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THE INTERACTION OF A TRYPSIN-DEPENDENT NEUTRAL PROTEASE AND ITS INHIBITOR FOUND IN TUMOUR CELLS

ANALYSIS OF COMPLEX KINETIC DATA INVOLVED IN A THIOL-DISULPHIDE EXCHANGE MECHANISM *

F.S. STEVEN, V. PODRAZKÝ and S. ITZHAKI

Department of Medical Biochemistry, Stopford Building, University of Manchester, Manchester M13 9PT (U.K.)

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Summary

Ehrlich ascites tumour cells contain a granule-derived zymogen which on trypsin activation yields a collegenolytic neutral protease. The preparation of the granule fraction by subcellular fractionation procedure results in the preparation of a second fraction referred to as the post-granule supernatant fraction. The post-granule supernatant fraction contains a latent form of the granule-derived neutral protease and an excess of cytoplasmic inhibitor for this enzyme. The inhibitor of neutral protease is also capable of inhibiting trypsin and in each case the chemical mechanism of enzyme · inhibitor complex formation has been shown to be a reversible thiol-disulphide exchange. The post-granule supernatant fraction exhibited complex kinetic data when the interactions between the inhibitor, the latent enzymes and trypsin were examined simultaneously by incremental analysis. The data were interpreted and quantitatively analysed by computer analysis. It was demonstrated that the conventional types of analysis could not have provided meaningful interpretations of the experimental data provided by these complex-interacting systems.

Introduction

Ehrlich ascites cells grown in mice [1] have been shown to contain the zymogen of a trypsin-dependent neutral protease located in the granule frac-

* Supplementary data to this article, giving details of mathematical analyses of fluorescent-labelled polymeric collagen fibrils as substrate, are deposited with, and can be obtained from: Elsevier Scientific Publishing Company, BBA Data Deposition, P.O. Box 1527, Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/072/68425/524 (1978) 170.

tion. This enzyme has been shown to cleave the telopeptide regions of tropocollagen molecules within polymeric collagen fibrils and can be followed by a sensitive fluorimetric assay [2] in a similar manner to trypsin [3] and the neutral protease derived from the granules of polymorphonuclear leucocytes [3]. Sonicates of these tumour cells contained a potent inhibitor of both the granule neutral protease and of trypsin [4]. During sonication, some of the granule zymogen escaped from disrupted granules and passed into the cytoplasm so that the resultant post-granule supernatant fraction [4] contained a mixture of the inhibitor plus a latent form of the neutral protease.

In this study, the complex kinetic data exhibited by the interaction of the tumour neutral protease and its inhibitor will be described and quantitatively analysed employing fluorescein-labelled polymeric collagen fibrils as substrate. This system can only be demonstrated when an activating agent such as trypsin is included in the assay [4]. It is considered that polymeric collagen fibrils approximate more closely to the substrate encountered physiologically during tumour cell migration than the more commonly used substrates such as synthetic amides, esters and peptides used for trypsin-like enzyme assays. Identical results were obtained in a parallel study in which casein was used as substrate and the course of enzymic digestion followed by fluram analysis of the solubilised products [5,6]. Recently, Sellers et al. [7,8] have suggested that extracellular latent collagenase was in fact an enzyme · inhibitor complex which could be re-activated by inclusion of a thiol-blocking agent in the test system or by treatment with trypsin or chymotrypsin which destroyed the inhibitor. The evidence presented in this paper demonstrates a similar re-activation of neutral protease by means of the mercurial drug mersalyl. We have defined the chemical nature of the interaction between the neutral protease and the inhibitor as a thiol-disulphide exchange reaction. This exchange mechanism also explains the re-activation of neutral protease by trypsin and chymotrypsin [9] and the inhibition of trypsin by the tumour inhibitor and other thiols [9].

Materials and Methods

Ehrlich ascites cells were grown in mice, collected, washed and sonicated in 0.34 M sucrose [4]. Normally, 10 mice provided approx. 10^{10} cells. Subcellular fractionation of the sonicate consisted of a low speed centrifugation step ($600 \times g$ for 10 min) to remove undisrupted cells and cell debris. The supernatant from this operation was then centrifuged at $20\,000 \times g$ for 15 min to produce a pellet of granules and the post-granule supernatant fraction [10]. The granule fraction contained a zymogen of neutral protease extractable in 10 ml 0.5 M KCl; the post-granule supernatant fraction (60 ml) contained excess inhibitor and a latent form of the neutral protease.

