins via three pathways:

(i) complex formation with liberated lysosomal or activated plasma proteinases,
(ii) inactivation by proteolytic degradation, and
(iii) inactivation by oxidative denaturation.

The latter mechanism deserves special interest in view of the proposed pathological effectiveness of neutrophil elastase. Although the oxidized α_1-proteinase inhibitor is still capable to react slowly with neutrophil elastase, the complex thus formed is readily dissociated by substrates exhibiting high affinity for elastase, for example, elastin, that means, the oxidized inhibitor cannot hinder elastase to digest natural target substrates. Remarkable, in contrast to the native inhibitor, oxidized α_1-proteinase inhibitor does not react at all with pancreatic elastase.

RELEASING PROCESSES AND MEASUREMENT OF LIBERATED LYSOSOMAL PROTEINASES

Most intriguing is the question concerning the mechanisms responsible for a more or less dramatic escape of lysosomal factors from within the cells (Fig. 4).

Whereas only small amounts should leak out during normal phagocytosis, relatively high amounts could be excreted during frustrated phagocytosis when the phagocyte is unable to take up a comparatively huge structural element, as for example a vascular plasma membrane or parts of cartilage. Disintegration processes caused by endogenous or exogenous endotoxins, eventually in combination with complement lysis, are most dramatic events leading to release of all lysosomal or phagolysosomal constituents.

Neutrophil elastase liberated under such conditions is present in the circulation primarily in form of the elastase-α_1-proteinase inhibitor (E-α_1PI) complex (Fig. 5). A small amount of neutrophil elastase may be bound also to α_2-macroglobulin (α_2M), however, compared to the E-α_1PI complex the E-α_2M complex is

much more rapidly eliminated from the circulation ($t_{1/2} \sim 10$ min versus $t_{1/2} \sim 1$ h) so that estimation of E-α_2M complexes in plasma needs special assays of extreme sensitivity.

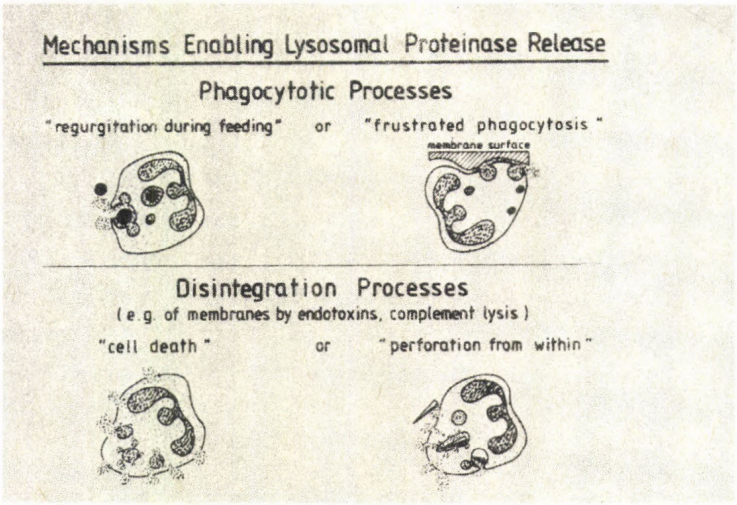

Fig. 4. Various releasing processes of lysosomal proteinases from within the cell.

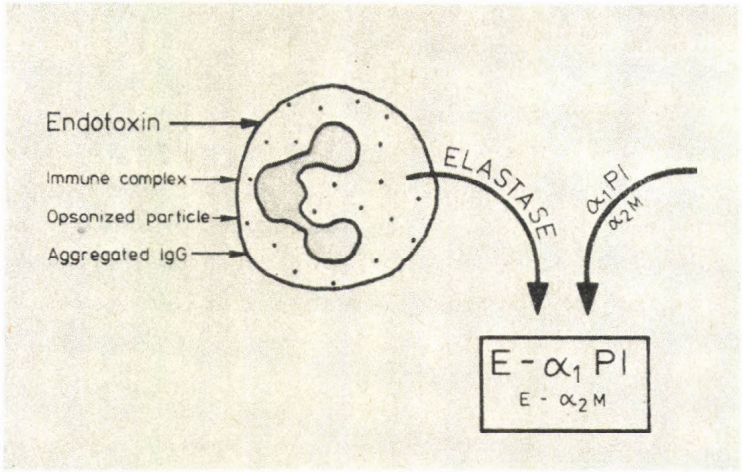

Fig. 5. Liberation of lysosomal elastase (E) due to different stimuli and complex formation with α_1-proteinase inhibitor (α_1PI) as well as (to a much less extent) α_2-macroglobulin (α_2M).

E-α_1PI levels (Fig. 6) can be measured exactly with a newly developed enzyme-linked immunoassay (Neumann et al., 1981). Briefly, plasma samples or other body fluids are incubated in plastic tubes coated with sheep antibodies directed against human neutrophil elastase. After two washing steps, the surface-fixed E-α_1PI complex is exposed to rabbit antibodies directed against human α_1PI. These antibodies are labelled with alkaline phosphatase. Under the conditions used, the amount of bound alkaline phosphatase is proportional to the concentration of E-α_1PI in the applied sample.

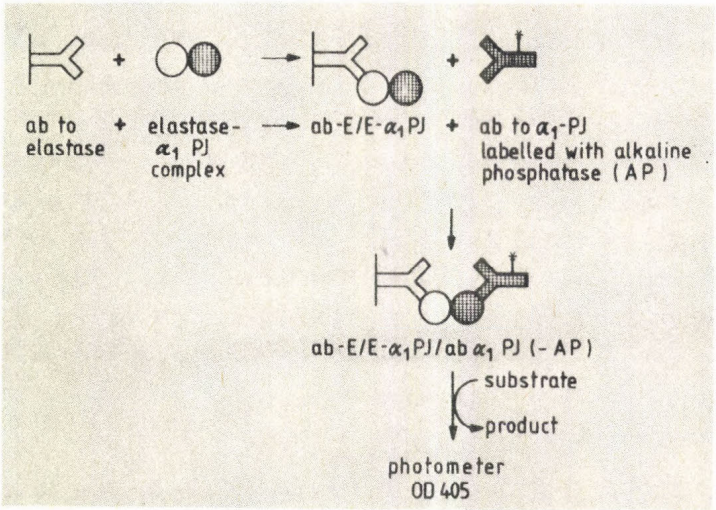

Fig. 6. Principle of the enzyme-linked immunoassay used for determination of the elastase-α_1-proteinase inhibitor complex (E-α_1PI).

ab-E = antibodies against human granulocytic elastase
ab-α_1PI (-AP) = antibodies against human α_1-proteinase inhibitor, labelled with alkaline phosphatase.

CLINICAL STUDIES

Multiple organ failure during severe inflammation or after polytrauma concerns primarily lungs, liver and kidneys. These organs are especially rich in the formerly mentioned cells containing high numbers of lysosomes. In addition, rapid accumulation of polymorphonuclear granulocytes in the lungs during the inflammatory response is also a well-known phenomenon. Hence, a connection between the clinically observed sequence of organ failure and the inherent lysosomal functional capacity of an organ should be seriously considered.

Neutrophil elastase release after major abdominal surgery

In our first prospective clinical study the plasma levels of the E-α_1PI complex were measured in suitable intervals in more than 120 patients subjected to major abdominal surgery followed either by uncomplicated recovery or infection. Of the latter group, only 30 patients fulfilled previously defined and generally accepted septic criteria (Jochum et al., 1983). Of these patients fourteen survived the infection (group B), whereas sixteen died due to severe septicemia (group C). The eleven patients of the control group A recovered from the operation without complications.

Compared to healthy individuals or preoperative values (below 110 ng/ml), the operative trauma caused an up to threefold increase of the E-α_1PI plasma levels (Fig. 7).

The elevated preoperative mean value of group C was caused by 6 patients being infected already before operation; the slight early postoperative decrease was probably due to surgical elimination of the infection focus.

In contrast to group A, group B and C patients maintained moderately elevated E-α_1PI levels for several days after operation. At the time of diagnosis of septicemia, highly significantly elevated E-α_1PI levels were measured corresponding to

an up to sixfold respectively tenfold increase in group B and C. Individual peak levels were found to be as high as 2 500 ng/ml in both groups.

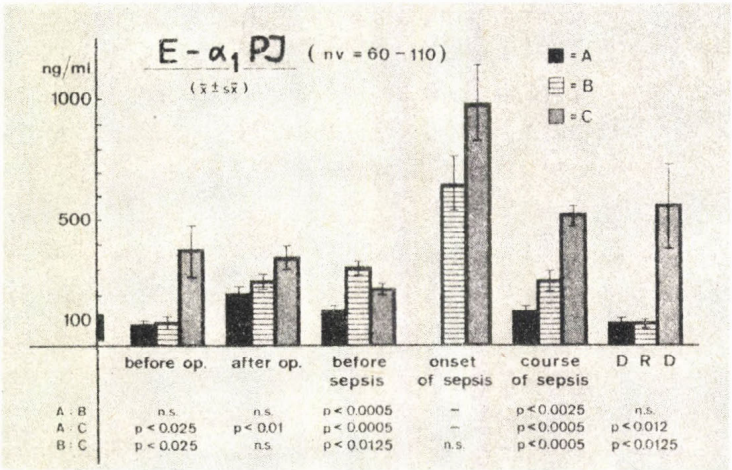

Fig. 7. Plasma levels of elastase-α_1-proteinase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery.

A = patients (n = 11) being without postoperative infection
B = patients (n = 14) surviving postoperative septicemia
C = patients (n = 16) dying as a result of septicemia.

The E-α_1PI levels are given as mean values ($\bar{x}$ SEM) for the day before operation, the day after operation as well as for the postoperative phase before sepsis, at onset of sepsis and during septicemia. Last determination were done on day of discharge (D) for group A, on day of recovery (R) for group B, and before death (D or †) for group C.

nv = normal value

Most interestingly, in patients with persisting septicemia the E-α_1PI levels remained high until lethal outcome (group C) whereas recovery from septicemia was reflected by a concomitant decrease of the E-α_1PI levels to the normal range.

Parallel to the E-α_1PI levels the plasma concentrations of other factors, expected to show a typical response to inflammatory stimuli, were also measured. The concentration pattern of antithrombin III, the most important regulatory inhibitor protein of the clotting cascade, turned out to be just opposite to that of the E-α_1PI complex (Fig. 8). Especially at onset of sepsis and during septicemia, antithrombin III levels are reached, which have to be considered as clinically critical as far as the risk of hypercoagulability or disseminated intravascular coagulation is concerned.

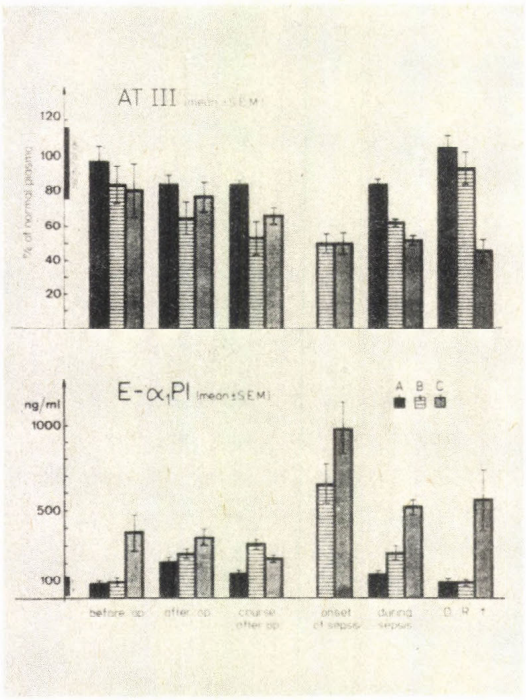

Fig. 8. Plasma levels of the inhibitory activity of antithrombin III (AT III) compared to the amount of the elastase-α_1-proteinase inhibitor complex (E-α_1PI) in patients subjected to major abdominal surgery.

For details, see legend to Fig. 7.

The concentration patterns found for coagulation factor XIII (F XIII), the fibrin stabilizing transglutaminase, and α_2-macroglobulin were very similar to that of antithrombin III, as summarized in Fig. 9:

Plasma levels, elevated (↑) or decreased (↓):
(↑↑) highly signif. (↑) signif. (n) normal

Parameter		Sepsis	Prefinal	Survival
E-α_1 PI complex	c	↑↑	↑↑	n
Antithrombin III	a	↓↓	↓↓	n
Factor XIII	a	↓↓	↓↓	n
α_2-Macroglobulin	a	↓↓	↓↓	n
	c	↓↓	↓↓	n-↓
C-reactive protein	c	↑↑	↑↑	n
α_1-Proteinase inhibitor	a	n-↓	n-↓	n-↓
α_2-Plasmin inhibitor	a	n	n	n
α_1-Antichymotrypsin	c	↓	n	n
C1 Inactivator	a	n-↓	n	n-↓
	c	n	n	n

[a] activity assay [c] concentration assay

Fig. 9. Correlation between plasma levels of E-α_1PI and other plasma factors in patients suffering from septicemia after major abdominal surgery.

High levels of E-α_1PI were found at onset of sepsis and prefinal; these values normalized in survivors. Simultanously, low levels of AT III, α_2M and F XIII occurred in sepsis and prefinal, whereas they normalized in survivors. The levels of the acute phase reactant C-reactive protein (CRP) paralleled mainly the E-α_1PI, but turned out to reflect the course of sepsis much less specifically.

In contrast, the other given proteinase inhibitors did not show significant correlations to E-α_1PI. This behaviour might be especially caused by the fact that these proteinase inhibitors

are acute phase reactants as well. Therefore, the consumption of the inhibitors due to complex formation with their target enzymes or other inactivation reactions might be compensated by the enhanced production of these proteins during inflammation.

Summing up, the results of this clinical study clearly showed that in inflammatory diseases a correlation exists between the release of a lysosomal enzyme marker, the neutrophil elastase, and the clinical situation of the patient, respectively the consumption of selected plasma factors. We take this as a clear indication that liberated lysosomal factors and especially neutrophil proteinases contribute significantly to the inflammatory response of the organism by unspecific degradation of plasma and other factors. This view is also supported by results of experimental animal studies performed more recently (Jochum et al., 1981b). Early application of potent exogenous proteinase inhibitors could prevent or at least diminish highly significantly endotoxin-induced consumption of various plasma factors including AT III and F XIII.

Neutrophil elastase release after polytrauma

In a preliminary study release of granulocytic elastase into plasma was followed up every 4 hours in patients who suffered from multiple trauma. The increase of the E-α_1PI complex up to 16 hours after accident coincided clearly with the severity of the injury (Fig. 10), the degree of injury being established on the basis of a hospital internal scale ranging from 1 - 20 points. Group I patients (5 to 7 points) showed a maximal increase of E-α_1PI up to 5-fold above normal values and group II patients (9 to 11 points) up to 10-fold. In group III (14 to 17 points) peak levels higher than 20-fold above normal were measured. During the further observation period (up to 100 h) a significant decrease of E-α_1PI plasma levels towards normal values was observed in all patients.

