Biochimica et Biophysica Acta, 568 (1979) 49—58

BBA 68734

THE REVERSIBLE THIOL-DISULPHIDE EXCHANGE OF TRYPSIN AND CHYMOTRYPSINOGEN WITH A TUMOUR-DERIVED INHIBITOR

KINETIC DATA OBTAINED WITH FLUORESCEIN-LABELLED POLYMERIC COLLAGEN FIBRILS AND CASEIN AS SUBSTRATES

FRANK S. STEVEN and VLADIMIR PODRAZKÝ *

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

(Received October 9th, 1978)

Key words: Trypsin, Chymotrypsinogen, Inhibitor complex, Disulfide exchange

Summary

Ehrlich ascites cells contain a cytoplasmic inhibitor of both trypsin and the granule neutral protease and possess a reactive thiol which interacts with an important disulphide bond in trypsin, resulting in the formation of the trypsin-inhibitor complex. When a fixed quantity of trypsin was completely inhibited by addition of the cytoplasmic inhibitor, the trypsin could be re-activated by the addition of either trasylol-trypsin or chymotrypsinogen. Since trasylol-trypsin, chymotrypsinogen (and any derived chymotrypsin) has no ability to solubilise fluorescein-labelled peptides from the substrate, the appearance of trypsin activity was probably due to a non-enzymic exchange reaction, in which these inactive forms displaced trypsin from the trypsin-inhibitor complex. Kinetic data suggest that this displacement was a time-dependent equilibrium reaction controlled by the relative concentration of the reacting species.

Introduction

We recently demonstrated the presence of a trypsin-dependent neutral protease and its cytoplasmic inhibitor in Ehrlich ascites tumour cells [1]. The inhibitor carries a reactive thiol group which interacts with an important disulphide bond in the neutral protease, resulting in the formation of an inactive enzyme-inhibitor complex, by means of a thiol-disulphide exchange reac-

* On leave of absence from the Research Institute of Food Industry, Prague, Czechoslovakia.

tion [2,3]. The inhibitor was prepared by a subcellular fractionation procedure applied to sonicated tumour cells [4]. This provided a granule fraction and a post-granule supernatant fraction. This post-granule supernatant fraction contained the inhibitor as well as the trypsin-dependent neutral protease [2].

Complex kinetic data were obtained when a known quantity of trypsin was added to increasing quantities of post-granule supernatant in the presence of a fixed quantity of substrate. The analysis of these data has been reported in detail [3,5]. Three characteristic regions of the data plots were defined as (a) the activation of a latent form of the trypsin-dependent neutral protease by the added trypsin: (b) the inhibition of both neutral protease and trypsin and (c) the inhibition of trypsin alone. We now wish to report that these complex plots of kinetic data can also be obtained when casein is used as substrate and the products of proteolysis followed by a fluorimetric assay procedure employing 'fluram' [6,7]. Since chymotrypsin is well-known to be able to digest casein it was necessary to demonstrate that an enzymically inactive form of chymotrypsin was capable of initiating the release of previously bound trypsin and neutral protease from their inhibitor complexes. This has been achieved in two ways, (1) with Tos-Phe-CH_2-Cl-inactivated chymotrypsin [2], and (2) with chymotrypsinogen followed by (i) the selective inhibition of the re-activated trypsin and followed by (ii) the selective inhibition of the re-activated neutral protease. Similar results were obtained when trasylol-trypsin was employed to release active trypsin and neutral protease from inhibitor complexes.

The results of this study are important in the context of one enzyme displacing a previously inactive enzyme from an enzyme-inhibitor complex which is a novel mechanism for the reversible inhibition of a proteolytic enzyme. The role of thiols and disulphides in the biological control of these reactions could be of considerable importance, for example, in tumour development; where it is claimed that certain tumours may be prevented from spreading by the presence of inhibitors of proteolytic enzymes [8].