Crystalline trypsin (EC 3.4.31.4), previously treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (Tos-PheCH_2Cl), was obtained from Worthington Biochemical Corp. with 244 units/mg trypsin activity [11]. Crystalline chymotrypsin (EC 3.4.21.1) was obtained from Sigma, 45 units/mg [11].

Mersalyl was supplied by Evans Medical Ltd., Liverpool, as a solution con-

taining 10% (w/v) of the mercurial. Cysteine hydrochloride and dithiothreitol were both purchased from B.D.H.

Enzyme assays. Two techniques were employed in this study. The most convenient proteolytic assay system for these kinetic studies was found to be the solubilisation of fluorescein-labelled telopeptides from polymeric collagen fibrils [2,3]. The basic assay system consisted of a series of screw cap plastic tubes each containing 7.0 mg substrate/5.0 ml buffer, pH 7.5 (50 mM Tris/ 40 mM EDTA/500 mM KCl). Appropriate additions of enzyme fraction, trypsin, inhibitor and other reagents (see figure legends for details) were made, the tubes were equilibrated in a shaking water bath at 37°C. At suitable time intervals 100-μl samples were withdrawn by microsyringe from each tube and mixed with 3.0 ml distilled water prior to fluorimetric analysis [2,3].

It was found that conventional analysis of the proteolytic activity of the post-granule supernatant fraction produced misleading results. This problem was overcome by applying incremental analytical techniques in which a range of added post-granule supernatant fraction concentrations were analysed simultaneously: the resulting analytical data were plotted and submitted to computer analysis [12].

In order to check the validity of the incremental analyses obtained with fluorescein-labelled polymeric collagen fibrils as substrate a second assay system was developed employing casein and reduced casein as substrates. In this system, the enzymic digestion was terminated after 2 h by addition of trichloroacetic acid to yield a final concentration of 5% (w/v). After a period of 18 h standing at 20°C, the solubilised peptides were filtered and 10 μl of each filtrate assayed with fluram in 1% $NaHCO_3$ [5,6].

Assay of granule extract. The granule extract was assayed for neutral protease activity (a) in the absence of trypsin or chymotrypsin and (b) in the presence of these enzymes. The effect of adding 4 mM cysteine and a range of mersalyl concentrations to the trypsin-activated neutral protease of the granule fraction was also examined.

Post-granule supernatant fractions I and II. Data from two different post-granule supernatant fractions are presented in this study. We have referred to these as I and II for convenience in the text and legends. Incremental analyses of the post-granule supernatant fractions were carried out with the basic assay system (see Methods) plus a known quantity of added trypsin (required to activate the latent neutral protease). A series of control incremental analyses were carried out in which the trypsin was omitted in order to detect the presence of free neutral protease activity which did not require enzymic activation. In all cases these controls demonstrated the absence of neutral protease activity. Incremental analyses were also performed, as above, with the inclusion of 0.12 μM mersalyl and a combination of 0.12 μM mersalyl plus 4 mM cysteine adjusted to pH 7.4.

Reduction and re-oxidation of post-granule supernatant fraction II. The reversible thiol-disulphide exchange between the trypsin-dependent neutral protease and its inhibitor was studied by incubating the post-granule supernatant with dithiothreitol (final concentration 5 mM) at 20°C for 1 h followed by dialysis and incremental analysis under the standard assay conditions. A sample of the reduced post-granule supernatant fraction was allowed to stand

at 20°C for 24 h with occasional shaking in an open beaker to facilitate atmospheric re-oxidation of the reduced material. Incremental analysis of the re-oxidised post-granule supernatant fraction was then carried out.

Results and Discussion

Results are presented in terms of proteolytic activity expressed as a percentage of the added internal standard trypsin and also as units of trypsin activity [11]. Each graph is a composite plot obtained from at least five similar experiments and many of the individual points have been omitted to simplify the presentation of data. The intervals between the points are indicated in the legends for each figure and the accuracy of each plot is within ±3% over the range of added granule fraction, post-granule supernatant fraction and mersalyl addition stated in the legends of the figures.