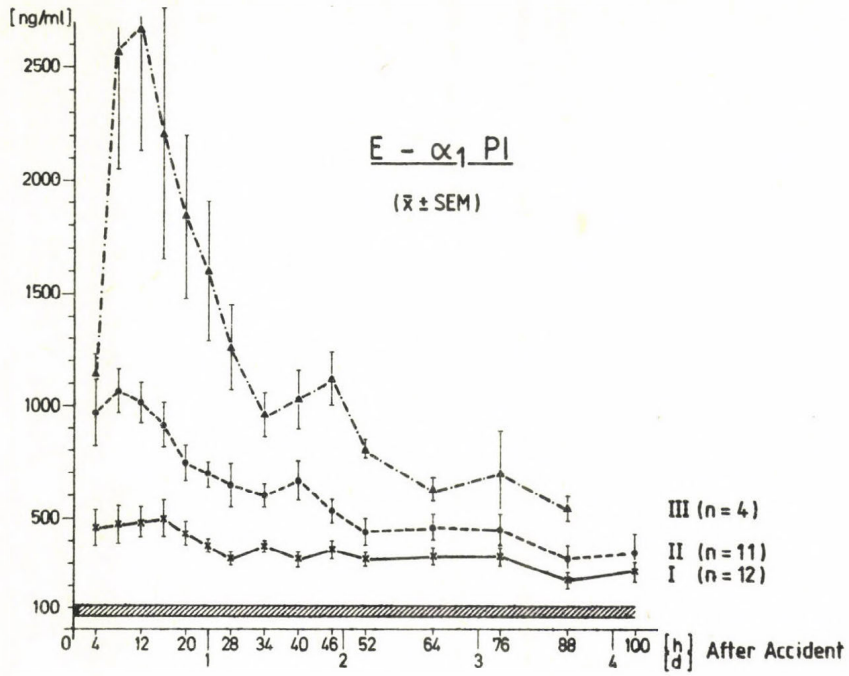

Fig. 10. Plasma levels of E-α_1PI complex in patients after multiple trauma. On the basis of a hospital internal scale with 20 points (HISP) patients were allied to 3 groups reflecting the severity of injury (I milde; II severe; III highly severe):

Group	patients (n)	HISP
I	12	6.3 ± 0.6
II	11	10.0 ± 1.0
III	4	15.3 ± 1.0

Normal range of E-α_1PI: 60 - 110 ng/ml

Surprisingly, no correlation between the amount of complexed elastase and other plasma factors, e. g. antithrombin III, factor XIII, prothrombin, α_2-macroglobulin, plasminogen, and α_2-antiplasmin could be demonstrated so far.

To see, whether this might be due to massive blood transfusions in polytrauma patients, we measured the activities of several plasma proteins in stored blood as a function of the storage period. The activities of the plasma proteins antithrombin III, prothrombin and α_2-macroglobulin were in the range of normal freshly drawn blood and stayed quite stable throughout the storage period, whereas the complexed elastase increased considerably up to more than 60-fold of normal within 35 days (data not shown). From these observations and the results seen in polytrauma patients we draw two conclusions:

(i) Due to massive blood transfusions and relatively long half life periods of the blood proteins probably also in the circulation, the concentration of these factors in patients'plasma are nearly not influenced by the early endogenous liberation of granulocytic elastase after polytrauma.

(ii) High levels of complexed elastase in patients' plasma might be also caused by blood transfusions.

However, in a more detailed investigation comparison of the E-α_1PI levels in patients' plasma with hypothetic levels - considering both: the amount of complexed elastase administered with the transfused blood and the elimination of the complex from the circulation ($t_{1/2}$ of approx. 1 h) - showed no coincidence at all. Probably, in patients receiving massive blood transfusions a relationship between neutrophil elastase release and consumption of humoral factors is measurable only locally, e. g. in lung lavage fluid or blood samples of the wounded or inflamed area. Nevertheless, the high E-α_1PI levels occuring in severely injured patients are a clear indication of a strong inflammatory response ongoing in the organism.

CONCLUSIONS

In severe inflammatory processes,multiple trauma or shock various cells like neutrophils, macrophages, endothelial cells and mast cells are stimulated or disintegrated. In this way a high

potential of lysosomal enzymes is released of which the proteinases are of special pathogenic effectiveness. Recent studies in our laboratory and by others (Egbring et al., 1977) indicate strongly that substrate-unspecific proteolysis by lysosomal proteinases, especially by the neutrophil elastase, contributes to a significant degree to the consumption and/or degradation of extracellular substances in such diseases. The amount of the E-α_1PI complex reflects the intensity of both the inflammatory stimulus and the response of the neutrophils. Hence, in most cases an increase of the E-α_1PI level in the circulation represents a systemic signal of a local inflammatory event. Therefore, the clinical situation of the patient has to be carefully considered for diagnostic interpretation of E-α_1PI levels in plasma. Nevertheless, to avoid a deleterious endogenous proteinase-proteinase inhibitor imbalance, early administration of convenient exogenous inhibitors directed against lysosomal enzymes should have a positive therapeutic effect also in humans.

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DISCUSSION

ELŐDI, S.:

Did you measure the amount of AT-III in septic patients? If the amount of antigen is in the normal range, one could differentiate whether the decreased activity is either due to consumption or to proteolytic damage on the inhibitor, since AT-III modified by proteolysis may still hold antigenic properties.

JOCHUM:

Unfortunately, immunological methods such as one- or two-dimensional immunoelectrophoresis can not differentiate between antithrombin III complexed with thrombin and antithrombin III degraded by elastase. We have done these experiments several years ago.

BOHLEY:

I wonder in what kind of lysosomes metallo-proteinases may occur.

JOCHUM:

It was reported that macrophages contain an elastase-like proteinase which was identified as a metallo-proteinase.

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DETERMINATION OF α_1 PROTEINASE INHIBITOR - ELASTASE (α_1PI-ELP) AND α_2 ANTIPLASMIN - PLASMIN (α_2AP-PL) COMPLEXES FOR DIFFERENTIATION OF HYPERFIBRINOLYTIC STATES IN PATIENTS WITH SEPTICEMIA

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Infectious diseases, with or without spreading of Gram-negative and Gram-positive bacteria or viruses in the blood, rarely lead to microcirculatory disturbances and subsequently to coagulation defects.

As a result of the impairment of the hemostatic balance microthrombosis and/or bleeding disorders can influence the course of the illness.

Depending on the bacterial involvement and its vehemence there are several pathological processes which may affect the hemostatic potential.

Blood cells, mainly thrombocytes, coagulation proteins and inhibitors may be reduced in the blood stream, not only by lack of synthesis, but frequently following increased consumption (7, 8, 10, 11, 12, 15, 24).

Consumption of coagulation factors and inhibitors may be due to:

1. extrinsic or intrinsic activation of blood coagulation
2. the enhancement of the fibrinolytic system

Abbreviations: α_2M: α_2-macroglobulin; α_1PI: α_1-proteinase inhibitor; ELP: elastase(-like proteinase); AT III: antithrombin III; α_2AP: α_2 antiplasmin; α_1PI-ELP: α_1 proteinase inhibitor-elastase complex; α_2AP-Pl: α_2 antiplasmin-plasmin complex; AT III-THR: antithrombin III-thrombin complex; DIC: disseminated intravascular coagulation; ETC: endotoxin; FDP: fibrin degradation products; FFP: fresh frozen plasma; PCC: prothrombin complex concentrate; PTT: partial thromboplasmin time; TT: thrombin time; PMN: polymorphonuclear leucocytes.

3. denaturation of plasma proteins and inhibitors by released cell proteinases, such as elastase and cathepsin G from mononuclear or polymorphonuclear leucocytes (Table 1).

To differentiate these polyetiological defects only indirect signs have been evaluated for diagnosis so far. For instance, the occurrence of fibrin degradation products or the decrease of substrates such as prothrombin and other coagulation factors, plasminogen or inhibitors as antithrombin III (AT III), α_2 antiplasmin (α_2AP) and, in the case of elastase, α_1 proteinase inhibitor (α_1PI) (2, 3, 4, 9, 20, 24).

Recently the diagnostic approaches have been improved by measuring neoantigen complexes of α_1 proteinase inhibitor-elastase (α_1PI-ELP) and α_2 antiplasmin-plasmin (α_2AP-Pl) (Table 1) (9, 10, 13).

The determination of antithrombin III-thrombin complexes (AT III-THR) is not possible since monospecific antibodies against this neoantigen are not yet available.

Therefore evidence of disseminated intravascular coagulation (DIC) has to be proven by assaying all the involved coagulation factors and inhibitors. In vivo hyperfibrinolysis can be demonstrated not only by the decrease of involved coagulation and fibrinolytic factors, but also via the appearance of α_2AP-Pl complexes (10).

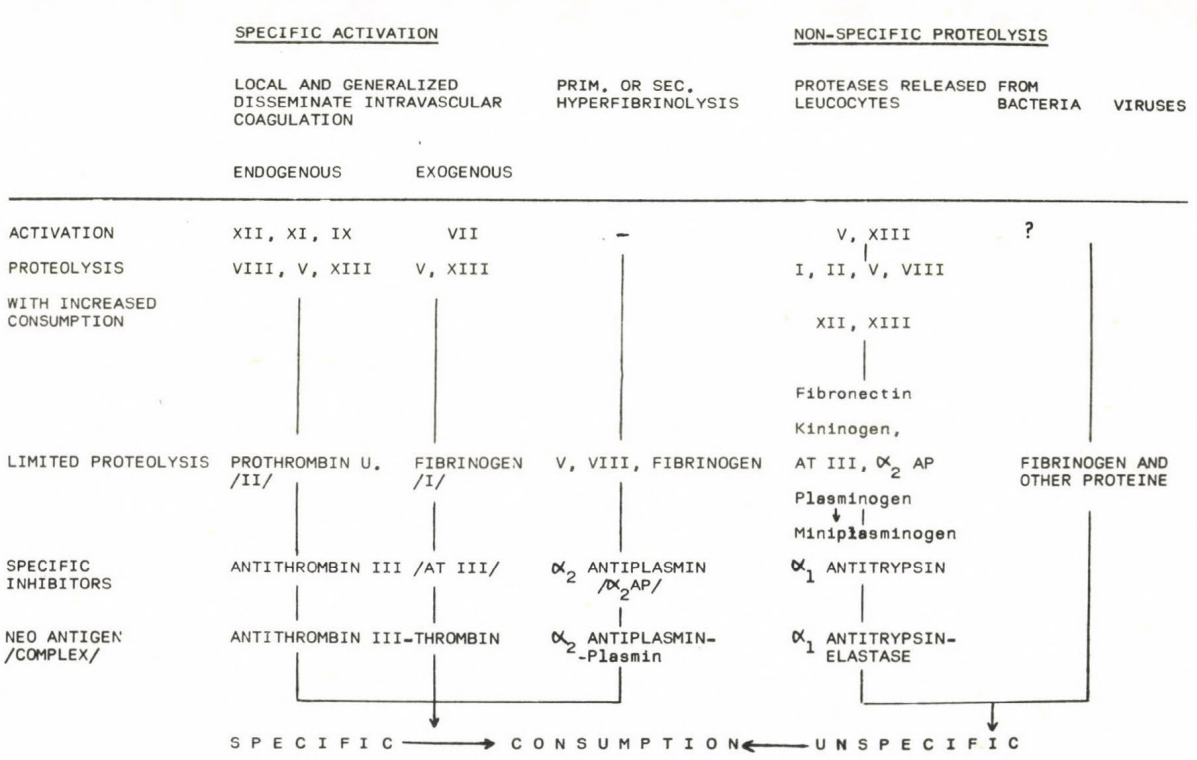
This is equally possible in the case of elastase release from polymorphonuclear granulocytes, because the neoantigen α_1PI-ELP complex is not only demonstrable by determining the inhibitor with two-dimensional immunoelectrophoresis, but also by estimation of α_1PI-ELP complexes with antisera against elastase alone (10).

The immuno-chemical estimation of the α_1PI-ELP complexes can be carried out by several methods: 1./ one- and two-dimensional immunoelectrophoresis (not a very sensitive estimation); 2./ ZIA (zonal immuno-assay); 3./ RIA (radioimmunoassay); 4./ ELISA (enzyme-linked immunosorbent assay).

With this highly sensitive enzyme-linked immuno-assay Duswald and Jochum showed elevated α_1PI-ELP complexes in patients after major abdominal surgery and, to a higher extent,

Table 1

Possible ways for activation and limited or unlimited proteolysis of coagulation factors in microcirculatory disturbances (10)



if they revealed septic complications (5, 16, 17).

Patients in a polytraumatic condition also contain elevated levels of ELP neoantigen (5)

Table 2

Natural substrates of neutral proteinases from polymorphonuclear leucocytes (24)

ELP	CLP	Nonspecific collagenase
Elastin		
Proteoglycan	Proteoglycan	Proteoglycan
Fibronectin	Fibronectin	
Collagen type I,II,III, IV	Collagen type II	Collagen type I,II,III
Fibrinogen/fibrin	Fibrinogen/fibrin	Fibrinogen
Factor V*,VII,VIII,XI, XIII (18)	Factor V,VII,VIII,XII, XIII	
Antithrombin III*		
α_2-plasmin inhibitor (19)		
C1s, C4, C2, C3*,	C1q, C1s, C4, C2, C3	C1s, C4, C3
C5*, C9, factor B*	C5, C9	C5
C1-inactivator		
IgG, IgM, Bence-Jones Protein	IgG, Bence-Jones Protein	
Kininogen	Angiotensinogen	
Specific collagenase		
Gelatinase	Specific collagenase*	
	Gelatinase	

*Substrates which become transiently activated

By the influence of endotoxin (ETC) on granulocytes, proteolytic enzymes (such as elastase and cathepsin G) are released into the circulation. These may destroy plasma proteins before their inhibition by proteinase inhibitors (α_1PI, α_2M). Elastase is able to degrade several coagulation factors, as shown in Table 2; cathepsin G, too, may degrade some of these coagulation proteins. Both enzymes destroy the above mentioned coagu-

lation substrates synergistically. The release of both enzymes from granulocytes is possibly responsible for in vivo denaturation of plasma proteins despite the apparently high antiprotease potential in the blood (7, 8, 22).

The inhibitor capacity may be reduced not only by building complexes with the enzymes, but also, according to Travis, if α_1PI is oxidized (23).

In order to demonstrate a correlation between elevated levels of α_1PI-ELP and coagulation defects in particular of factor XIII deficiency we examined in 1977 seven typical patients with different forms of septicemia and could demonstrate that factor XIII activity and subunit A+S was decreased, if α_1PI-ELP was elevated (7, 8).

Here five out of seven patients with severe septicemia displayed a factor XIII activity and concentration of below 50 % of normal. In these patients subunit A and activity as well as the carrier protein subunit S were found to be diminished (Table 3).

In six out of seven patients α_1PI-ELP complexes could be demonstrated. Therefore we had expected a correlation between factor XIII deficiency and these complexes because other signs of DIC were negative.

In 75 patients with different infections, investigated between 1974 and 1978, we were able to demonstrate a correlation between factor XIII deficiency and α_1PI-ELP in plasma and/or endotoxin in the blood was found. As can be seen in Table 4: In one third of the patients endotoxin and α_1PI-ELP complexes could be found. Of these, eleven patients have shown factor XIII activity below 50 % of normal, while patients without complexes and endotoxin showed normal or only moderate deficiency of factor XIII. Patients with endotoxin alone showed factor XIII deficiency in one third of the cases, but no α_1PI-ELP complexes could be detected using one-dimensional immunoelectrophoresis.

This method is not sensitive enough to evaluate enzyme inhibitor complexes in the range of 80-390 ng/ml. For this range a radioimmunoassay (RIA) for ELP has to be used, and

Table 3

Plasma-bound ELP, clinical data and coagulation factor levels in 7 patients with septicemia (7)

Patients	Diagnosis	Exciter[M]	Endo-toxin	µg ELP/ /ml Plasma	α_1 AT /mg/dl/	α_2 M /mg/dl/	Throm-bocytes /x10^3/	Leuko-cytes /x10^3/	Fever	Bleeding Tendency	Factor XIII /%/	Factor XIII Subunits /%/ A	Factor XIII Subunits /%/ S	Fibrin-ogen /mg/dl/	FDP /µg/ml/	Pro-thrombin Time /%/
Normal /n = 15/		-	ø	ø	188	224	300	4-9	-	-	100	100	100	256	4	100
Fie	Multiple liver obscesses, E.colisepsis	E.coli	ND	51	650	62	340	30.8	+++	ø	25	22	27	180	ND	93
MUI	Cholecystitis, liver obscess, septicemia	K.pneum.	ø	3.9	550	158	350	10.7	++	ø	44	43	47	680	30	60
Wol	Septicemia, acute renal failure, pneumonia	E.coli	+	25.0	355	120	4.5	42.6	+	++	35	38	40	380	80	39
Rom	Septicemia, putrid endome-tritis, residue of decidua, paralytic ileus	E.coli	ø	6.5	215	81	ND	14.0	++	+	50	40	25	182	ND	22
Jun	Septicemia, acute renal failure, endo-carditis	S.aureus	ø	ø	605	180	4..0	14.1	++	ø	70	65	75	770	70	92
Ina	Septicemia, pyelonephritis	E.coli	+	0.16	425	240	114	15.5	++	ø	75	57	93	640	70	56
Min	Septicemia, bronchopneumonia, pericarditis	E.coli	+	0.10	440	108	176	16.8	+	ø	48	17	47	764	70	45

Table 4

Factor XIII activity in 75 patients with septicemia correlated to endotoxin and $\alpha_1$antitrypsin-elastase complexes (10)

Plasma contains	Factor XIII activity (6)		
	> 50 %	< 50 %	< 25 %
α_1AT-Elastase and endotoxin	17	7	4
Endotoxin alone	21	5	3
none	12	4	-
	50	18	7

another highly sensitive enzyme-linked immunoassay (ELISA) is now available for this purpose as proposed by Jochum (16, 17). Some further example should demonstrate the possible role of elastase in coagulation disturbances and in particular in factor XIII deficiency (6). During urokinase therapy no one would expect a change in factor XIII activity, since plasmin is known not to degrade factor XIII. But in a patient treated with urokinase, factor XIII decreased to 53 % twelve days after starting the therapy (Table 5) (10).