Materials

Crystalline Tos-Phe-CH_2-Cl-trypsin, trypsin (EC 3.4.31.4) treated with Tos-Phe-CH_2Cl, containing 244 units enzyme/mg [9] and crystalline chymotrypsinogen A were purchased from Worthington Biochemical Corporation, NJ. Crystalline chymotrypsin (EC 3.4.21.1), 45 U/mg [9], was purchased from Sigma, London. The Kunitz trypsin-inhibitor was a gift from Bayer, F.R.G., (U.K. subsidiary, Haywards Heath, Sussex) and described as 'trasylol'. Trasylol-trypsin was prepared by reacting 100 μg trypsin with 1 ml trasylol (2000 K.I.U.) for 10 min at 20°C followed by exhaustive dialysis against distilled water at 4°C for 2 days. The source of the tumour inhibitor and neutral protease was the post-granule supernatant fraction which has been fully described [7,10]. Casein was obtained from Fisons Scientific Apparatus, and 'fluram' was purchased from Roche Products, London.

Methods

The assays of neutral protease and added trypsin were based upon the determination of fluorescein-labelled peptides solubilised from insoluble fluorescein-

labelled polymeric collagen fibrils, or on the assay of the fluorescent peptides produced by reacting 'fluram' with the trichloroacetic acid-soluble peptides derived from casein as the substrate. In each experiment, the incubation conditions were slightly different in order to achieve different objectives, thus only the basic concept of the methods will be described here.

The basic assay system employing fluorescein-labelled polymeric collagen fibrils contained 7 mg substrate, appropriate quantities of an activating enzyme (e.g trypsin) plus the postgranule supernatant fractions and buffer 50 mM Tris-HCl/500 mM KCl/40 mM EDTA (pH 7.5) to bring the total volume to 5 ml. The inclusion of EDTA was to ensure the complete inhibition of mammalian collagenase [11] which has been shown to be present in tumour cells [12]. After suitable periods of incubation at 37°C, the samples were centrifuged for 2 min., 100 μl of supernatant was mixed with 3 ml distilled water and the solubilised fluorescein-labelled peptides assayed by spectrofluorimetric analysis [7]. Immediately after the 100 μl samples had been removed, the samples were returned to the water bath for a further period of digestion. Control tubes were included which contained, (a) no added enzyme, (b) activating enzyme, and (c) activating enzyme plus sufficient post-granule supernatant to ensure total inhibition of the added activating enzyme.

Casein was also used as a substrate, solubilised in 0.01 M NaOH, 1% (w/v) $NaHCO_3$ at pH 7.5 adjusted with 0.1 M HCl. This solution was filtered and 1 ml added to each tube. Enzyme and post-granule supernatant were added and the mixture shaken at 37°C for the specified time. The reaction was then terminated by adding 1 ml 25% (w/v) trichloroacetic acid. The tubes were allowed to stand at room temperature for 18 h prior to filtration, 25-μl samples were removed mixed with 1 ml 1% (w/v) $NaHCO_3$ (adjusted to pH 8.4) and 0.5 ml acetone containing 300 μg 'fluram' added [6,7]. The contents of the tubes were rapidly mixed and the solubilised peptides estimated fluorimetrically.

Results and Discussion

The tumour neutral protease present in the granule fraction and the post-granule supernatant fraction required activation by trypsin or chymotrypsin [3]. Since both trypsin and these granule-derived neutral proteases are capable of cleaving only two fluorescein-labelled telopeptides per molecule of tropocollagen within the polymeric collagen fibrils [10], it was convenient to quantitate both these proteolytic enzymes by a common unit of enzymic activity. The unit of trypsin activity defined by Hummel [9] was chosen for this purpose, 1 μg of the trypsin being equivalent to 0.244 units trypsin activity. The plots of the experimental data are calibrated for proteolytic activity on two vertical axes, (1) the total proteolytic activity expressed as a percentage of the control (internal or external) of added trypsin and (2) the corresponding equivalent units of trypsin activity.

Re-activation of neutral protease in post-granule supernatant by trypsin addition

Fluorescein-labelled polymeric collagen fibrils as substrate. The proteolytic activity observed when a fixed quantity of trypsin (1 μg) was employed to

re-activate neutral protease, present in incremental additions of post-granule supernatant, is presented in Fig. 1.