Analysis of the granule extract

The granule extract was shown to contain a trypsin-dependent neutral protease capable of cleaving the telopeptide regions of fluorescein-labelled polymeric collagen fibrils [3] (Fig. 1). This neutral protease activity could also be activated by addition of chymotrypsin in place of trypsin, but was completely inhibited in the presence of 4 mM cysteine or by pre-incubation with dithiothreitol followed by dialysis prior to assay. The added cysteine (4 mM)

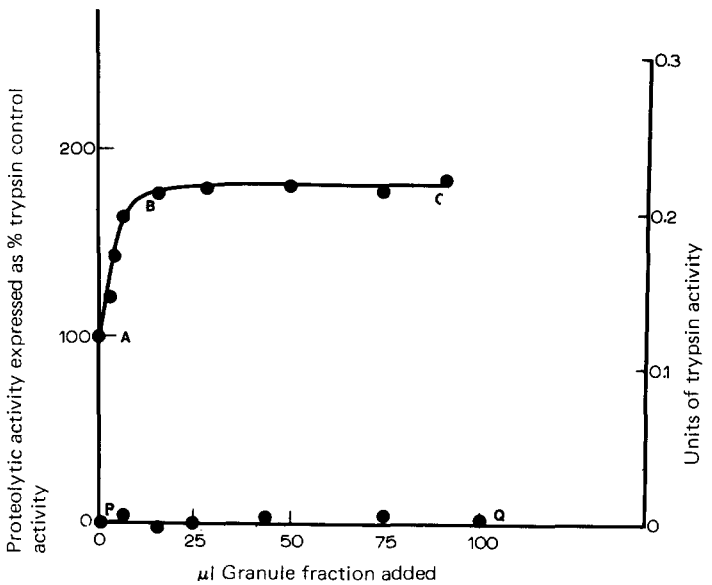

Fig. 1. Trypsin-dependent neutral protease of the granule extract. The assay system contained 7.0 mg fluorescein-labelled polymeric collagen fibrils, 0.5 μg trypsin, 5.0 ml buffer plus 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 50, 75, 100 μl of added granule extract. 100-μl aliquots from each reaction tube were removed after 1 and 2 h for fluorimetric analysis and the results presented as a composite plot with the trypsin alone represented by 100%. The curve ABC represents trypsin-dependent neutral protease activity in the presence of 0.5 μg trypsin, the line PQ represents the lack of neutral protease activity in the absence of trypsin.

had no detectable effect on the trypsin activity included in the test system for the assay of the trypsin-dependent neutral protease. This evidence suggested that the trypsin-dependent neutral protease contained an essential disulphide bond required for the maintenance of the active conformation of this enzyme. In contrast, addition of 3 μl mersalyl, to provide a final concentration of 0.12 μM (see below) to the granule extract had no effect on the neutral protease or on the activity of the included trypsin. From this evidence it was concluded that the trypsin-dependent neutral protease extracted from the granules was activated by tryptic modification of a zymogen to produce the active enzyme. Thus any effect which mersalyl is shown (see later) to have on the post-granule supernatant fraction proteolytic activity must be confined to the action of mersalyl on the free inhibitor and on the bound inhibitor held in the form of enzyme · inhibitor complex.

Analysis of the post-granule supernatant fractions I and II

Six different preparations of post-granule supernatant fractions were examined in this study. In each case a complex pattern of interaction between the trypsin and the substrate, trypsin plus neutral protease and the inhibition of both enzymes was observed over the period 1—6 h. Data from two of these preparations (I and II) will be presented here.

A typical set of data obtained over 3 h digestion of fluorescein-labelled polymeric collagen fibrils is presented in Fig. 2 for preparation I. The solubilisation of fluorescein-labelled telopeptides by the trypsin control in the absence