This decrease, however, is caused by a septic complication during the therapy. After the onset of fever, induced by septic thrombosis, α_1PI-ELP complexes have been evaluated (Table 5). Therefore, besides disseminated intravascular coagulation, ELP destruction of factor XIII must be considered. The behavior of α_2AP-Pl and α_2AP is demonstrated in Fig. 1.

The next example shows the course of a severe septic complication in a seventeen-year-old girl with bleeding as a result of bone marrow aspiration over a period of 20 hours. In this case no α_2AP-Pl, but α_1PI-ELP could be shown in the one-dimensional immunoelectrophoresis (Fig. 2) (10).

Table 5

Some coagulation data and the results of α_1PI-ELP level in a patient exhibiting septic complications in the second week of urokinase treatment

Sample	Date	Time	Transglut. 70-100 % F XIII	EDJE 100 % α_2AP	EDJE 100 % α_2AP-Pl	Partigen AT III	Partigen Plasminog.	EDJE Ø Elastase	Treatment with Urokinase=U Heparin=H
0	18.7.80	10^{00}	96	100	Ø	33.6	15.0	Ø	Ø
1	18.7.80	18^{00}	96	81	100	33.6	12.6	Ø	U
2	18.7.80	18^{00}	92.5	57	100	26,6	10.4	Ø	U
3	19.7.80	9^{00}	76.25	49	92	33.6	8.0	Ø	U
4	19.7.80	11^{00}	85	46	100	28.8	7.6	Ø	U
5	19.7.80	18^{00}	106.5	41	84	24.4	6.4	Ø	U
6	19.7.80	24^{00}	78.75	41	77	28.8	6.8	Ø	U
7	20.7.80	11^{15}	81	36	77	26.6	5.4	+	U
8	20.7.80	23^{00}	65.75	25	63	28.8	7.6	+	U
9	21.7.80	11^{00}	81	36	89	31.2	6.4	+	U
10	21.7.80	23^{00}	79.75	38	77	28.8	6.8	+	U
12	22.7.80	1^{15}	60.5	38	84	36.0	5.6	+	U
13	22.7.80	11^{00}	55.75	52	Ø	46.2	8.0	+	Ø
14	23.7.80	11^{00}	71	81	Ø	31.2	9.4	Ø	Ø
15	24.7.80	11^{00}	53.5	89	Ø	36.0	13.8	Ø	Ø
16	25.7.80	9^{00}	63.3	100	Ø	48.8	21.8	Ø	Ø
17	25.7.80	18^{00}	67.85	76	100	36.0	12.8	+	U
18	26.7.80	11^{00}	57.5	44	84	28.8	10.0	+	U
19	27.7.80		95	27	56	26.6	6.4	Ø	U
20	28.7.80		74	25	56	36.0	8.4	Ø	U
21	29.7.80		57	20	84	26.6	7.6	Ø	U
22	29.7.80	11^{00}	71	25	89	18.0	6.0	Ø	U

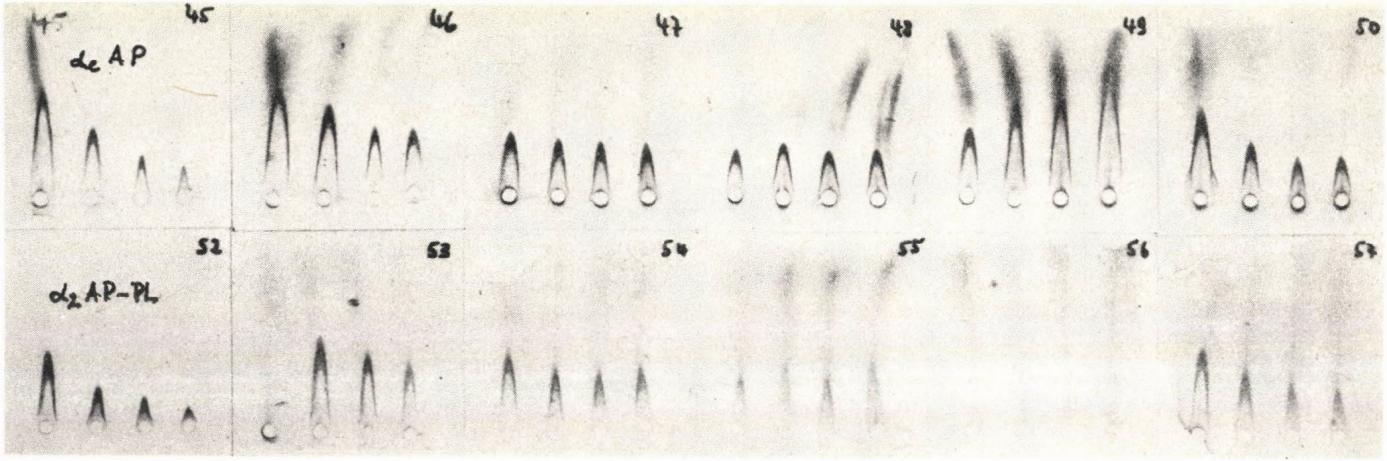


Fig. 1: Behavior of α_2AP-Pl complexes (1b) and α_2AP (1a) during urokinase therapy. After starting the therapy α_2AP-Pl complexes arose and the specific inhibitor decreased. During an interruption of the therapy, α_2AP increased (a 13-16) and the neoantigen α_2AP-Pl (b 13-16) disappeared promptly (2).

Table 6/a

Coagulation data of a 17 year-old girl with chronic hepatitis and severe septic complication

Date	F XIII (%)	Fibri-nogen %	AT III (%)	α_2-AP (%)	ELP (µg/ml)	α_2AP-Pl-Neo.	Plasmi-nogen (%)	Platelet count
22.4.	37.5	14	50	38	0.5	Ø		67.000
23.4	37.5	60	62	38	0.3	Ø	100	
24.4.		64	70	63	0.3	Ø		151.000
25.4		58	82	50	0.3	Ø		108.000
26.4	37.5	60/78	100	57	0.3	Ø	90	132.000
27.4	37.5	100	100	59	0.3	Ø	88	154.000
28.4	50	121	100	69	Ø	Ø	82	
29.4	50	140	70	63	Ø	Ø		275.000
30.4	50	120	80	69	Ø	Ø	63	305.000
1.5.		95						
3.5.		140	67	75	Ø	Ø		377.000
4.5.				64	Ø	Ø		364.000
6.5.	50	178	74	64	Ø	Ø	92	
7.5.	50	220	75				100	
8.5.	50	220	75				100	
10.5.	37.5	170	80	76	Ø	Ø	100	
12.5.				76	Ø	Ø		
14.5.	50	167	82				50	
17.5.	75	178	64	76	Ø	Ø	84	
18.5				81	Ø	Ø		286.000
19.5.	75	175	65				120	
26.5.	50	230	100	84	Ø	Ø	110	
2.6.				88	Ø	Ø		

Table 6/b

Coagulation data of a 17 year-old girl with chronic hepatitis and severe septic complication

Date	Quick (%)	PTT (s)	TT (s)	F II (%)	F V (%)	F VII (%)	F VIII (%)	F IX (%)	F X (%)	F XI (%)	F XII (%)
22.4.	12	119	180	17	30	29	44	62	23	40	30
23.4.	48	119	180	51	70	45	100	43	44	40	48
25.4.	26	80		36/44							
26.4.	49/51	84/80	43	35/44	37/40	32/34	50/68	34	42	40	47
27.4.	70	52	22	45	95	53	100	70	66	77	100
28.4.	75	58.3	28.3		90		100				
29.4.	100	41.9	22								
30.4.	80	46.5	21.7								
1.5.	60	37	22.6								
3.5.	81	46.4	21.2								
4.5.	81	40.8									
6.5.	75	40.4	20		100	58	100				
7.5.	75	36.8	19.3				100				
8.5.	75	36.8	19.3				100				
10.5.	65	34	20				100				
14.5	90	34	19.2			65					
17.5	82	37	22.2			68					
19.5.	85	34.3	17.6				100				
26.5	75	33	18.4			84					

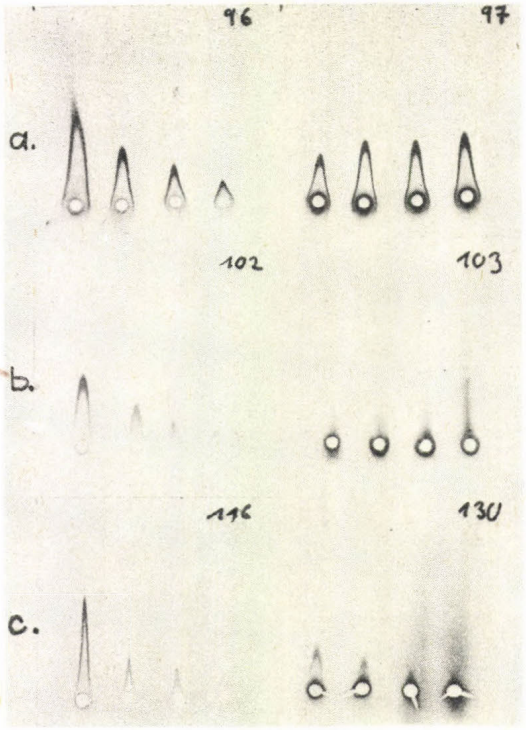


Fig. 2:
Effect of α_1AT-ELP complexes in a 17 year-old girl during a severe septicemia (a), α_2AP, (b) α_2AP-Pl, (c) α_1AT-ELP complexes in one dimensional immuno-electrophoresis with anti-sera against the inhibitor (a), the neoantigen α_2AP-Pl (b) and against elastase (c).

In this patient fibrinogen was found to be diminished to 20 mg% and factor XIII to 30 % of normal activity (Table 6a, b). Unspecific and/or thrombin induced consumption of factor XIII and other coagulation factors and inhibitors makes a substitution with plasma fractions and AT III necessary. As shown in Fig. 3, nearly 6 litres of plasma equivalents and 6500 U/AT III had to be substituted to prevent bleeding tendency and coagulation defects.

During replacement therapy, fibrinogen, factor XIII and antithrombin increased. Despite the fact that no platelets were substituted, the thrombocytes increased during the substitution with AT III and plasma equivalents to normal values. The elevated level of α_1PI-ELP decreased in a few days to normal values. With one-dimensional immunoelectrophoresis this neoantigen was no more detectable.

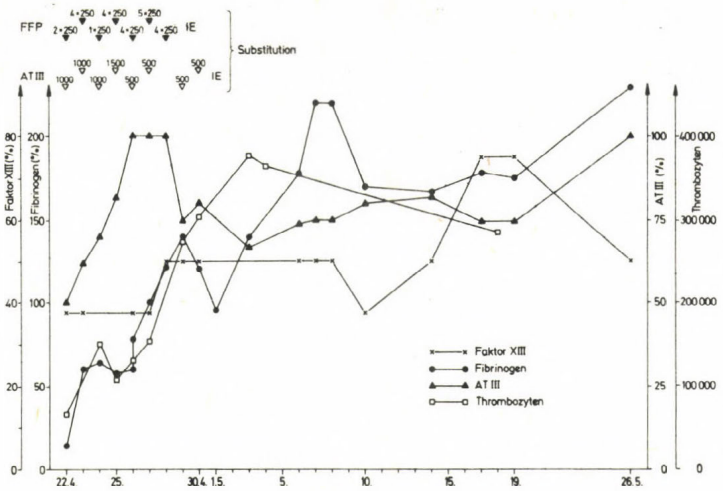


Fig. 3: Behavior of factor XIII, fibrinogen, AT III and thrombocytes under substitution with fresh frozen plasma and antithrombin III concentrates. Under this treatment not only the substituted factors but also platelet count increased (from 17.000 to about 200.000, within 9 days).

In contrast, in 3 patients with meningococcal septicemia not only α_1PI-ELP but also α_2AP-Pl could be detected simultaneously. Therefore besides DIC degradation by elastase and plasmin may be responsible for consumption of coagulation factors.

Early substitution with plasma derivates as AT III and FFP, cryoprecipitate and PCC is successful in preventing further consumption of coagulation factors and severe hemorrhage (1).

Malaria tropica is often complicated by severe "DIC". This could be confirmed in one young patient with malaria tropica (Pl. falciforme and Pl. ovale) who had low levels of coagulation factors, inhibitors and thrombocytopenia. The patient was oliguric and severely ill. On admission only α_1PI-ELP was elevated to 400 ng/ml. Although no α_2AP-Pl complexes could be detected plasminogen was decreased to 15 % of normal. This could be the cause of reduced fibrinolytic activity in the kidney.

Therefore besides application of AT III concentrate we also have substituted with plasminogen (Fig. 4). Under this substitution therapy coagulation factors, inhibitors and platelets returned to normal levels. The bleeding tendency discontinued and the patient became poliguric.

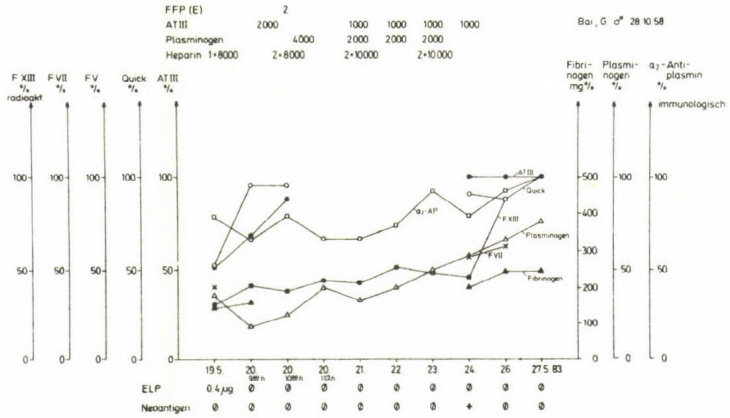


Fig. 4: Behavior of factor XIII, fibrinogen and plasminogen under substitution with fresh frozen plasma, antithrombin III concentrates and plasminogen. Under the treatment not only the amount of substituted factors, but the platelet count increased (from 20.000 to about 200.000, within 4 days, patient with tropical malaria) (20).

Disorders with Factor XIII deficiency possibly caused by elastase like proteases released from granulocytes (11)

1./ Erosive hemorrhagic gastroduodenitis with fibrinolysis and low Factor XIII

2./ Generalized proteolysis in a young woman with Weber-Christian disease

3./ Factor XIII dificiency in Hennoch-Schönlein's purpura

4./ Acute myelocytic leukemia

5./ Promyelocytic leukemia

6./ Other myeloproliferation diseases

7./ In Septicaemia
8./ Major abdominal surgery
9./ Combustio
10./ Multiple fractures
11./ Fatal iron intoxication

DISCUSSION

The main function of granulocytes is providing protection against microorganisms by phagocytosis and intracellular killing. Besides this function these cells may apparently digest alien proteins. From 1968-1975 it became apparent that the granulocytes are secretory cells, which, by secreting neutral proteinases, interfere with the regulation of the inflammatory response. In case of a failure of the protective proteinase inhibitor system, the granulocyte proteinases are responsible for severe tissue destruction under several clinical conditions, as seen in Table 7:

The destruction of elastin containing structures in lung connective tissue and of joint tissue and of the glomerular basement membrane in patients with ARDS syndrome and pulmonary emphysema, rheumatoid arthritis and nephrotic syndrome may be due to a pathological effect after the release of lysosomal enzymes from granulocytes (24).