Re-activation of neutral protease and trypsin by chymotrypsinogen addition

Casein as substrates. An excess of a post-granule supernatant was added to a fixed quantity of trypsin to completely inhibit the trypsin. This was to ensure that the re-activating agent (in this case, chymotrypsinogen) actually displaced the trypsin and neutral protease from the inactive enzyme-inhibitor complexes, rather than protecting the trypsin from inhibition (as would be the case if the chymotrypsinogen was added to the post-granule supernatant simultaneously or prior to the addition of trypsin to the test system). After 5 min, pre-incubation of the trypsin and post-granule supernatant, chymotrypsinogen and substrate were added and incubated at 37°C for 2.5 h. Analyses of samples removed from each tube resulted in the plot presented in Fig. 2.

Chymotrypsinogen alone had no action on casein; the action of 1 μg trypsin alone is shown as 100% for an external control containing no post-granule supernatant. When increasing amounts of chymotrypsinogen were added to the trypsin inhibitor complex, the total proteolytic activity of the test system increased markedly and then levelled off at higher concentrations of chymotrypsinogen (Fig. 2).

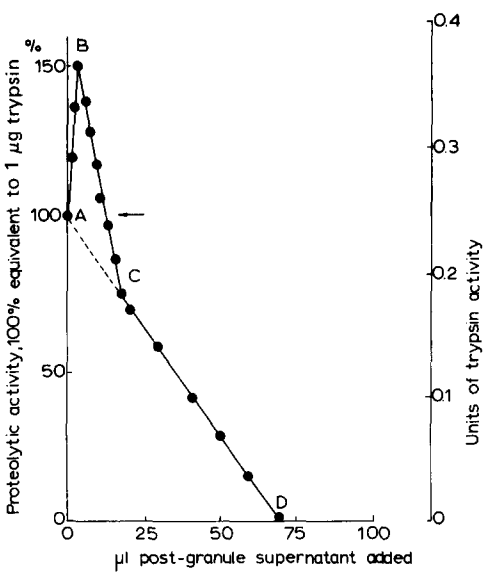

Fig. 1. Re-activation of neutral protease in post-granule supernatant by trypsin with fluorescein-labelled polymeric collagen as substrate. The basic assay system was used with 1 μg (0.244 units) of trypsin added to each tube with incremental additions of post-granule supernatant. The plot is a composite drawn from readings taken after 1 and 2 h, the accuracy of the analysis is within ±3%. The arrow marks the degree of proteolysis exhibited in the trypsin control and is represented by 100% on the left-hand vertical scale. The region AB represents trypsin activation of neutral protease from a latent form (either a zymogen or enzyme-inhibitor complex). The region BC represents net proteolytic activity of trypsin plus neutral protease when both enzymes are being inhibited by increasing quantities of inhibitor. The region CD represents the declining activity of trypsin alone in the presence of increasing concentrations of inhibitor.

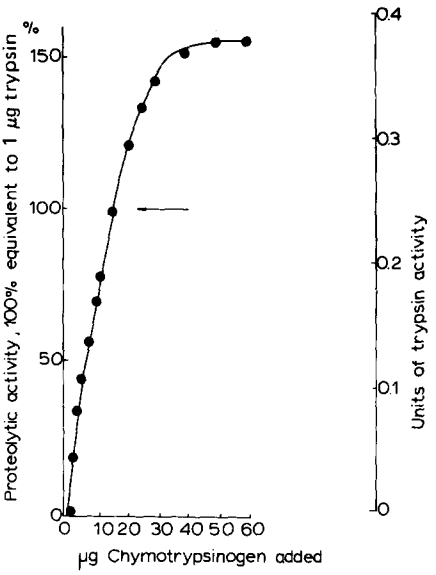

Fig. 2. Re-activation of neutral protease and trypsin by chymotrypsinogen with casein as substrate. The test system consisted of 1 µg trypsin plus 100 µl post-granule supernatant in 3 ml 1% $NaHCO_3$, 40 mM EDTA buffer (pH 7.5). After 2 min, incremental additions of chymotrypsinogen were made to each tube, followed by 1 ml casein solution (14.2 mg casein/tube). The tubes were incubated at 37° C for 2.5 h and the reaction stopped by the addition of 1 ml 25% (w/v) trichloroacetic acid. After 18 h, the contents of the tubes was filtered and 25 µl removed for 'fluram' analysis. The arrow represents the trypsin control.