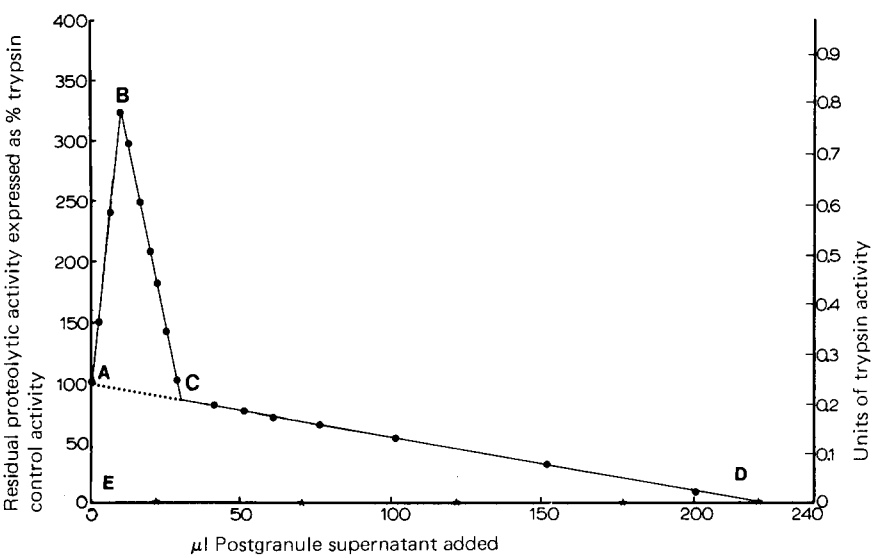

Fig. 2. Incremental analysis of post-granule supernatant fraction I. The graph ABCD represents the solubilisation of fluorescein-labelled telopeptides from the fluorescent substrate by both trypsin and neutral protease activity observed when increasing quantities of post-granular supernatant fraction I were added to the substrate plus 1.0 μg trypsin (0.244 unit) in 5 ml buffer incubated over 3 h. The results are presented as a composite plot with 1.0 μg trypsin alone represented by 100%. The line ED, following the abscissa, represents a further set of control experiments in which no trypsin was added.

of added post-granule supernatant fraction is represented by 100% at the point A on Fig. 2. A linear decline of product formation [4] was observed with added post-granule supernatant fraction over the volume range 30—220 μl (line CD in Fig. 2) with complete inhibition of the added trypsin at 220 μl post-granule supernatant fraction indicated by the point D. This linear relationship (CD) is extrapolated over the region 0—30 μl as the dotted line AC to join the axis at A, at which point the trypsin had 100% activity and no post-granule supernatant had been added. It is evident that the line AD represents the progressive inhibition of the added 1 μg trypsin (0.244 unit [11]) by increasing quantities of the inhibitor in the added post-granule supernatant fraction.

The marked increase in product formation over the range AB (Fig. 2) represents the solubilisation of the substrate by increasing amounts of measurable neutral protease activity added with increasing quantities of post-granule supernatant fraction added to the original 1 μg of trypsin in the test system. We use the word "measurable" to represent the sum of latent neutral protease and active neutral protease which is capable of attacking the substrate in the presence of the added trypsin and the inhibitor present in the added post-granule supernatant fraction. The trypsin concentration controls the percentage of this "measurable" neutral protease activity which is actually active as neutral protease (see later and also see region BC in Fig. 2).

In Fig. 2, the maximum solubilisation of fluorescein-labelled telopeptides was observed with 9 μl post-granule supernatant fraction added to 1 μg trypsin. In the absence of added trypsin, 9 μl of post-granule supernatant had no action on the substrate, emphasising the requirement of trypsin for the demonstration of neutral protease activity in the post-granule supernatant fraction. On increasing the volume of post-granule supernatant fraction from 9 to 30 μl, the resultant neutral protease activity exhibited a marked decline which was linearly related to the amount of added post-granule supernatant over the range BC in Fig. 2. The region BC is the result of the trypsin-activated neutral protease becoming increasingly inhibited by further additions of inhibitor for neutral protease in the post-granule supernatant fraction. It is clear that the slope of the line BC is markedly different from the slope of the line ACD. Since the line ACD represents the inhibition of trypsin, the line BC must represent the summation of the inhibition of both trypsin and the neutral protease in the presence of increasing quantities of inhibitor. Further experiments demonstrated that the failure to observe neutral protease activity in the region CD is controlled by the presence of excess inhibitor rather than the lack of trypsin required to activate latent neutral protease (Steven, F.S. and Podrazký, V., unpublished data).

Quantitation of data

The complex kinetic data obtained in this study clearly indicate the problems which may be encountered when conventional analysis of proteolytic activity is attempted with such a complex system as the post-granule supernatant fraction. For example, no two chosen volumes of post-granule supernatant fraction, when submitted to the normal assay procedures, would provide confirmatory results for either the enzyme or inhibitor content. We have resolved this problem by combining the data obtained from incremental

TABLE I

QUANTITATION OF NEUTRAL PROTEASE AND INHIBITOR PRESENT IN A TYPICAL PREPARATION OF MOUSE EHRLICH ASCITES TUMOUR SONICATE (FIG. 2) OBTAINED FROM APPROX. $1.2 \cdot 10^{10}$ CELLS

Units of enzyme activity expressed as trypsin units [11] or units/mg protein [13].