In the early state of the white cell maturation blast cells do not contain any lysosomal proteinases. Later matured cells produce these enzymes up to the polymorphonuclear granulocytes. In some forms of acute granulocytic leukemia the release of these proteinases contributes to the hemostatic defects in the patients as we demonstrated in the early seventies (7). As was subsequently found in 1975, the proteinase release from granulocytes plays a more important role in patients suffering from various infectious diseases (10).

Later others also found (16, 17) that elastase and cathepsin G were responsible for the severe coagulation defects in patients after major surgery with and without septic complications.

Table 7

Possible pathophysiological role of neutral proteinases from polymorphonuclear leucocytes (24)

Disease	Symptoms	Possible aethiology	Substrates
Septicemia	Coagulation defects	Enzyme release by endotoxin, immune complexes	Fibrinogen, factor XIII and other coagulation factors and inhibitors
ARDS-syndrome (polytrauma, septicemia)	Fluid lung (capillar injury, alveolitis)	Accumulation of aggregated PMN in the lung	Lung connective tissue + basement membrane (collagen, elastin, proteoglycan, fibronectin)
Pulmonary emphysema	Destruction of elastin containing structures	Genetic α_1PI deficiency: inactivation of α_1PI by tobacco smoke	Lung connective tissue
Rheumatoid arthritis	Synovitis, destruction of joint tissue	IgG altered by the enzymes → formation of rheumatoid factor IgG - immune-complexes → enzyme release	Joint connective tissue (collagen, proteoglycan)
Gout	Synovitis, destruction of joint tissue	Enzyme release by phygocytosis of urate cristalls	Joint connective tissue (collagen, proteoglycan)
Immune complex nephritis	Nephrotic syndroma, renal failure	Local accumulation of immune complexes → glomerular injury by PMN enzyme release	Glomerular basement membrane

The actual effect of proteinases such as elastase or cathepsin G on blood proteins such as coagulation factors and inhibitors has not yet been established (14, 16). The action on these proteins must be possible despite high antiprotease potential in the plasma. There are several hypotheses which point to the fact that degradation of proteins may indeed occur in vivo.

1./ The inhibitor is complexed with proteinases
2./ The inhibitor is oxidized and no longer effective
3./ The inhibitor may be consumed, in particular at local sites of inflammation where very high enzyme activities may be present.

There are many possible indirect signs that degradation of coagulation factors and inhibitors is due to proteinase action.

1./ The appearance of fibrin degradation products distinct from plasmin digestion.
2./ The reduction of both subunits A and S of factor XIII. During coagulation only subunit A is consumed (7, 8, 10).
3./ The diminution of AT III cannot only be related to thrombin mediated consumption, because AT III is a substrate of ELP and is cleaved near the active site of the molecule (16). Despite the small immunochemical denaturation of the single chain molecule, AT III becomes inactive.

The coagulation examinations in 75 patients (selected patients from 1974-1978) revealed in one third severe, in one third moderate and in one third no coagulation defects. The relatively high percentage of patients with no coagulation defects may be a result of the mild course of the infectious disease or the supposed increased turnover of clotting factors may be compensated by all inhibitors present. As demonstrated in Table 4, factor XIII deficiency may be correlated to the appearance of α_1PI-ELP in the patients plasma.

Besides the demonstration of elastase three patients with meningococcal septicemia revealed α_2AP-Pl complexes. Thus we had to assume that in these cases two distinct reactions were

responsible for the degradation of some coagulation factors. It is necessary to mention the fact that with one dimensional immunoelectrophoresis α_1PI-ELP complexes could only be demonstrated above 390 ng/ml ELP (21).

In the six examples where we demonstrated the course of the illness we found, using one dimensional immuno-electrophoresis, elevated α_1PI-ELP in a well demonstrable concentration.

While the patients under urokinase treatment and in three patients with septicemia due to n-meningitis infection revealed both antigens, the 17-year-old girl and the young patient with malaria demonstrated only, but highly elevated α_1PI-ELP complexes. As demonstrated in the figure showing the courses of the illness, the severe coagulation defects had to be treated with plasma derivates such as PCC, AT III and FFP, which contained not only factor V but α_1PI and α_2AP.

Only the patient with malaria became oliguric and demonstrated additionally low plasminogen levels. In this case additional plasminogen substitution as proposed by Dr. Karges, was applied in order to treat the microcirculatory disturbances close to the glomerular apparatus in the kidneys.

In conclusion

1./ The determination of the neoantigens α_1PI-ELP and α_2AP-Pl is suitable to differentiate the cause of hyperfibrinolytic states.

2./ DIC is not well understood if one considers only thrombin and/or plasmin mediated proteolysis, degradation and increased consumption of coagulation factors and inhibitors.

3./ To affect coagulation disturbances in DIC anticoagulant treatment with heparin alone is not sufficient to stop the circulus vitiosus of protein consumption or in particular the "abnormal proteolysis syndrome" (11) as it occurs in patients with septicemia (10).

4./ Besides coagulation proteins and fibronectin probably inhibitors such as AT III, α_1-PI and α_2AP have to be substituted additionally with concentrates or - if not available with FFP to avoid bleeding tendency and to improve the hemostatic balance at the same time.

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PROTEINASES OF PLANT AND MICROBIAL ORIGIN

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ISOLATION AND CHARACTERISTICS OF STREPTOMYCES RIMOSUS PROTEASES

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INTRODUCTION

Streptomycetes are known to synthesize a variety of proteases but aside from Streptomyces griseus, proteolytic enzyme system of other Streptomyces species has not been extensively described. In our study of S. rimosus extracellular enzymes we have detected at least eight proteolytically active components (Pokorny et al., 1979). We have also isolated three of them. Serine alkaline proteinase was characterized to the greatest extent (Renko et al., 1981a) while trypsin-like and elastase-like enzymes were partially described (Vitale et al., 1980; Renko et al., 1981b).

In continuation of S. rimosus proteolytic enzymes study trypsin-like proteinase, alkaline metallo-proteinase and leucine aminopeptidase were purified and their properties determined.

MATERIALS AND METHODS

The source of enzymes was culture filtrate of S.rimousus grown under conditions for oxytetracycline production, clarified by centrifugation (2400xg, 30 min) and concentrated 20-fold by ultrafiltration through Diaflo hollow fiber H1P10. Concen-

Abbreviations: PMSF: phenylmethane-sulphonylfluoride;
TLCK: L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone;
TPCK: L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone

trated filtrate was dialyzed against 0.01 M Na-acetate buffer, pH 5.8, and applied to a CM-Sephadex C-50 column, or was directly fractionated by acetone precipitation.

Enzyme assay

Hydrolysis of hemoglobin, casein, azocoll, gelatine and elastin was determined as previously described (Pokorny et al., 1979). Hydrolysis of N α-benzoyl-2-naphthylamide (BANA), amino acid- and peptide-2-naphthylamides (2-NA) was estimated by colorimetric method of Nagatsu et al. (1970)

Trypsin-like activity towards N α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N α-benzyloxycarbonyl-norleucyl-prolyl-arginine-p-nitroanilide (Z-Nle-Pro-Arg-Nan; kind gift of Prof. P. Elődi, Debrecen, Hungary) was measured in 0.05 M TrisHCl buffer, pH 8.0, containing 10^{-3} M $CaCl_2$, at 30°C, with continuous recording of absorbancy at 405 nm. The same method was used for the determination of leucine aminopeptidase. The reaction mixtures contained $2x10^{-3}$ leucine-p-nitroanilide (Leu-p-Nan) in 0.1 M TrisHCl buffer, pH 8.2, supplemented with $5x10^{-3}$ M $CaCl_2$. The temperature was 37°C. Inhibitors and activators were incubated with the enzymes for 5 to 15 minutes prior to the start of the reaction.

Hydrolysis of peptides was followed by thin-layer chromatography on silica gel plates with n-butanol-acetic acid-water (60:15:25) as solvent.

Polyacrylamide gel electrophoresis, molecular weight determination and protein assays were performed as previously described (Renko et al., 1981a,b).

RESULTS AND DISCUSSION

Enzyme isolation

Two proteinases and one aminopeptidase were isolated from S.rimosus culture filtrates following the procedure outlined in Fig. 1.

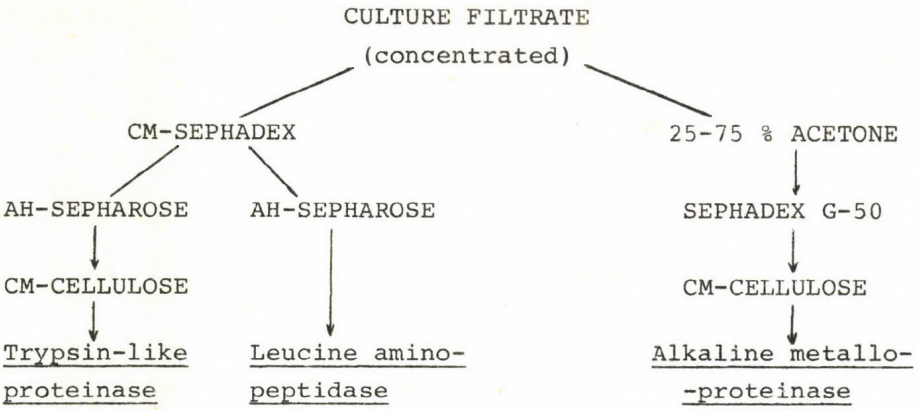


Fig. 1: Purification scheme of S. rimosus proteinases

Chromatography on CM-Sephadex C-50 in 0.01 M Na-acetate buffer, pH 5.8, yielded trypsin-like and leucine aminopeptidase activity in separate fractions. The first one was eluted from the column with the starting buffer and the second one upon the addition of 0.4 M NaCl. AH-Sepharose 4B (Aminohexamethylene-Sepharose) chromatography of both enzymes was performed in 0.01 M Na-acetate buffer, pH 5.5, with a linear gradient of NaCl (0.0-1.0 M).

Final purification ot trypsin-like proteinase was achieved on a CM-cellulose column in 0.001 M Na-acetate buffer, pH 4.5, using 0.0-0.3 M NaCl gradient as eluant.

This procedure gave 550-fold purification with a yield of 19 % (Table 1). Thus, it represented an improvement over one used earlier (Renko et al., 1981b).

AH-Sepharose chromatography was the final step for the leucine aminopeptidase purification summarized in Table 2. At the end of the fermentation process aminopeptidase activity unlike proteolytic activity was very low. This made culture filtrates poor sources for the enzyme isolation, which contributes to the low yield.

Table 1

Purification of S. rimosus trypsin-like proteinase

Purification step	Vol. (ml)	Protein (mg/ml)	Specific activity*	Yield (%)
Culture filtrate	10,000	10.000	0.054	100
Dialyzed concentrate	500	17.50	0.360	58
CM-Sephadex	1,150	0.52	2.960	33
AH-Sepharose 4B	420	0.11	29.740	25
CM-Cellulose	160	0.22	29.790	19

*Determined with BAPNA at pH 8.0, and expressed as ΔA_{410}/min mg, measured at pH 2.0.

Table 2

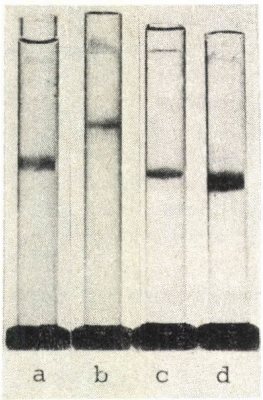
Purification of S. rimosus leucine aminopeptidase

Purification step	Vol. (ml)	Protein (mg/ml)	Specific activity*	Yield (%)
Culture filtrate	18,100	6.90	-	-
Dialyzed concentrate	1,130	18.00	0.85	100.0
CM-Sephadex C-50	1,550	2.80	0.76	19.0
AM-Sepharose 4B	35	0.13	103.90	2.7

*Determined with Leu-2-NA at pH 7.4 in the presence of 5×10^{-3} M $CaCl_2$, and expressed as ΔA_{530}/min mg.

The isolation of the metallo-proteinase started with acetone precipitation. The precipitate formed at 25-75 % acetone was dissolved and subjected to Sephadex G-50 gel filtration in 0.1 M Na-phosphate buffer, pH 8.0. Fractions containing low molecular weight material with proteolytic activity were chromatographed on CM-cellulose in 0.1 M Na-phosphate buffer, pH 8.0, with 0.00-0.15 M NaCl. The purified metallo-proteinase was treated with 10^{-2} M EDTA and dialyzed against 0.01 M NaCl for storage in an inactive form. Determination of the yield was hindered by the presence of other proteinases in the culture filtrate.

All three isolated enzymes were electrophoretically homogeneous as illustrated in Fig. 2.



(a) Trypsin-like proteinase (100 µg)
(b) Leucine aminopeptidase (20 µg)
(c) Alkaline metallo-proteinase (90 µg)
(d) Alkaline metallo-proteinase (300 µg)

Fig. 2: Polyacrylamide gel electrophoresis of S.rimosus proteases; (a) pH 8, 7 % gel. (b-d) pH 4.4, 15 % gel.

Properties of the enzymes

Molecular and catalytic properties of the enzyme isolated first, are presented in Table 3.

Table 3

Properties of S. rimosus trypsin-like proteinase

Activity:	Digests proteins, BANA, BAPNA
pH optimum:	pH 8.4-8.8
Temp. optimum:	45°C (pH 8.4)
Stability:	pH range 5.5-9.0
Mol. weight:	27,000 (gel filtration), 29,000 (SDS PAGE)
pI:	4.5
Inhibitors:	PMSF, TLCK, soybean trypsin inhibitor, leupeptin

In view of its ability to hydrolyze characteristic trypsin substrates and its susceptibility to inhibitors, the enzyme is classified as a trypsin-like serine proteinase. Unlike "paromotrypsin" isolated from S.rimosus by Chauvet et al. (1976) and pancreatic trypsin, it has a low pI and correspondingly higher content of the acidic amino acids (Table 4).

Table 4

Amino acid composition of S. rimosus trypsin-like proteinase

Lysine	16	Glutamic acid	24	Methionine	2
Histidine	3	Proline	9	Isoleucine	8
Arginine	4	Glycine	37	Leucine	16
Aspartic acid	23	Alanine	35	Thyrosine	5
Threonine	16	Cysteine	2	Phenylalanine	6
Serine	25	Valine	12	Tryptophan	ND

Another noteworthy characteristic is the presence of two cysteine residues versus 12 in trypsin. This low content of disulphide linkages seems to be a common feature of trypsin-like proteinases fr n streptomycetes irrespectively of their pI (Yoshida et al. 1971).

The specificity of the enzyme, estimated semiquantitatively with different peptides as substrates, showed its requirement for arginine at S_1 position (Table 5).

Table 5

Specificity of S. rimosus trypsin-like proteinase

Substrate, split bond	Relative rate*
Arg$\overset{\downarrow}{-}$Phe	+
Arg$\overset{\downarrow}{-}$Phe-Ala	++
Met-Arg$\overset{\downarrow}{-}$Phe	+++
Leu-Trp-Met-Arg$\overset{\downarrow}{-}$Phe-Ala	+++++
Arg$\overset{\downarrow}{-}$Val-Tyr-Ile-His-Pro-Phe	++
Asp-Arg$\overset{\downarrow}{-}$Val-Tyr-Ile-His-Pro-Phe	++++
Z-L-Arg$\overset{\downarrow}{-}$2-NA	++++

*Estimated visually by thin layer chromatography.

The results also revealed the ability of the S.rimosus trypsin-like proteinase to split off amino-terminal arginine, which could not be demonstrated for bovine trypsin under the same conditions. The possibility of contamination with arginine aminopeptidase was ruled out by the constancy of endo- and exo-peptidase activity ratio in the fractions obtained by gel filtration and polyacrylamide gel electrophoresis of the enzyme. The pH optima for the hydrolysis of casein, BANA, and Arg-2-NA were also identical.

Substrate preferences of the trypsin-like proteinase and pancreatic trypsin were compared on the basis of equal caseinolytic activities (Table 6).

Table 6

Relative specificity of trypsin-like proteinase and trypsin

Substrate	Relative reaction rate (%)	
	Trypsin-like	Trypsin*
Casein	100.0	100.0
Bz-DL-Arg-2-NA	19.4	0.5
Bz-DL-Arg-p-Nan	65.6	3.7
Z-Nle-Pro-Arg-p-Nan	2074.0	344.7
L-Arg-2-NA	0.2	0.0075

*Bovine pancreas, TPCK-inhibited (Serva). **Activity determined as μmoles of liberated amino groups.