Chymotrypsinogen may have displaced a portion of the bound trypsin and this free trypsin then may cause the conversion of chymotrypsinogen to the enzyme chymotrypsin. This chymotrypsin could then cause the observed solubilisation of casein (Fig. 2). It is probable that chymotrypsin may be a contributory factor. However, the levelling-off of product formation at high concentrations of added chymotrypsinogen is not consistent with this view, since it is known that approx. 1% of the available substrate was solubilised at this point (unpublished data).

Fluorescein-labelled polymeric collagen fibrils as substrate

Using fluorescein-labelled polymeric collagen fibrils as substrate, the kinetic data for the re-activation of trypsin and neutral protease by incremental additions of chymotrypsinogen are presented in Fig. 3. It can be seen that chymotrypsinogen re-activated previously inhibited proteolytic activity.

The data of Fig. 3 suggest that two factors influence the rate of exchange of previously-bound trypsin and neutral protease by added chymotrypsinogen. These factors are the concentration of the competitive chymotrypsinogen molecules and the time of incubation at 37° C. Higher concentrations of chymotrypsinogen and longer periods of incubation promote this exchange. It should be noted that chymotrypsinogen alone had no detectable action on this substrate.

The initial lag period (Fig. 3) observed over the first 2 h incubation prevents the test system being strictly compared to the control value of 100% trypsin

activity. In the latter case, there was no lag period and the enzyme was active throughout this period. This problem can be overcome by using a pre-incubation period prior to adding the substrate. However, this modification has the disadvantage that the time dependence of the exchange reaction (Fig. 3) cannot be observed (Fig. 4). An advantage is that the newly-released trypsin can be quantitated specifically.

Re-activation of trypsin and neutral protease after pre-incubation with chymotrypsinogen

Fluorescein-labelled polymeric collagen fibrils as substrate. Results of the re-activation of proteolytic activity by addition of chymotrypsinogen under three different experimental conditions are presented in Fig. 4. The difference between (a) and (b) in Fig. 4 should represent the quantity of trypsin re-activated by added chymotrypsinogen; if such a differential curve were plotted, this would approximate to curve (c). In fact, curve (c) was obtained