Preparation	Volume (ml)	Neutral protease activity		Inhibitor	
		(a) Total units	(b) Units/mg protein	(a) Total units	(b) Units/mg protein
Post-granule supernatant fraction I	60	1290	4.8	420	1.6
Granule extract	10	600	15.8	0	0

analysis with a computer analysis [12] and a typical analysis is presented in Table I. These quantities are expressed in equivalent trypsin units [11] and the inhibitor is also quantitated in the same manner, one unit of inhibitor being defined as sufficient inhibitor to inhibit one unit of trypsin activity. These values are expressed as units of enzyme or inhibitor per mg of protein [13].

The significance of this incremental analysis is simply that the technique allows all components of the test system to be analysed quantitatively [12]. It has the added advantage that the effect of agents such as mersalyl and cysteine on the components of the post-granule supernatant fraction can be unambiguously defined, since each of these components is represented by one of the straight lines obtained from a plot of the experimentally determined values (Fig. 2). The addition of an internal control (trypsin) to each assay enables the data obtained over a period of 3 h to be presented as a composite plot in which the control represents 100% of the initial trypsin activity in the absence of neutral protease or inhibitor activity.

Effect of increasing concentrations of mersalyl on fixed quantities of post-granule supernatant fraction II

The effect of mersalyl addition to the test system is shown in Fig. 3 (see legend for details). The maximal effect of mersalyl re-activation of neutral protease was achieved at 3 μl or 0.12 μM concentration of the mercurial. It had previously been shown that the concentration of mersalyl had no effect on the trypsin-activated neutral protease obtained from the granule fraction, nor did the drug have any effect on the control trypsin.

Effect of 0.12 μM mersalyl on the incremental analysis of post-granule supernatant fraction II. The result of this analysis was a progress curve similar to Fig. 1 and is not shown in order to conserve space. No inhibition of the re-activated neutral protease in the presence of excess post-granule supernatant fraction was observed. Thus all the inhibitory action observed in Fig. 2 had been destroyed by the addition of mersalyl.

Effect of 0.12 μM mersalyl plus 4 mM cysteine on the incremental analysis of post-granule supernatant fraction II. When incremental analysis was carried out in the presence of 0.12 M mersalyl plus 4 mM cysteine, the composite plot obtained after 0.5, 1.0, 1.5 and 2 h is presented in Fig. 4. It can be seen that

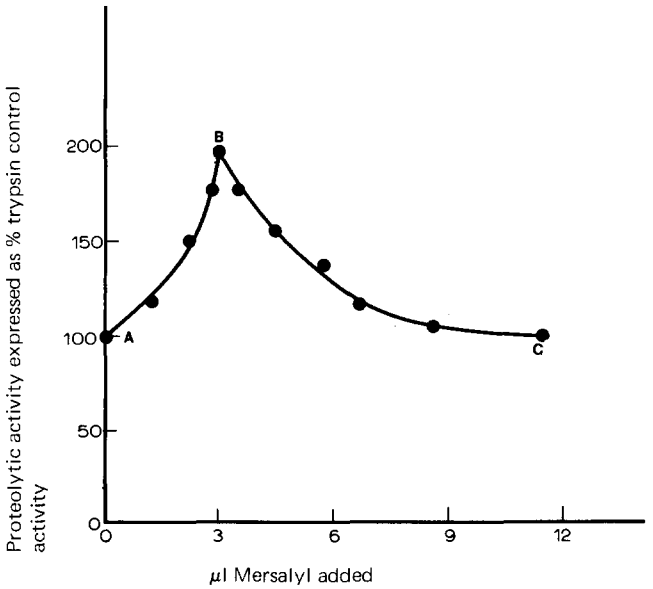

Fig. 3. Effect of increasing concentrations of mersalyl on fixed quantities of post-granule supernatant fraction II. This is a composite plot obtained with 3-, 15- and 30-μl quantities of post-granule supernatant employing a range of mersalyl concentrations. In each case the maximum re-activation of the trypsin-dependent neutral protease activity took place with 3 or 0.12 μM mersalyl. The point A represented as 100% is the resultant proteolytic activity observed when 0.5 μg trypsin was incubated with 3, 15 or 30 μl post-granule supernatant fraction in the absence of mersalyl.