Z-Nle-Pro-Arg-Nan (Pozsgay et al., 1981) was by far the best substrate for both enzymes. However, relative activities toward synthetic substrates were higher for the trypsin-like proteinase than for trypsin. Concomitantly, Km values of S.rimosus enzyme were lower than that of bovine trypsin, indicating its higher affinity for them. Similar observations were made for S. erythreus, S. fradiae and S. griseus trypsin-like proteinases (Yoshida et al., 1971). This relative preference for amidase substrates seems to be a general characteristic of streptomycetes "trypsins".

Characteristics of the second enzyme, isolated as leucine aminopeptidase (LAP), are shown in Table 7.

Table 7

Properties of S. rimosus leucine aminopeptidase

Substrate:	Leucine-p-nitroanilide, Km: 7.9×10^{-4} M
pH optimum:	pH 8.0-8.2
Temp. optimum:	55°C (pH 8.0)
Stability:	pH range 5-10; temperatures to 70°C
Mol. weight:	27,500 (gel filtration)
pI:	7.3
Inhibitors:	Ca^{2+}, EDTA, o-phenanthroline, amastatin, bestatin, puromycin
Activators:	Ca^{2+}, (Co^{2+})

The enzyme needed Ca^{2+} ions for its full activity, and showed good thermal and pH stability like other Streptomyces leucine aminopeptidases. Smaller molecular weights than that of mammalian LAP might also be a common property of these enzymes (Uwajima et al., 1973; Vosbeck et al., 1973; Kastrikina, 1980).

S. rimosus leucine aminopeptidase was more sensitive to amastatin than to bestatin, whereas hog kidney LAP has greater susceptibility to bestatin (Suda et al., 1976; Aoyagi et al., 1978). At the same time inhibitory activity of bestatin against S.rimosus LAP was much lower ($IC_{50}=2.1 \times 10^{-4}$ M) than activity against hog kidney enzyme ($IC_{50}=3.4 \times 10^{-8}$ M) determined in parallel. This difference indicates structural differences at the active site.

The specificity of the enzyme was determined with amino acid naphthylamides as substrates. It hydrolyzed only Leu-2-NA, Phe-2-NA and Met-2-NA significantly. Activities toward these substrates could not be separated by gel filtration or polyacrylamide gel electrophoresis, and their ratio was 1:0.51:0.37 (Co^{2+} as activator). The preference for the bulky and hydrophobic residues of leucine and phenylalanine was reported for LAP from S. peptidofaciens (Uwajima et al., 1972), and S. griseus which also hydrolyzed prolyl, tyrosyl, alanyl and

methionyl bonds (Vesbeck et al., 1975).

Table 8 summarizes the properties of the third enzyme from S. rimosus culture filtrates.

Table 8

Properties of S. rimosus alkaline metallo-proteinase

Activity:	Digests casein, gelatine, hemoglobin
pH optimum:	pH 8.0-9.0
Temp. optimum:	50°C (15 min incubation, pH 8.5)
Stability:	pH range 5.0-9.5, temp. to 30°C
Mol. weight:	12,500 (SDS-PAGE)
pI:	9.9
Inhibitors:	EDTA-o-phenanthrolin
Activators:	Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}

The high pH optimum, inactivation by 10^{-3} M EDTA and restoration of activity by divalent cations allow the enzyme to be classified as an alkaline metallo-proteinase. The most efficient cation was Zn^{2+}, which could restore full enzyme activity at a concentration of $5x10^{-5}$ M, followed by Mn^{2+}, Co^{2+} and Ca^{2+}. At higher concentrations Zn acted as an inhibitor.

The alkaline metallo-proteinase hydrolyzed gelatine, azo-casein and azocoll, but had no activity on elastin or collagen. It did not split collagenase substrate of Wunsh and Heidrich (1963), hexapeptide Leu-Trp-Met-Arg-Phe-Ala, N-glutaryl-Gly--Gly-Phe-2-NA, Leu-Gly-Gly-2-NA or Gly-Pro-Leu-2-NA.

The enzyme is thermolabile and is one of the smallest proteolytic enzymes described so far. It resembles Myxobacter strain Al-1 protease I (Morihara, 1974). To our knowledge similar enzymes have not been isolated from Streptomyces species previously.

The isolation and characterization of the trypsin-like proteinase, leucine aminopeptidase and alkaline metallo-proteinase from S. rimosus provide data necessary for elucidation of the proteolytic enzyme system of this industrially important microorganism. Knowledge of their properties could also contribute to the better understanding of the entire classes of these enzymes.

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HÜTTER:

Did you assay whether your enzyme (leucine aminopeptidase) is able to hydrolyze dipeptides and leucine amide? Can this enzyme be activated by manganese ions? How long was the preincubation time in your experiments? In the case of human LAP you would need 1-2 hours.

VITALE:

S. rimosus leucine aminopeptidase hydrolyzes dipeptides, whereas leucine amide was not tested. The enzyme was activated by Mn^{2+} to a very small extent. Preincubation time ranged up to 20 min.

STEPANOV:

How did you measure the molecular weight of the metallo proteinase which seems to be unusually low?

VITALE:

It was assayed by SDS-polyacrylamide gel electrophoresis and gel filtration. The enzyme also passed PM-10 ultrafilter.

GLAUMANN:

Are Streptomyces proteinases synthesized as proforms?

VITALE:

As far as I know, they are not. It is believed that these proteinases are extruded into the medium as unfolded chains resuming tertiary structure outside the cell.

GLAUMANN:

Are they glycoproteins?

VITALE:

No, they aren't.

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ACID PROTEINASES OF MICROBIAL ORIGIN

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INTRODUCTION

There are many reasons for the growing interest in proteolytic enzymes of microorganisms over the past ten years. Microbial proteinases are widely used in various industrial processes, e.g. in food industry, tanning industry and in production of detergents. They are also valuable reagents in clinical and research laboratories. The study of proteolytic enzymes of various microorganisms also contributed to our understanding of the role played by these enzymes in processes of intracellular protein turnover, digestion, protein translocation, selective modification of proteins by limited proteolysis, etc. The pathogeneicity of some microorganisms to animals and plants may involve proteolysis in penetration of the host organism and in counteracting its defence. Therefore the study of proteinases may help us to understand pathogenesis and its possible control. Some of the microbial proteolytic systems are very complex and contain numerous enzymes

Abbreviations: DAN: diazoacetyl-D,L-norleucine methyl ester; EPNP: 1,2-epoxy /p-nitrophenoxy/ propane; pCMB: p-chloro-mercuribenzoate; TLCK: tosyl-lysyl chloromethyl ketone; TPCK: tosyl-phenyl-alanyl chloromethyl ketone; PMSF: phenylmethane-sulphonylfluoride.

/Spady and Gaertner, 1978; Achstetter et al., 1981/. Proteinases of eucariotic microorganisms have been recently reviewed by North /1982/.

Our work involves two microorganisms. Aspergillus niger is used for industrial production of citric acid, and Claviceps purpurea for the production of physiologically important ergot alkaloids. Besides the desired product, both microorganisms also secrete proteolytic enzymes. The possible use of waste culture broth as a source of enzymes for industrial application was the main reason for studying A. niger C. purpurea production of ergot alkaloids depends on tryptophane as a precursor. This amino acid may also be derived from processes of protein degradation in which proteinases are involved /Rehaček et al., 1971; 1972/. A detailed knowledge of the proteinases would help to clarify their possible role in alkaloid biosynthesis.

MATERIALS

A. niger ATCC 10577 was grown as a surface culture for industrial citric acid production. At the end of the fermentation the biomass was separated from the culture liquid and resuspended in tap water. After 24 hrs it was filtered and the filtrate was used as the source of enzymes.

C. purpurea was grown under conditions required for ergot alkaloid biosynthesis in a submerged culture. After 240 hrs fermentation, broth was withdrawn and mycelia removed by centrifugation.

RESULTS AND DISCUSSION

Separation of concentrated culture filtrates of A. niger and C. purpurea on Sephadex G-100 showed that many proteinases were present which differed in molecular weight and in optimal pH for hydrolysis of substrates. Subsequent testing with proteinase inhibitors revealed in both cases the presence of aspartic proteinases and proteinases which were sensitive to phenylmetane-sulphonylfluoride /PMSF/ Kregar et al., 1981;

Suhar et al., 1982/. We developed a purification procedure for the isolation of enzymes that have an acidic pH optimum but differ in their catalytic mechanism. The purification scheme for C. purpurea culture liquid is shown in Fig. 1, and for A. niger culture filtrate in Fig. 2.

CULTURE LIQUID

SUPERNATANT

CONCENTRATE

SEPHADEX G-100

FRACTION 1 — FRACTION 2

CM-SEPHADEX pH 8.0 — PEPSTATIN-SEPHAROSE

FRACTION 1A — FRACTION 1B — CM-SEPHADEX pH 5.0

CM-SEPHADEX pH 6.0 — ASPARTIC PROTEINASE

SEPHADEX G-150

SERINE PROTEINASE

Fig. 1. Purification scheme of C. purpurea proteinases.

Aspartic proteinases

The isolation of aspartic proteinases was achieved by affinity chromatography on the immobilized pentapeptide inhibitor, pepstatin with additional gel and ion exchange chromatography steps /Kregar et al., 1981; 1983/. The enzymes isolated from both microorganisms were electrophoretically homogenous. Biochemical characterization revealed that both proteinases have very similar properties /Table 1/. The results obtained show that A. niger excretes an aspartic proteinase, also under conditions for citric acid production. Aspartic proteinases from A. niger have been isolated by Bosmann /1973/, and from A. niger var. macrosporus by Iio and Yamasaki /1976/. Although both aspartic proteinases differ from our enzyme in molecular weight, they are very similar in

other properties and appear to be essentially the same enzymes. Aspartic proteinases isolated from other Aspergilli show many similarities with our enzyme /Tsujito and Endo, 1977/, particularly in respect to pH optimum, isoelectric point and pH stability. A. niger proteinase is also similar to other aspartic proteinases of microbial origin /Matsubara and Feder, 1971/.

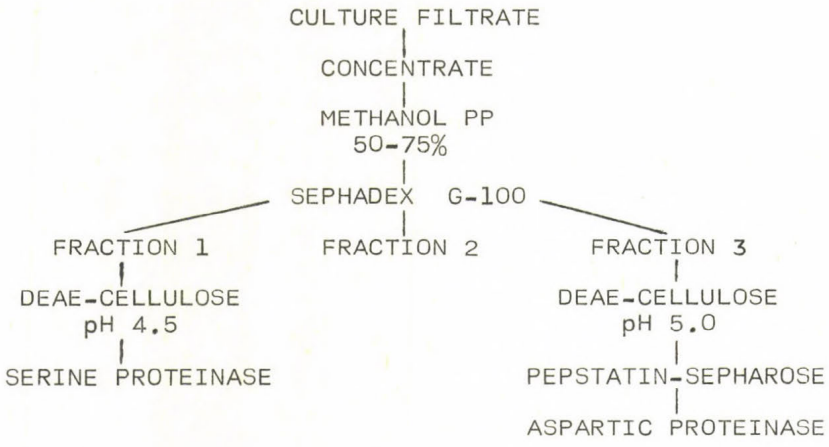

Fig. 2. Purification scheme of A. niger proteinases.

C. purpurea enzyme also exhibits the typical properties of an aspartic proteinase. A pronounced similarity can be found with C. albicans acid proteinase /Rüchel, 1981/. Both enzymes have almost identical molecular weight, isoelectric point, are completely inhibited by pepstatin and show low reactivity with diazoacetyl-D,L-norleucine methyl ester /DAN/ and 1,2-epoxy/p-nitrophenoxy/propan /EPNP/. The majority of pepstatin sensitive aspartic proteinases of microbial origin were found to be very sensitive to these active site directed reagents. On the other hand, cathepsin D, a mammalian aspartic proteinase which is extremely sensitive to pepstatin, also showed a low reactivity with DAN and EPNP /Kregar et al., 1977/. Preliminary experiments on the determination of secondary structure by CD measurements revealed a similarity between A. niger and C. purpurea aspartic proteinases.

Table 1

Comparison of some properties of aspartic proteinases from A. niger and C. purpurea

	A. niger	C. purpurea
Molecular weight:		
gel chromatography	38 000	43 000
SDS electrophoresis	43 000	41 500
Isoelectric point	4.0; 4.6	4.6
pH optimum	3.5	3.5
Inhibition:		
pepstatin 20 μM	100%	100%
PMSF 5 mM	0%	0%
DAN 5 mM	n.d.	40%
EPNP 50 mM	n.d.	40%

Serine proteinases

The next group of acid proteinases eluted in the 90 000-100 000 molecular weight range. Using additional ion exchange chromatography on DEAE-cellulose at pH 4.5, an electrophoretically pure enzyme from A. niger was obtained. This was not possible with C. purpurea enzyme, where two ion exchange chromatography steps and rechromatography on Sephadex G-150 yielded an incompletely pure enzyme. The characterization of both enzymes showed that they have an acid pH optimum for hydrolysis of hemoglobin at pH 4.0 /Fig. 3/, but they were not inhibited by pepstatin. A molecular weight of 115 000 was determined by gel chromatography for both proteinases, and 100 000 by SDS electrophoresis for A. niger enzyme. The enzymes were stable between pH 3.0-8.0, and were inhibited by soman and by PMSF to the extent of approximately 60%. Some

other potential inhibitors were tested on C. purpurea enzyme but they showed no effect /Table 2/. A. niger proteinase was stable to 60°C and this temperature was also optimal for hydrolysis of hemoglobin. Whereas A. niger enzyme exhibited proteolytic activity toward N-α-benzoyl-D,L-arginine-2-naphthylamide, C. purpurea proteinase, on the other hand, did not cleave either this substrate or N-α-benzoyl-Arg-p-nitroanilide.

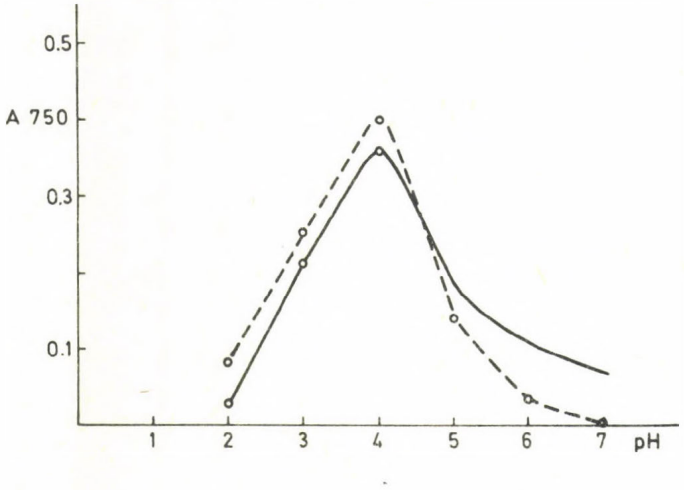

Fig. 3. pH dependence of hemoglobin hydrolysis by A. niger /----/ and C. purpurea /——/ serine proteinase.

The summarized properties of the proteinases from both microorganisms again display many similarities /Table 3/. According to their inhibition with soman and PMSF, they can be classified as serine proteinases, although they are different from the majority of other serine proteinases which show their maximal activity in the neutral or alkaline pH range. There is a report, however, on a serine proteinase from the maize root which degraded hemoglobin maximally at pH 4 /Shannon, 1979/, and on Streptomyces rimosus serine proteinase exhibiting an acid and an alkaline pH optimum for hydrolysis of hemoglobin /Renko et al., 1981/. Also, serine

Table 2

Effect of some potential inhibitors on C. purpurea serine proteinase

Effector*	% inhibition
Pepstatin 20 μM	2
EDTA	0
pCMB	0
PMSF	66
DAN	2
TPCK	8
TLCK	6
Benzamidine	0
p-NH_2-benzamidine	17

*5 mM final concentration

proteinases are generally of low molecular weight, usually around 25 000. Larger enzymes have been reported, e.g. in A. niger /Bosmann, 1973/, A. nidulans /Stevens and Stevens, 1980/, Phycomyces blakesleeanus /Fiecher and Thompson, 1979/ and the already mentioned proteinase from the maize root /Shannon, 1979/. A serine proteinase with a molecular weight of 126 000, the largest one reported so far, was found in Blakeslea trispora /Govind et al., 1981/. The serine proteinase isolated from A. niger by Bosmann /1973/ had a molecular weight of 68 000, but it degraded acetylhemoglobin optimally at pH 7.4.