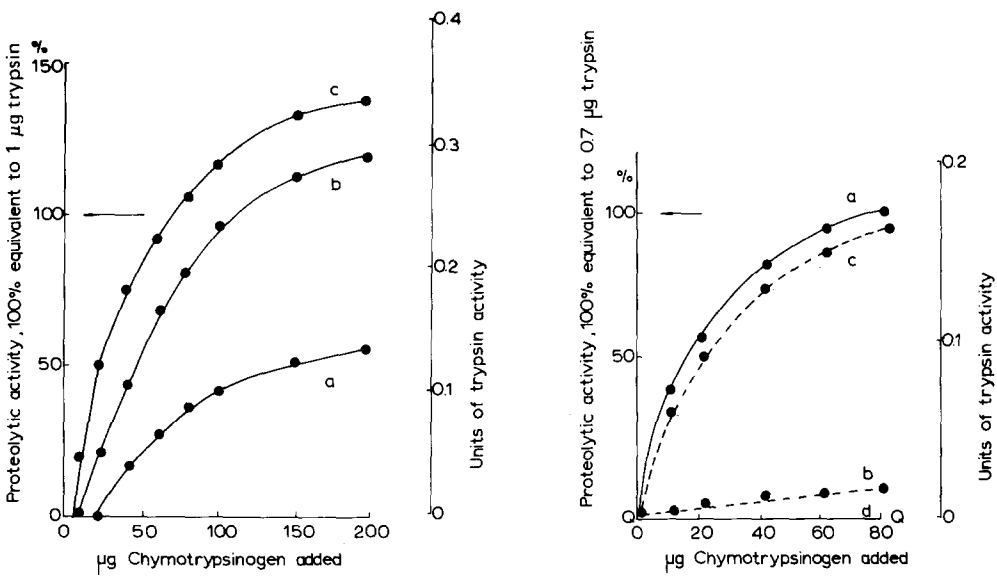

Fig. 3. Re-activation of neutral protease and trypsin by chymotrypsinogen with fluorescein-labelled polymeric collagen as substrate. 5 ml Tris-HCl/KCl/EDTA buffer (pH 7.5) was mixed with 1 µg trypsin and 50 µl post-granule supernatant and left at room temperature for 2 min. Incremental additions of chymotrypsinogen were then made, followed by 7 mg substrate. The tubes were shaken at 37°C and 100-µl samples removed at various time intervals for spectrofluorimetric analysis. The position of the arrow represents the trypsin control at each time interval, viz. a, 20 min; b, 1 h; c, 2 h.

Fig. 4. Re-activation of trypsin and neutral protease after pre-incubation with chymotrypsinogen employing fluorescein-labelled polymeric collagen as substrate. Each tube contained 0.7 µg trypsin plus 50 µl post-granule supernatant in 0.2 ml Tris-HCl/KCl/EDTA buffer (pH 7.5). Series (a—d) contained the same incremental additions of chymotrypsinogen, each series was incubated at 37°C for 1.5 h prior to adding 4.8 ml additional buffer and 7 mg substrate. In series a, the buffer had no additions; in series b 100 µg soya bean trypsin inhibitor was added and, in series c, the buffer contained 50 mM cysteine to selectively inhibit neutral protease activity. The results obtained after 1 h digestion are plotted, with the arrow indicating the trypsin control. In a control series d each tube lacked trypsin but was otherwise identical to tubes in series a. The results of this series followed the axis QQ.

when the system contained 50 mM cysteine to inhibit neutral protease activity but not trypsin (2).

The re-activated proteolytic activity was at least partly due to the trypsin added initially to the test system and subsequently inhibited with post-granule supernatant. It was important to show that this proteolytic activity was not due to either chymotrypsin or a contaminant enzyme present in the zymogen preparations. These facts were shown by including a duplicate series of tubes containing buffer alone (a) and buffer plus an excess of soya-bean trypsin inhibitor (b) in order to destroy selectively the re-activated trypsin. The proteolysis observed in (a) is largely trypsin activity, and this accounts for approx. 94% of the control trypsin activity when 80 μg chymotrypsinogen were used. These results prove that the 1 μg trypsin was first inhibited completely by the post-granule supernatant, and then re-activated by exchange with chymotrypsinogen (a) to be ultimately inhibited by soya-bean trypsin inhibitor (b).

It is possible that both trypsin and neutral protease were inhibited by the soya-bean trypsin inhibitor. Studies on the mechanism of inhibition of these enzymes showed that inhibition is through a thiol-disulphide interaction, as the enzyme has a disulphide bond essential for its activity, [2,3]. It has been established that 50 mM cysteine completely inhibits the neutral protease, but that 250 mM cysteine is required to inhibit trypsin. It was, therefore, possible to include 50 mM cysteine in the test system to inhibit neutral protease, but not trypsin (Fig. 4(c)). These results prove that the observed re-activation was not due to collagenolytic activity associated with the chymotrypsinogen preparation.

After 4—6 h incubation, the curve (a) of total proteolytic activity exceeded the trypsin control, but returned to this value when 50 mM cysteine was included in the assay. This evidence suggests that neutral protease was released from the inhibitor after all the trypsin had been released. This agrees with the data in Figs. 1 and 3 in which the slope for the neutral protease inhibition is steeper than that for trypsin, indicating that the inhibitor has a preference for binding neutral protease rather than trypsin. The inhibitor would, therefore, preferentially exchange trypsin with chymotrypsinogen and retain neutral protease in the enzyme-inhibitor complex, only releasing the neutral protease after long periods of incubation with high concentrations of competitive agents (Fig. 3). It is probable that the binding affinity of the inhibitor for neutral protease is greater than trypsin, which will also be greater than that for chymotrypsin and chymotrypsinogen. Increasing concentrations of the more weakly-bound proteins (e.g. chymotrypsinogen) were required to displace the more firmly-bound enzymes from the inhibitor, by altering the equilibrium position in favour of the more concentrated species.