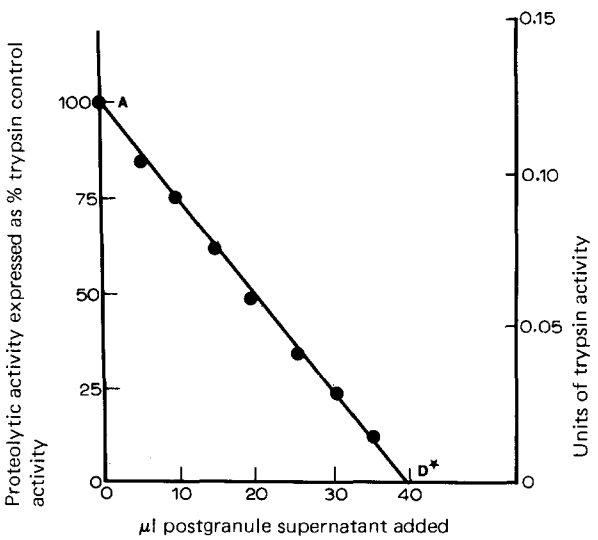

Fig. 4. Incremental analysis of post-granule fraction II plus cysteine. The basic test system contained 0.5 μg trypsin, 3 μl mersalyl and cysteine to produce a final concentration of 4 mM cysteine plus increasing quantities of post-granule supernatant fraction. The results, presented as a composite plot over a 2 h incubation period, are expressed as a percentage of the original trypsin activity in the absence of added post-granule supernatant fraction.

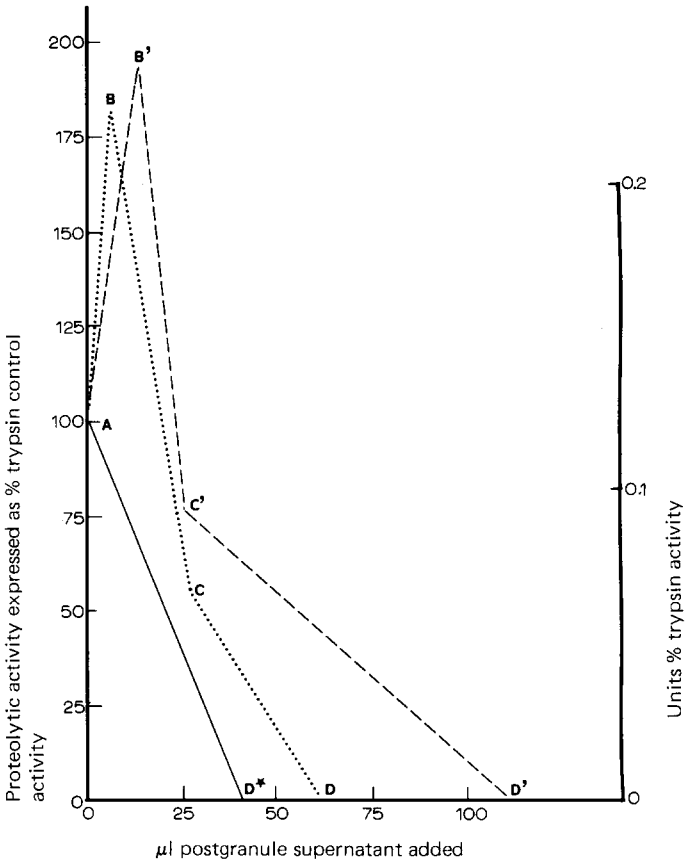

Fig. 5. Incremental analysis of post-granule supernatant fraction II, reduced fraction II and re-oxidised fraction II. The basic test system contained 0.5 μg added trypsin plus a range of post-granule supernatant fraction II (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 75, 100, 125 μl). The results are presented as composite plots obtained over a 2 h incubation period and are expressed as percentages of the activity of the 0.5 μg trypsin alone, denoted by 100% at point A. The dotted plot ABCD represents the original post-granule supernatant fraction, the linear plot AD* represents the reduced fraction II and the dashed plot AB'C'D' represents the regain of original properties after re-oxidation of fraction II.

the mersalyl re-activation of neutral protease (reported above) has been abolished by the addition of cysteine and all that remains in Fig. 4 is the modified inhibition line AD*, denoting inhibition of trypsin by the added post-granule supernatant. At 4 mM cysteine addition the mercurial mersalyl is inactivated and can no longer re-activate the latent neutral protease. At the same time the cysteine reacts with the trypsin-activated neutral protease (region AC, Fig. 2) to form an inert, reduced from of the enzyme, as previously shown with the granule extract and cysteine (see above). Cysteine could be replaced by dithiothreitol, glutathione and mercaptoethanol at suitable concentrations with identical results.