Our results indicate pronounced similarities in proteinases excreted by two microorganisms of the Ascomycete class. They are found in two different subclasses, however, but the classification is based on morphological criteria only.

Table 3

Comparison of some properties of serine proteinases from A. niger and C. purpurea

	A. niger	C. purpurea
Molecular weight:		
gel chromatography	115 000	113 000
SDS electrophoresis	100 000	n.d.
pH optimum:		
hemoglobin	4.0	4.0
casein	2.0	n.d.
pH stability	2 - 8	3 - 8
Inhibition		
pepstatin 20 μM	0%	0%
soman 5 mM	60%	n.d.
PMSF	n.d.	65%

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DISCUSSION

BARRETT:

Does your serine acid proteinase have any resemblance to the pepstatin resistant acid proteinase that has been described for another microorganism by Japanese workers?

KREGAR:

Yor are probably referring to Scytolidium proteinases. They are not inhibited by pepstatin, but they have very low molecular weight /around 22 000/.

STEPANOV:

What about the inhibition actually?

KREGAR:

We preincubated the enzyme with pMSF and soman /a DFP analogue/ for 30 min. The residual activity toward hemoglobin was determined afterwards. We have not gone into the details of this inhibition yet.

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ENDO-PROTEASES AS REGULATORS OF VIRUS REPLICATION

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ABSTRACT

Picornaviruses suppress cellular protein degradation and produce their own highly specific endo-protease. The viral enzyme processes viral precursors to yield the coat proteins, which then assemble. Options for inhibiting viral proteases are discussed, including the role of serum protease inhibitors in blocking viral proteolysis. One such inhibitor was used to purify and characterize the poliovirus protease. A virulent and an attenuated strain of poliovirus were compared with regard to their abilities to induce cleavage of the subunit of a serum protease inhibitor; the virulent virus was able to process the inhibitor with greater efficiency.

INTRODUCTION

Viral protein synthesis and processing in cells infected by picornaviruses has been extensively studied. A favorable feature for these studies is the drastic inhibition of cellular protein synthesis /and protein synthesis of most other coinfecting animal viruses/ by the small positive-stranded RNA viruses. This effect permits radioisotope labeling solely of viral proteins shortly after infection.

The extensive proteolytic processing used in forming the proteins of picornaviruses has made them a useful model for the study of biological regulation by proteases. In this

communication, the ability of the virus to block cellular protein degradation is compared with shut-off of cellular protein synthesis following infection. Alpha-1-macroglobulin, a serum protease inhibitor, was then used to identify the poliovirus-coded protease, and characteristics of the enzyme are presented. It was also found that poliovirus strains differing in their degree of virulence for man show variability in their interaction with a serum protease inhibitor, and clinical implications of this are discussed.

MATERIALS AND METHODS

Growth of poliovirus and Hela cells in culture has been described previously /Korant, 1972/. Labeling of viral proteins and subsequent analysis in polyacrylamide slab gels has also been published /Korant et al., 1979/. Details of individual experiments are given in the text. Isotopes used in these studies were purchased from New England Nuclear Corp., Boston, Massachusetts, USA.

RESULTS

Inhibition of Cellular Protein Degradation Following Virus Infection. The shut-off of host protein synthesis by picornaviruses has been of long-standing interest as a dramatic example of translational control. Following infection there is usually, but not always, viral-induced inactivation of factors required in translation of cellular proteins, particularly the binding of cellular mRNA to the small ribosomal subunit. We previously carried out cell-free studies on the suppression of ribosome-associated proteolytic activity following viral infection /Korant and Lonberg-Holm, 1981; Langner et al., 1982/.

We have examined the in vivo effect of infection of transformed human epithelial cells /WISH/ by poliovirus type 2 on degradation of cellular proteins. The cells were cultured for 17 hours in medium supplemented with /^{14}C/ labeled amino

acid mixtures either in the absence of amino acid analogs, or in their presence. They were then challenged with 10 infectious virus particles per cell and protein degradation assayed by precipitation of labeled intracellular and extracellular proteins with trichloroacetic acid. The virus used was highly purified by zonal and equilibrium centrifugation followed by dialysis, to avoid non-specific effects.

Table 1

Protein degradation in virus-infected cells

Sample	Rate of Degradation /% per hr/
WISH cells	1.4
WISH cells + poliovirus 2	0.8
WISH cells + amino acid analogs	2.6
WISH cells + analogs + polio 2	0.9

The results of the study are shown in Table 1. The ability of WISH cells to degrade proteins is significantly impaired following virus challenge, a result which conforms to our earlier results on cell-free degradation /Korant and Lonberg-Holm, 1981/. The virus suppresses turnover of analog-containing proteins to an even greater extent, when the percentage of degradation is compared with that of "normal" proteins; however, a basal level of degradation continues after virus challenge for analog-containing as well as unmodified proteins. Similar results have been reported previously in bacteria infected with viruses /Simon et al., 1978/.

Based on the experiment shown in Table 1, we have compared the viral inhibition of cellular protein synthesis with the suppression of degradation. The results are summarized in Table 2. There are clearly many parallels between the two events, a result which implies that the same mechanism and possibly the same hypothetical viral product may be involved.

It would be helpful in studying the mechanism if viral mutants were available which were not able to suppress these cellular events. It is not straight - forward to select such mutants, since virus production is not markedly affected by an inability of the virus to inhibit cellular translation. We are devising a scheme presently to try to identify mutants which do not block degradation.

Table 2

Inhibition of protein synthesis or degradation by picornavirus

Characteristic	Synthesis	Degradation
Time after infection	Immediate	Immediate/early
Dependent on multiplicity	+	+
Requires protein synthesis	+	+
Requires viral genome replication	-	-
Inactivation of cap-binding protein	+	?
Required for virus production	-	?
Viral mutant known not to inhibit production	-	-

Proteolytic Processing of Viral Polypeptides. The mRNAs of picornaviruses appeared at one time to be unique in the sense that they coded for several proteins, but used only a single translation start signal. Their translation product is a polyprotein containing several domains, each with a distinct function. Gene expression is controlled by elaborate post-translational proteolytic events, which generate viral structural proteins, polymerases, etc. /reviewed in Perez-Bercoff, 1978/. However, it has become apparent that this type of regulation is not limited to picornaviruses. In fact, most animal viruses use some degree of proteolytic processing for structural proteins. And it has recently become clear that

there are also polyprotein precursors for some classes of cellular proteins. The neuropeptide hormones provide several very clear examples of large mRNAs with single initiation sites, producing a number of different protein/peptide products by cleavage.

While many animal viruses /and others infecting insects, plants and bacteriophages/ use proteolytic processing during virion assembly, the picornaviruses are the most outstanding example, because they use cleavages to form all viral polypeptides.

With picornaviruses, the cleavages of the structural precursors do not cause the products to separate. To the contrary; there is alteration in conformation, antigenic structure and isoelectric point and the cleavage products assemble into larger and more complex structures, culminating in the viral capsids. When the RNA associates with the capsids it is locked in by a final protein cleavage which also places the virion into a specific native configuration which recognizes cellular receptors. Proteolytic cleavages regulate RNA synthesis as well, by activating and deactivating the viral polymerase, and also by generating the genome-linked peptide. For a review of protein cleavage pathways of picornaviruses see Rueckert in Perez-Bercoff, 1978.

Although the phenomenon of protein cleavages in virus-infected cells is thoroughly documented, the actual function or role of the cleavages is not clear. For example, the extensive proteolysis of the picornavirus structural precursor does not cause dissociation of the products, but rather alters their folding. Why this cannot be accomplished without cleavage remains to be understood. It seems likely that cleavage is used for modulation of protein function in a system where transcriptional and translational controls are not favored.

Cleaved Sites in Viral Precursor Proteins. In the past several years the amino acid sequences of the cleavage recognition sites have become known for several picornaviruses.

This data has in part been provided by end-group analyses of viral proteins, but more information has been deduced from sequencing of viral genomes, mRNA, or DNA complementary to the viral RNA. The latter technique has permitted analysis of large portions of picornavirus RNAs and the entire sequence of a poliovirus /Kitamura et al., 1981/.

Table 3

Protein cleavage sites

Poliovirus /nascent/	leu thr thr tyr/gly phe gly his
	asp ala met tyr/gly thr asp gly
Poliovirus /intermediate/	pro arg leu gln/gly leu pro val[x]
	ala leu ala gln/gly leu gly gln[x]
	ala leu phe gln/gly pro leu gln[x]
	ala met gln gln/gly ile thr asn
	val ile lys gln/gly asp ser trp
	ala gly his gln/gly ala tyr thr
Poliovirus /maturation/	pro met leu asn/ser pro asn ile
FMDV[xx] /structural/	pro ser lys gln/gly ile phe pro
	pro arg thr gln/thr thr ser thr
	lys gln leu leu/asn phe asp leu
FMDV /non-structural/	glu/gly leu ile val
FMDV /maturation/	ala leu leu ala/asp lys lys thr

[x] rapid

[xx] foot and mouth disease virus

Comparisons of the cleaved sites in precursor proteins of two picornaviruses are shown in Table 3. The picornavirus sequences have several rather distinct classes of cleaved sites. First are the nascent cleavages sites, processed almost instantly during translation. They are reminiscent of chymotryptic cleavage sites; the new carboxyl termini being donated by aromatic /tyrosine/ or hydrophobic residues. Next are the rapidly processed sites, such as those in structural precursors /half-life of 5 minutes in infected cells/. For

the picornaviruses these contain glutamine-glycine or glutamic acid-X residues, often surrounded by hydrophobic /leucine, isoleucine, valine/ sequences. Sites which are more slowly cleaved also contain glutamine or glutamic acid, but are in more neutral or hydrophilic regions of the precursors, which may alter their affinity for the participating proteases. Lastly, there are distinctly specific sites which are processed during combination of the viral RNA and the coat proteins, the so-called maturation cleavage. The occurrence of highly conserved peptide regions at processing sites of viral precursor proteins may offer an avenue for design of selective inhibitors.

Origin of the Processing Proteases. At the outset, it should be emphasized that the important cleavages of picornavirus precursor proteins in infected cells do not resemble the normal degradation of intracellular proteins. As a rule, the proteolytic cleavages of viral proteins are at rare, highly site-specific regions /see Table 3/, and no soluble peptides or amino acids are released.

From a number of lines of evidence, it appears that initial cleavages of nascent viral polypeptides are carried out by cellular proteases associated with the translational apparatus. These may be the membrane-bound proteases which process signal sequences from nascent cellular secretory proteins. Another candidate is a ribosomal protease of animal cells /Langer et al., 1982/. The cleavage specificity is reminiscent of chymotrypsin, a serine-active site protease. Both the nascent cleavage reactions and the ribosomal protease are blocked by diisopropylphosphfluoridate /DFP/ a classic inhibitor of serine proteases.

A body of evidence from several laboratories established that the intermediate protein cleavages of picornavirus precursors, some of which yield the viral structural proteins, are carried out by a virus-coded protease. The enzyme activity is not detected in extracts of uninfected cells, but is found in lysates of infected cells in quantities which increase in

time after infection and with the amount of infecting virus /Korant et al., 1979/. Cell-free protein synthesizing systems, programmed with picornaviral RNA, produce a specific proteolytic activity which also processes the capsid polypeptides /Pelham, 1978/. In both infected cells and cell-free extracts it was shown that the protease is not efficient at cleaving proteins of heterologous viruses or cellular proteins /Korant, 1977; Shih et al., 1982/.

The picornavirus protease is apparently a member of the class of proteases with cysteine at the active site, based on inhibitor sensitivity /Korant, 1973; Pelham, 1978/. In general, cysteine proteases are not highly specific with regard to substrates cleaved, and thus the specifity determinants of the viral enzymes are of innate biochemical interest. Glutamine or glutamic acid cleaving proteases are described in bacteria, but have not been well-characterized.

Inhibitors of Viral Protein Cleavages. This topic has been reviewed recently /Korant, 1981/. The approach seems to be promising for design of antiviral agents for those animal viruses which produce their own specific proteolytic enzymes, that is picornaviruses, retroviruses and adenoviruses, and possibly alphaviruses, myxo- and paramyxoviruses, poxviruses, and herpesviruses.

The mode of action of cleavage inhibitors can be classified in either of two general categories; alteration of the substrate to prevent its proper cleavage, or inhibitors of the protease catalytic activity. The first category contains certain protein modifying reagents, amino acid analogs, high or low temperature and some metal ions, zinc being the most often reported. Inspection of this category reveals that none of these treatments is likely to lead to specific alteration of viral proteins.

The second approach is targeted at the proteolytic enzymes. The picornavirus protease is very sensitive to inhibitors of thiol proteases, eg mercurials, N-ethyl maleimide, and iodoacetate. We have begun to design inhibitors which

mimic the structure of the cleavage sites, based in part on their peptide sequences. For example, we found that carbobenzoxy leucine chloromethyl ketone was an irreversible inhibitor of poliovirus protease /Korant et al., 1979/ and showed also that it blocked viral protein cleavages and virus production /Korant, 1981/.

Inhibitors of viral protein cleavages are readily tested for picornaviruses. The test can be made at a time after infection when the cellular protein synthesis is completely inhibited and only virus proteins are labeled with radioactive amino acids. The viral proteins are size-separated in polyacrylamide gels and any inhibition of cleavage can readily be detected. Variations on the assay involve mixing purified viral protease with radioactive viral precursors obtained from cell-free translation or with synthetic substrates. The results may then be visualized by autoradiography of dried gels. A spectrophotometer may also be used if the cleaved substrate is chromogenic.

We have recently found that certain natural protease inhibitors prevent viral protein cleavages /Korant and Lonberg-Holm, 1981/. Human alpha-2-macroglobulin or rat alpha-1-macroglobulin blocked poliovirus protease action in extracts, and covalently bound the enzyme. This finding has been useful in characterizing the viral protease. The identity of the picornavirus protease has been a matter of some controversy. An initial report claimed that a structural polypeptide of EMC virus possessed protease activity /Lawrence and Thach, 1975/. However, a subsequent study indicated that a non-structural translation product was required and it was then proposed that a polioviral non-structural polypeptide of approximate molecular weight 40 000 was the polioviral processing protease /Korant et al., 1979/. This result was then challenged by three groups, based on independent purification methods or antibody studies /Palmenberg et al., 1979; Gorbalenya et al., 1981; Havecak et al., 1982/.

We have re-examined this question by analyzing the amino

acid sequence of the poliovirus protein bound to a serum protease inhibitor. Cells infected with poliovirus type 1 for three hours were placed in amino-acid free medium and the viral proteins were labeled with single radioactive amino acids for 90 minutes. At that time, cell extracts were prepared by mechanical breakage of the cell membrane, and the cytoplasmic fraction, containing the labeled viral proteins, were incubated with purified rat alpha-1-macroglobulin, a large protein protease inhibitor present in rat serum. The incubation at 37° was continued for 60 minutes, and then further reaction was prevented by adjusting the samples to contain 1% sodium dodecyl sulfate /SDS/, mercaptoethanol and 8 M urea, and boiling. The extracts were then separated on 7.5% polyacrylamide gels in SDS, and dried directly without chemical fixation. Radioactive complexes of viral protein and alpha-1-macroglobulin subunit were identified in autoradiographs of the dried gels, cut from the gels, and eluted by electrodialysis. The sequence of the amino-terminal portion of the bound poliovirus protein was determined by automated Edman degradation and liquid-scintillation counting of the fractions after each Edman cycle. The results will be presented in detail elsewhere, but clearly identify the bound viral protein with a segment of the coding region of the viral RNA. The RNA sequence is from nucleotide 5430 to 5978 /Kitamura et al., 1981/. The protease amino acid composition was deduced from the RNA sequence and is presented in Table 4. The enzyme is apparently of the thiol class of proteases, and there are three cysteines present in the sequence. A poliovirus variant is known in which the third cysteine is converted to a methionine, leaving the enzyme functional /Cann et al., 1983/. We believe the active site cysteine in the protease of poliovirus type 1 is at position 153 /our unpublished results/. Further studies are in progress.

Table 4

Amino acid composition of poliovirus type 1 protease

Amino Acid	Mole Percent*
Arginine	5
Lysine	4.5
Aspartic Acid	4.5
Glutamic Acid	5
Asparagine	5
Glutamine	4
Glycine	9.8
Alanine	8.5
Serine	5
Threonine	9.8
Methionine	2.9
Leucine	4.5
Isoleucine	7.4
Valine	8
Histidine	2.2
Phenylalanine	4
Tyrosine	2.2
Tryptophan	0
Proline	4.5
Cysteine	1.7

* from RNA sequence

Interaction of Viral Proteases with Serum Protease Inhibitors. In using the binding of poliovirus protease to alpha-1-macroglobulin as a purification step, we noted that there was breakdown of the inhibitor during the course of incubation. This has been examined in detail, using /^{125}I/ labeled rat alpha-1-macroglobulin. As shown in Figure 1, after even a 60 minute exposure to extracts of cells infected with a virulent strain of poliovirus, breakdown by limited

proteolysis of the alpha-1-macroglobulin subunit can be observed. By comparison, uninfected cell extracts show no ability to process the inhibitor. Surprisingly, extracts of cells infected with an attenuated, oral vaccine strain of type 1 poliovirus were deficient in the ability to cleave the protease inhibitor subunit. Only prolonged reaction /24 hours/ showed that the attenuated virus could cleave the inhibitor, but this was also observed to a degree in extracts of uninfected cells.

The genetic and biochemical basis of poliovirus attenuation is not understood, but it is possible that an altered viral protease is co-selected with the loss of virulence. It is also possible that infection by the virulent strain, but not the attenuated, activates cellular proteases which process the alpha-1-macroglobulin subunit. However, there is little evidence to suggest leakage of lysosomal hydrolases into the cytoplasm of infected cells, nor are cellular proteins non-selectively degraded following infection /Korant et al., 1980/.

DISCUSSION

The study of proteolysis associated with replication of viruses is crucial to understanding the function and assembly of viral proteins. It may also lead to a fuller appreciation of the various controls of protein degradation in uninfected cells. As shown in this study, viral infection suppresses the usual turnover of proteins which occurs in cells in culture. The data presented indicate the role of a viral product in blocking the degradative pathway/s/. This function would appear to be of obvious advantage to the virus, since in principle it would protect viral structural proteins from attack by cellular protease activities. We are hopeful that characterizing the mechanism of viral suppression of degradation will help to clarify the basis of selection of certain classes of proteins for intracellular destruction.

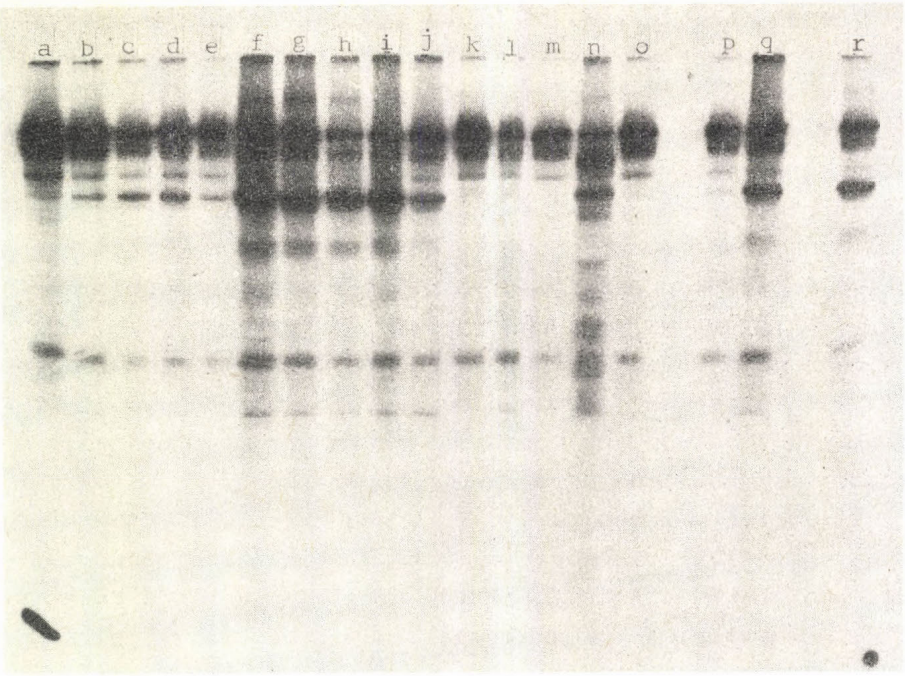

Figure 1 Legend. Cleavage of alpha-1-macroglobulin subunit in cell extracts

Purified rat serum alpha-1-macroglobulin was radioiodinated and added to homogenates of Hela cells. After incubation at 30° for the times indicated, the samples were treated as described in Results and the proteins analyzed on a polyacrylamide slab gel in SDS-containing buffer. After electrophoresis, gels were dried and used to expose x-ray film for 24 hours.

Lanes a through e - polio 1 /virulent/ infected for increasing times /hourly intervals/; expose alpha-1-macroglobulin for 60 min. Lanes f through j - same, but expose alpha-1-macroglobulin for 24 hours; k - alpha-1-macroglobulin, no incubation; l - incubated 24 hours, no extract added; m - incubated 60 min. with uninfected Hela cells; n - incubated 24 hours with uninfected Hela cells; o - polio 1 /attenuated/ 60 min. after infection, 60 min. incubation; p - 4 hours after infection, 60 min. incubation; q - polio 1 /attenuated/, 60 min. after infection, 24 hour - incubation; r - 4 hours after infection, 24 hour - incubation.

The picornaviruses, of which poliovirus is a prototype, ensure proper processing of their structural proteins by encoding a proteolytic enzyme of exquisite specificity. Only a very few sites are cleaved by this enzyme in viral precursor proteins, and virtually no activity of the enzyme is detected on heterologous proteins. The primary sequences recognized by viral proteases are becoming known, but there may well be even more extended regions of protein-protein recognition in order for the viral protease to act. The viruses seem to provide a good model for beginning the study of high-specificity endoproteases involved in regulatory pathways. Cellular analogs may include the proteases which process the neuropeptide hormones from precursor polyproteins.

Ultimately, our major goal is specific inhibition of the virus replication process. Inhibitors of clinical importance will possibly be synthetic mimics of cleavage sites /Barrett, 1980/. We have also found an ability of naturally-occurring serum protease inhibitors, such as alpha-2-macroglobulin, to block viral protease action in cell lysates. Such endogenous inhibitors may play an important role in limiting pathogenesis arising from certain infections, particularly those where a unique, virus-coded enzyme is present. In this regard, we find it intriguing that a highly virulent virus is able to induce degradation of a serum protease inhibitor. It is possible that, to a degree, virulence may result from the virus' ability to inactivate such a host line of defense. Moreover, the fragments of serum protease inhibitors generated by exposure to infected cell lysates may themselves have toxic properties which contribute to the clinical severity of the disease. Further comparisons of wild-type viruses and their attenuated derivatives, focusing on their interaction with endogenous protease inhibitors, may help to clarify the biochemical basis of viral pathogenicity.

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DISCUSSION

BARRETT:

Have you prepared diazomethyl ketones to inhibit the virus protease, as cysteine proteases should be sensitive?

KORANT:

Yes, we have tried, but we have had no luck at finding antiviral activity. The experiments were all done with infected cells.

GLAUMANN:

What was your protocol for microinjection of the alpha-1-macroglobulin into cells?

KORANT:

The cells are fused with PEG, then injected with virus and finally microinjected with the inhibitor. Labelled methionine is added to visualize the viral proteins.

BARRETT:

It's good to see that someone is finding a use for the ability of certain antiproteases to form covalent bonds with proteases.

KORANT:

Yes, the reaction has been of great use to us.

BARRETT:

You should be interested in the antiviral activity of endogenous cysteine protease inhibitors.

KORANT:

Yes, we are very interested in them, and plan to examine their activity against selected viruses.

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CATALYTIC PROPERTIES OF PLANT PROTEINASES. A COMPARISON BETWEEN SERINE AND THIOL ENZYMES: SPINACH LEAF PROTEINASE, PAPAIN, FICIN AND BROMELAINS

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INTRODUCTION

Over the past 50 years, a number of proteinases have been found and/or isolated from fruits, seeds, leaves and latex of several plants, but only recently their role in cellular processes and plant metabolism has been studied.

Most plant proteinases appear to be sulfydryl enzymes with pH optima ranging between 5 and 8. However, in recent years several endopeptidases that do not involve a sulfydryl group for catalytic activity have been reported in plant tissues. The latter enzymes fall into two groups: an acid group (pepsin-like), having pH optima at 3-4, and an alkaline group, with pH optima at 7.5-8.5, that appears to resemble the serine proteinases found ubiquitously in animals and microorganisms. It has always been puzzling that plants seem to use primarily sulfydryl proteinases for protein degradation, whereas microorganisms and animals utilize primarily serine proteinases (see: Ryan and Walker-Simmons, 1981 and references sited therein).

The aim of the present study is to compare side by side the catalytic and inhibitor binding properties of the leucine specific serine proteinase from spinach leaves with those of sulfydryl proteinases, notably papain, ficin and bromelains.

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As in the case of mammalian proteinases, both serine and thiol plant proteinases catalyze the hydrolysis of low molecular weight substrates *via* a mechanism involving a minimum of two intermediates:

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E\cdot P \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E+P_2$$
$$+P_1$$

In the case of reactions catalyzed by papain, ficin and bromelains, the intermediate E·P is considered to be an ester formed between the acyl moiety of the substrate and the cysteine-SH group (Lowe, 1977), whereas in the case of the spinach leaf leucine specific serine proteinase, E·P is assumed to be a hydroxyl-acyl-enzyme intermediate.

The knowledge of catalytic and inhibitor binding properties, supplemented with structural data so far available for plant and mammalian proteinases, allows a rather detailed description of their catalytic behaviour.

Catalytic properties of the spinach leaf leucine specific serine proteinase

The presence of proteinases and related specific inhibitors in spinach leaves (*Spinacia oleracea* L.) has been recently reported (Satoh and Fujii, 1982; Watanabe and Kondo, 1983). On the other hand, no gelatinolytic and/or proteolytic activities have been found by Yamaguchi et al. (1982).

The presence of proteinases in the crude juice of spinach leaves has been assayed measuring the hydrolysis of the following p-nitroanilides and p-nitrophenyl esters of (L-)amino acids[+]: N-α-L-leucine p-nitroanilide (LeuNHNp), N-α-L-alanine

[+]All reagents, substrates and inhibitors used in the present study were of the highest purity commercially available or synthesized according to literature methods (see: Ascenzi et al., 1982). Leupeptin and the porcine pancreatic secretory trypsin inhibitor (PSTI) were generous gifts of Dr. G. Borin and Prof. E. Menegatti, respectively.
The enzymatic assays and inhibitor binding measurements were performed as previously reported (Ascenzi et al., 1982; Antonini et al., 1983). In order to ensure the homogeneity of the system, 3% ethanol/water (v/v) or 3% acetonitrile/water (v/v) reaction mixtures were employed. No effect of etha or acetonitrile (up to 5%) in the reaction mixtures has been observed.

Table 1

Relative activity for the catalyzed hydrolysis of a number of p-nitroanilides and p-nitrophenyl esters of (N-α-substituted L-)amino acids by the crude juice of spinach leaves (pH 7.5, phosphate buffer, I=0.1 M; T=23.5±1 ^{o}C)

Substrates	Relative activity (%)
AlaNHNp	23.9
LeuNHNp	100.0
LysNHNp	8.9
BzLysNHNp	10.2
BzArgNHNp	10.9
SPheNHNp	0
AcTyrNHNp	0
ZGlyONp	5.0
ZAlaONp	25.1
ZValONp	33.1
ZIleONp	31.6
ZLeuONp	100.0
LysONp	10.0
ZLysONp	10.1
ArgONp	11.2
ZArgOnp	9.5
ZPheONp	0
ZTyrONp	0

Substrate saturating concentration was $1x10^{-2}$M.

p-nitroanilide (AlaNHNp), N-α-benzoyl-L-arginine p-nitroanilide (BzArgNHNp), N-α-benzoyl-L-lysine p-nitroanilide (BzLysNHNp), N-α-L-lysine p-nitroanilide (LysNHNp), N-α-succinyl-L-phenylalanine p-nitroanilide (SPheNHNp), N-α-acetyl-L-tyrosine p-nitroanilide (AcTyrNHNp), N-α-carbobenzoxy-L-leucine p-nitrophenyl ester (ZLeuONp), N-α-carbobenzoxy-L-alanine p-nitrophenyl ester (ZAlaONp), N-α-carbobenzoxyglycine p-nitrophenyl ester (ZGlyONp), N-α-carbobenzoxy-L-isoleucine p-nitrophenyl ester (ZIleONp), N-α-carbobenzoxy-L-valine p-nitrophenyl ester (ZValONp), N-α-carbobenzoxy-L-lysine p-nitrophenyl ester (ZLysONp), N-α-L-lysine p-nitrophenyl ester (LysONp), N-α-carbobenzoxy--L-arginine p-nitrophenyl ester (ZArgONp), N-α-L-arginine p-nitrophenyl ester (ArgONp), N-α-carbobenzoxy-L-phenylalanine p-nitrophenyl ester (ZPheONp), N-α-carbobenzoxy-L-tyrosine p-nitrophenyl ester (ZTyrONp) and p-nitrophenyl p-guanidinobenzoate (pNGB).

At fixed pH (between pH 5 and 9.5), ionic strength (0.1 M) and temperature (23.5±1 ^{o}C), the leucine derivatives show the highest hydrolysis rate, indicating that a leucine specific proteinase is the most representative enzyme in the crude juice of spinach leaves. Lower hydrolytic activities towards glycine, alanine, valine, isoleucine, lysine and arginine derivatives have been observed. Moreover, the hydrolysis of tyrosine and phenylalanine derivatives is not catalyzed by the crude juice of spinach leaves (see Table 1).

In order to check the possibility that the catalytic activities observed depend on the same proteinase, the hydrolysis of some p-nitroanilides of L-amino acids was measured after polyacrylamide gel electrophoresis of the spinach leaf crude juice. Fig. 1 shows the dependence of the relative activity for the hydrolysis of LeuNHNp, AlaNHNp, BzLysNHNp, LysNHNp, and BzArgNHNp on the relative electrophoretic mobility. For all the p-nitroanilides examined, the maximum of their catalyzed hydrolysis corresponds to a value of μ equal to 0.58±0.05. Moreover, in order to check the hypothesis of various proteinases present with different specificities but with the same relative electrophoretic mobility, the catalyzed

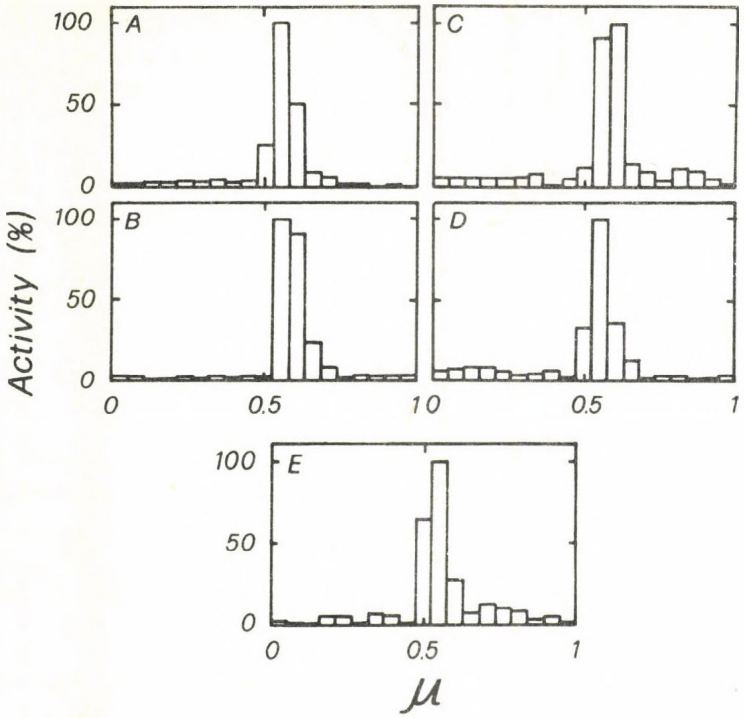

Fig. 1: Dependence of the relative enzyme activity for the hydrolysis of LeuNHNp (A), AlaNHNp (B), BzLysNHNp (C), LysNHNp (D) and BzArgNHNp (E) by the crude juice of spinach leaves on the relative electrophoretic mobility (μ). The enzymatic assay was performed on sliced polyacrylamide gels. The data have been obtained at pH 7.5, phosphate buffer, I=0,1 M; T=23.5±1 °C.

hydrolysis of all p-nitroanilides examined was determined after the selected inhibition of the leucine specific activity by N-α-tosyl-L-leucine chloromethyl ketone (TosLeuCK) (see below). In the presence of TosLeuCK (100 μM), not only the hydrolysis of LeuNHNp was inhibited, but also that of the other anilides derivatives. These findings suggest that possibly one leucine specific proteinase is involved in the hydrolysis of all the synthetic substrates here employed. Nevertheless, the small but significant catalyzed hydrolysis of anilides of

(L-)amino acids by the crude juice of the spinach leaves at μ values different from the maximum (0.58±0.05) (see Fig. 1) should indicate the presence of lower concentrations of other amidase activities.