Incremental analysis of reduced and re-oxidised post-granule supernatant fraction II. Incremental analysis was carried out on (a) freshly prepared post-granule supernatant fraction, (b) reduced and (c) re-oxidised samples and the composite plots presented in Fig. 5. In each case 0.5 μg of trypsin was included

in each tube. It can be seen that the reduced post-granule supernatant fraction demonstrates the complete absence of neutral protease activity whilst the trypsin inhibition was exhibited over the whole range of post-granule supernatant added. After atmospheric re-oxidation, the post-granule supernatant fraction regained the typical plot of the original material. The peak of neutral protease activity in the re-oxidised post-granule supernatant fraction moved to a greater volume of added post-granule supernatant fraction than in the original material. The quantity of re-oxidised post-granule supernatant fraction required to inhibit both neutral protease and added trypsin was also greater than in the original material (Fig. 5). These two increases denote a decline in inhibitor activity in the re-oxidised as compared to the original fraction. This could well be explained by atmospheric oxidation of the reactive thiol essential for inhibitory activity.

The interaction of the inhibitor with the neutral protease is a reversible process as indicated by the re-activation of the enzyme by mersalyl and the data presented in Fig. 5. These facts can be correlated to define the chemical mechanism of the enzyme · inhibitor complex formation by means of a reversible thiol-disulphide exchange reaction as illustrated in Fig. 6. The individual reactions are numbered for ease of description. The thiol of cysteine (4 mM) disrupts a significant disulphide bridge (I) essential for the maintenance of the conformation of the neutral protease in an enzymically active form. The inhibitor contains an essential thiol group (II) capable of disulphide exchange with the significant disulphide bridge of the neutral protease, resulting in the formation of a disulphide bond between the enzyme and the inhibitor to produce the inactive enzyme · inhibitor complex (II). This complex lacks enzymic activity since the significant disulphide bridge for enzymic activity has now been cleaved by a disulphide exchange reaction (this type of inhibition is shown in Figs. 2 and 5, line BC). The action of the mercurial, mersalyl (abbreviated to R-Hg-OH in Fig. 6) is to disrupt the inactive enzyme · inhibitor complex (III) with subsequent re-activation of the neutral protease activity and inactivation of the inhibitor (IV). Such an explanation of the observed re-activation would also fit the results reported by Sellers et al. [7,8] for the activation of a latent form of collagenase by mercurials.

In the scheme presented in Fig. 6, the excess inhibitor present in the post-granule supernatant fraction would also react with mersalyl to form an inactive complex (IV). Addition of cysteine to the mersalyl re-activated neutral protease destroys the effect of mersalyl (see Fig. 4) and is shown in reaction V: the added cysteine also destroys the neutral protease activity by complex formation (VII). Thus the result of adding mersalyl plus 4 mM cysteine is to leave the inhibitor (VI) and trypsin as the only species which can now combine and be measured in the assay (Fig. 4). It has previously been pointed out that trypsin was not directly affected by either mersalyl or by 4 mM cysteine.

The proliferation of tumour cells is thought to be mediated by proteolytic enzymes [14,15] and it has been claimed that cytoplasmic inhibitors of neutral proteases cause the regression of certain types of tumour [16]. One approach to gaining an understanding of cellular control mechanisms involved in tumour growth is to attempt to isolate and characterise these cytoplasmic inhibitors. The present paper suggests that a simpler approach to the control of neutral

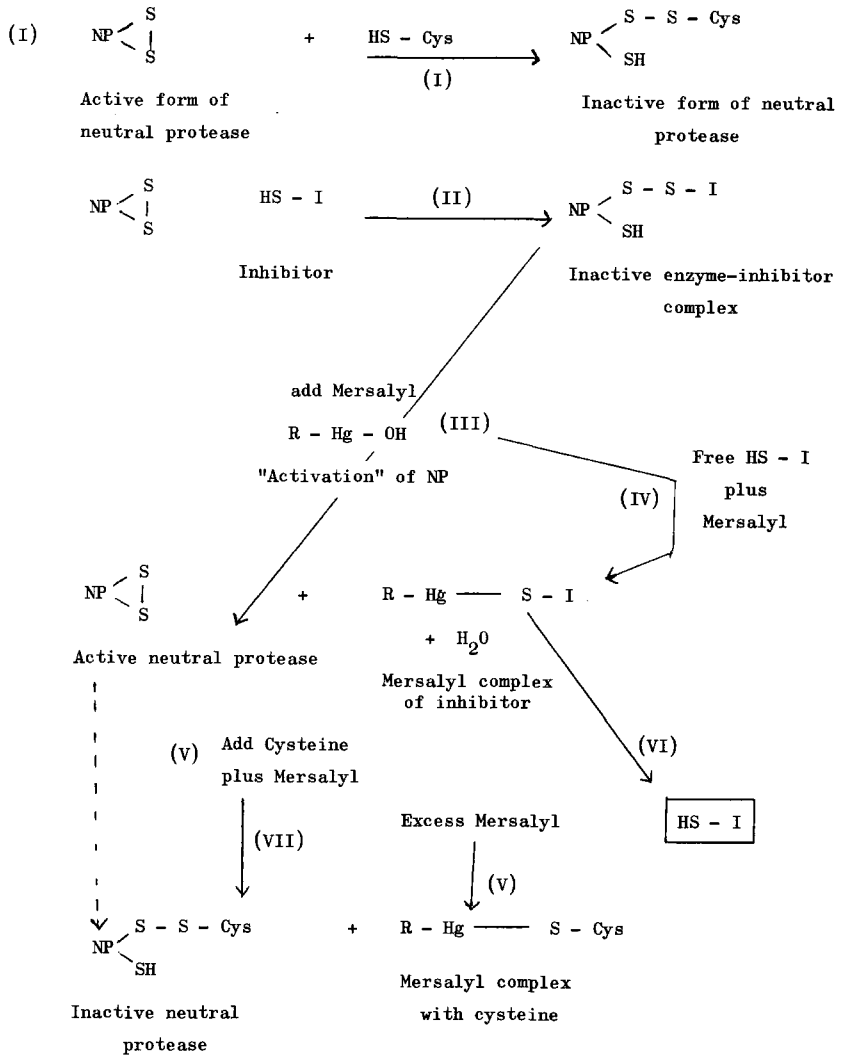

Fig. 6. Proposed mechanism for thiol-disulphide exchange described in the text and figures.

protease activity might be by means of regulating the extracellular concentration of thiol agents (Fig. 5).

It was of interest to observe that soya bean trypsin inhibitor could not be dissociated from trypsin by the addition of mersalyl or cystine which would suggest an entirely different mechanism of enzyme-inhibitor binding from that described in this study.

The suggestion that latency [7,8], associated with certain proteolytic enzymes, may be due to enzyme · inhibitor complexes has now been confirmed for the tumour neutral protease and its cytoplasmic inhibitor. The mechanism of inhibition has now been defined as a reversible thiol-disulphide exchange, the inhibitor carrying a reactive thiol group which can exchange with a significant disulphide bond within the neutral protease. The disulphide

linkage is readily reduced and re-oxidised in a reversible manner with the corresponding changes to inactive and active forms of the enzyme. Trypsin has also been shown to possess a similar significant disulphide bond, cleaved by higher concentrations of thiols [9] than are required to disrupt the disulphide bond of the tumour neutral protease described above. The inhibition of trypsin by thiols was demonstrated to exhibit similar enzyme kinetics to those exhibited by the neutral protease although the disulphide bond of the neutral protease was cleaved at lower concentrations of thiols and inhibitor (see Fig. 2) than was that of trypsin.

The fact that the highly purified crystalline enzyme trypsin behaved in the same manner as the crude neutral protease in the presence of (a) thiols; (b) the inhibitor, or (c) disulphide protecting agents such as cystine and oxidised glutathione [9], demonstrates the validity of the results obtained with the crude neutral protease-containing extracts used in this study. In spite of the lack of a pure enzyme and inhibitor being available, the mechanism of inhibition can be elucidated by means of kinetic analysis. It is also of interest that the inhibitor can be selectively adsorbed from the post-granule supernatant fraction employing oxidised glutathione affinity columns (Steven, F.S. and Podrazký, V., unpublished data).

We feel the application of incremental analysis, employing fluorescein-labelled polymeric collagen fibrils as substrate to these complex reactions has enabled us to define the chemical mechanism involved in the formation of the enzyme · inhibitor complex. Such an analysis would not have been possible by means of conventional analytical techniques which depend upon the repeated analysis of multiple samples using a predetermined fixed volume of enzyme-containing solution to be assayed. These incremental analyses have now been confirmed with casein, in place of polymeric collagen fibrils, as substrate (Steven, F.S. and Podrazký, V., unpublished data), invalidating the possible criticism that the fluorescein-labelled polymeric collagen fibrils employed in the present study were an unsuitable substrate.

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