The absence of any effect of substitutions at the N-α-position on the hydrolysis rate of both esters and anilides suggests that the spinach leaf leucine specific proteinase is an endopeptidase.

According to the relative hydrolysis rate catalyzed by the partially purified spinach leaf leucine specific proteinase[≠] as well as by the crude juice, the specificity towards the substrates considered can be arranged as follows:

Gly < Ala < Val ~ Ile < Leu > Arg ~ Lys

over the whole pH range explored (5-9.5) (see Table 1 and Fig. 2). According to general views (Kraut, 1977; Steitz and Shulman, 1982), the effect of the amino acid side chains on the hydrolysis rate suggests that the acylation step is rate limiting in catalysis between pH 5 and 9.5.

From the inspection of Fig. 2 it may be observed that at all pH values, p-nitrophenyl esters show a systematic higher hydrolysis rate as compared to that of the corresponding p-nitroanilides. This finding may be related to the fact that the leaving group present in p-nitrophenyl esters is very good as compared to that of p-nitroanilides, which is very poor.

The values of steady-state parameters (V_{max} and K_m) for the spinach leaf leucine-specific proteinase catalyzed hydrolysis of LeuNHNp have been determined at pH 7.5 (see Fig. 3). Under all experimental conditions, the dependence of the initial velocity upon substrate and enzyme concentrations and the time course of the reaction follow simple Michaelis-Menten kinetics.

[≠]The spinach leaf leucine specific proteinase has been partially purified by ion-exchange chromatography on DEAE-BIOGEL A (100-200 mesh) (Bio-Rad, Richmond, USA).

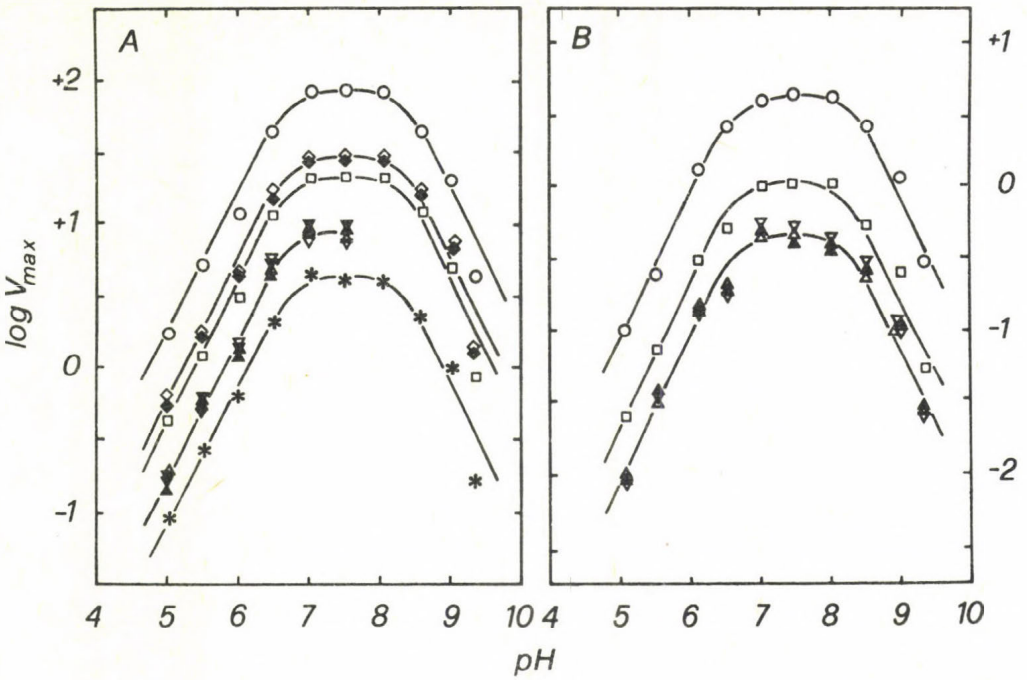

Fig. 2: Effect of pH on the maximal velocity for the spinach leaf leucine specific serine proteinase catalyzed hydrolysis of ZLeuONp (A,○), ZGlyONp (A✱), ZAlaONp (A,□), ZIleONp (A,◆), ZValONp (A,◇), ZLysONp (A,△), LysONp (A,▲), ZArgONp (A,▽), ArgONp (A,▼), LeuNHNp (B,○), AlaNHNp (B,□), LysNHNp (B,▲), BzLysNHNp (B,△) and BzArgNHNp (B▽). The velocity is expressed as micromoles of substrate hydrolyzed per minute per milliliter (1 milliliter contains 0.04 mg of total proteins). The solid lines are the theoretical curves for two ionizing groups of pK_a values of 7.1 and 8.2, respectively. ZLysONp, LysONp, ZArgONp and ArgONp show high alkaline hydrolysis rates and therefore may be usefully employed as substrates only up to pH 8 (Ascenzi et al., 1982). Substrate saturating concentration was $1x10^{-2}$ M at all pH values. The data have been obtained in acetate buffer, pH 5-6; phosphate buffer, pH 6-8.5; and borate buffer, pH 8.5-9.5, all at I=0.1 M (sodium salts); T=23.5±1 °C. No specific ion effects were found by using different buffers overlapping in pH.

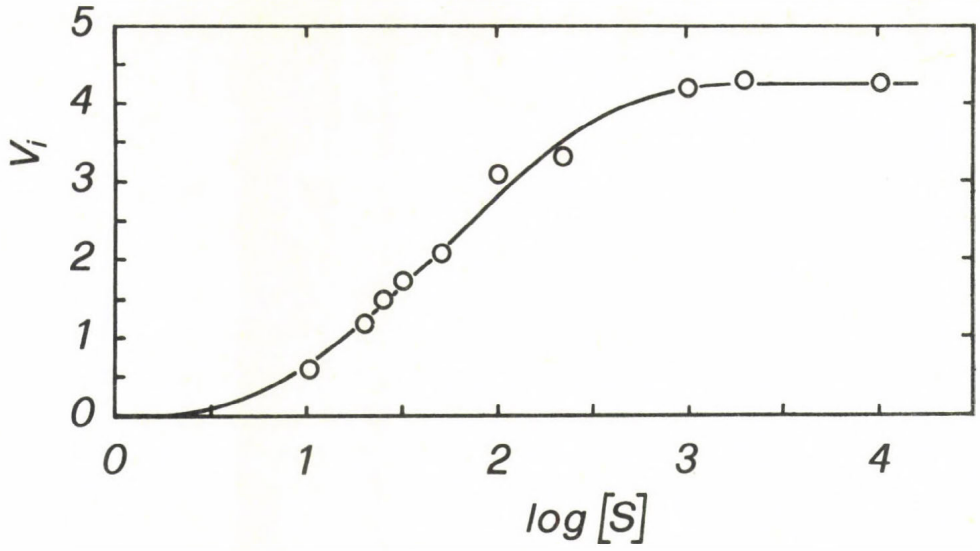

Fig. 3: Dependence of the initial velocity for the spinach leaf leucine specific serine proteinase catalyzed hydrolysis of LeuNHNp on the substrate concentration. The velocity is expressed as micromoles of substrate hydrolyzed per minute per milliliter (1 milliliter contains 0.04 mg of total proteins). The solid line is the theoretical curve calculated with the following values of steady-state parameters: K_m=50.1 μM V_{max}=4.25 micromoles of substrate hydrolyzed per minute per milliliter. The data have been obtained at pH 7.50, phosphate buffer, I=0.1 M; T=23.5±1 °C.

In agreement with the observed peculiar specificity of the spinach leaf leucine specific proteinase, the inhibitor effect of the bovine basic pancreatic trypsin inhibitor (BPTI), PSTI, soybean trypsin inhibitor (STI), leupeptin, TosPheCK, TosLysCK and of the active site titrant pNGB, all containing a cationic or aromatic side chain at the reactive site, on the catalyzed hydrolysis of esters and anilides is negligible as

compared to that of TosLeuCK (inhibitor concentration was 100 μM).

For all the substrates examined, the pH dependence of the hydrolysis rate, catalyzed by the spinach leaf leucine specific proteinase, depends on two ionizing groups with pK_a values of 7.1 and 8.2 respectively (see Fig. 2). In this respect, the pH dependence of the spinach leaf leucine specific proteinase catalyzed hydrolysis of anilides and esters is closely similar to that observed for animal serine proteinases, notably bovine β-trypsin and bovien α-chymotrypsin (see: Antonini and Ascenzi, 1981; Ascenzi et al., 1982; and references cited therein) as well as for the proteolytic activity present in the juice of alfalfa (Medicago sativa L.) (Tozzi et al., 1981). In animal proteinases, the observed ionizations have been assigned tentatively to the acid-base equilibria of His57, involved in the catalytic triad, and of the N-terminal Ile16. The deprotonation of Ile16 is accompanied by the cleavage of the salt bridge between the N-terminal residue and the Asp194, and induces the inactivation of the enzyme (see: Kraut, 1977; Steitz and Shulman, 1982 and references cited therein).

The presence of a histidine residue involved in the catalytic site of the spinach leaf leucine specific proteinase has been checked by inactivating the enzyme with N-α-tosyl chloromethyl ketones of N-α-L-amino acids. As reported above, only TosLeuCK inhibits the spinach leaf leucine specific proteinase, suggesting that an imidazole ring is involved in the active center.

The amidase and esterolytic activities of the spinach leaf leucine specific proteinase are essentially insensitive to some cations (Ca^{2+}, Fe^{2+}, Cu^{2+}), chelating agents (ethylenediaminetetraacetate disodium salt: EDTA) and reduced and oxidized sulfydryl group reagents (L-cysteine, p-chloromercuribenzoic acid), whereas they are sensitive to low concentrations (100 μM) of phenylmethylsulfonyl fluoride (PMSF).

The insensitivity to thiol reagents and cations, as well as the sensitivity to PMSF, suggests that the spinach leaf leucine specific proteinase contains a high nucleophilic serine residue at the active site.

The molecular weight of the spinach leaf leucine specific proteinase is 60,000±6,000 as measured by gel filtration on UltroPac TSK column (7.5x600 mm, G2000 SW, purchased from LKB, Bromma, Sweden). The presence of more than one active site in the macromolecule cannot be excluded; also the possibility that the enzyme is in an aggregation state cannot be ruled out. In these respects, it is of interest to recall that several plant and animal serine proteinases, as human urokinase, either with similar or higher molecular weights, are composed of a single polypeptide chain with only one active site (Ryan and Walker-Simmons, 1981; Ascenzi et al., 1982).

Serine and sulfydryl plant proteinases: a brief comparison

All known animal and plant serine proteinases are thought to employ a charge relay system to hydrolyze both natural and synthetic substrates, although this is not entirely accepted (Kraut, 1977; Steitz and Shulman, 1982). In this system, Ser195 hydroxyl group becomes highly nucleophilic through hydrogen bonding with the side chain of His57 which is also hydrogen-bonded with the carboxyl of Asp102. During catalysis the appropriate side chain of an internal amino acid at the P_1 site of the substrate fits into the principal specificity site (or pocket or cleft) or the enzyme near Ser195 and the nucleophilic serine oxygen attacks the carbonyl carbon of the scissile peptide bond. A tetrahedral intermediate results that is subsequently converted to an acyl enzyme and finally hydrolyzed to products (see: Kraut, 1977; Steitz and Shulman, 1982).

Also plant sulfydryl proteinases employ a charge relay system to hydrolyze substrates (see: Lowe, 1977; Ryan and Walker-Simmons, 1981; and references cited therein). In this system Ser195, present in serine proteinases, is replaced by the highly nucleophilic Cys25 which is in a grove close to the imidazole ring of His159. The negative charge of the side chain of Asp158 promotes removal of the charged products of hydrolysis.

As expected from the presence of different nucleophilic residues at the active site of serine and thiol proteinases, these enzymes show different reactivities towards specific sulfydryl and hydroxyl reagents. On the other hand, they show the same reactivity towards histidyl reagents.

In general, three classes of specificities are ascribed to serine proteinases: trypsin-like enzymes that split proteins at internal peptide bonds where lysine or arginine occupy the P_1 residue of the substrate; chymotrypsin-like proteinases that cleave at bulky hydrophobic P_1 residues of the substrate (i.e., tyrosine, phenylalanine and tryptophan); and elastase-like proteinases that cleave the alanine residue if present at the P_1 site. In this respect, the spinach leaf leucine specific serine proteinase should be considered the prototype of a new class of specificity among serine proteinases.

Papain, ficin and bromelains show a broader specificity as compared to that of serine proteinases. In this respect papain hydrolyzes amides of N-α-benzoxyl-L-arginine and L-lysine readily and those of glutamine, histidine, glycine and tyrosine at reduced rates (see: Lowe, 1977; and references cited therein). However, the enzyme splits peptide bonds having a wide variety of amino acids at the carboxyl, or P_1, site of the scissile peptide bond, including alanine, asparagine, serine, glycine, histidine, aspartic acid and leucine. Different specificities of serine and thiol proteinases are also reflected in the different binding of natural and synthetic inhibitors (Ryan, 1981; Laskowski and Kato, 1980).

In spite of different specificities, serine proteinases exhibit generally pH optima around 8 (Kraut, 1977; Steitz and Shulman, 1982). On the other hand, thiol proteinases show a broad pH optimum, ranging between 5 and 8 (Lowe, 1977). Different effects of pH on the catalytic steps for the serine and thiol proteinase catalyzed hydrolysis of synthetic substrates are known. In this framework, the acylation step (k_{+2}) is rate limiting in the serine proteinase catalyzed hydrolysis of synthetic substrates at pH values higher than 5. On the other hand, in thiol proteinases the deacylation step (k_{+3}) seems to be rate limiting in catalysis around neutrality. Thus, in the

ficin-catalyzed hydrolysis of N-α-benzoylglycine p-nitrophenyl ester, k_{+2} is rate limiting at acid pH values (3.9-5.9) whereas, at pH values higher than 5.9, k_{+3} becomes rate limiting in catalysis (Hollaway et al., 1971). Since, the effect of the side chain of (L-)amino acids on the proteinase catalyzed hydrolysis of synthetic and natural substrates can only be detected under conditions where k_{+2} is rate limiting in catalysis, the findings reported above suggest that serine and thiol plant proteinases could be differently distributed in the same plant tissue. In this respect, it is known that in plant cells areas at different pH values (vacuole and cytoplasm) are present and must play a role in the regulation of metabolism, as many enzymatic activities are so sensitive to pH (Smith and Raven, 1979).

A direct study of the relations between proteinase compartmentation and different pH values of cell organelles should be of wide interest for a better knowledge of the role of both serine and thiol proteinases in cellular processes and plant metabolism.

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DISCUSSION

STEPANOV:

Was the used spinach leaf proteinase purified?

ADUCCI:

The leucine specific spinach leaf proteinase has only been partially purified by ion exchange chromatography. The data presented have been obtained either with the crude juice or with the partially purified enzyme.

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SUBJECT INDEX

MEMBRANE DYNAMICS AND TRANSPORT OF NORMAL AND TUMOR CELLS

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