The chymotrypsinogen used could have been contaminated with either trypsin or chymotrypsinogen, and, thus we employed chymotrypsinogen which had been pre-incubated with excess Tos-Lys-CH_2-Cl and Tos-Phe-CH, followed by exhaustive dialysis of these inhibitors prior to use. The results obtained with this preparation confirmed the data reported in this study and invalidate this possible criticism.

Re-activation of trypsin and neutral protease by pre-incubation with trasylol-trypsin

Fluorescein-labelled polymeric collagen fibrils as substrate. Two preliminary steps were necessary: (a) preparation of enzymically-inactive trasylol-trypsin and (b) addition of a calculated quantity of tumour post-granule supernatant to a known quantity of trypsin to produce complete inhibition of the trypsin. With these two preparations of inhibited trypsin, it was possible to design a suitable experimental system to demonstrate that the trypsin and neutral protease linked to the tumour inhibitor could be exchanged for trasylol-trypsin. This exchange resulted in the subsequent regain of trypsin and neutral protease activity (Fig. 5).

1 μg trypsin was added to 5 ml buffer plus 50 μg post-granule supernatant fraction (which contained latent neutral protease and sufficient tumour inhibitor to inactivate both the neutral protease and added trypsin). After 2 min equilibration at room temperature, the solutions were mixed with 0—10 μg trasylol-trypsin and after a further 2 min 7 mg fluorescein-labelled polymeric collagen fibrils was added to each tube. The tubes were capped and transferred to a shaking water-bath at 37°C; 100-μl samples were withdrawn from each tube at hourly intervals for analysis. Control tubes contained (a) substrate only; (b) 1 μg trypsin and substrate and (c) 1 μg trypsin plus 50 μl post-granule supernatant fraction and substrate.

The data (Fig. 5) shows re-activation of proteolytic activity with incremental additions of trasylol-trypsin over 5 h. The results are presented as percentages

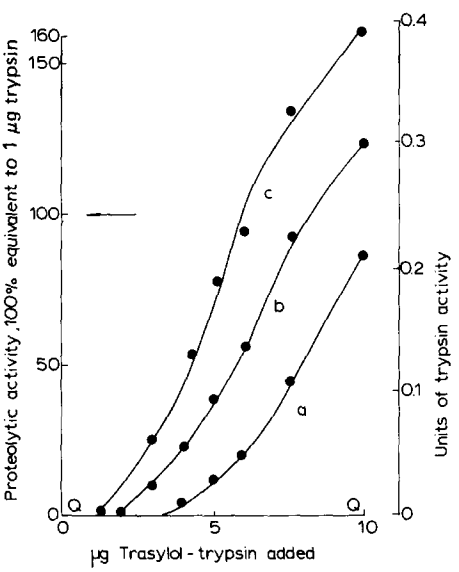

Fig. 5. Displacement of trypsin and neutral protease from the enzyme-inhibitor complex by trasylol-trypsin. The basic assay system was used with 50 μl post-granule supernatant fraction. The arrow indicates the action of 1 μg trypsin alone and the axis QQ represents complete inhibition of (i) trypsin observed with inhibitor at the start of the experiment in the absence of trasylol-trypsin, and (ii) 10 μg trasylol-trypsin alone. The effect of the inclusion of trasylol-trypsin is shown after 2 h plot (a); 4 h plot (b) and 5 h plot (c).

of the proteolytic activity exhibited by 1 μg of trypsin [control (b)] and also as equivalent trypsin units, 1 μg being equivalent to 0.244 units trypsin [9]. The control (c) showed no proteolysis over 5 h and a corresponding control with 10 μg trasylol-trypsin plus the substrate also showed no proteolysis.

Addition of trasylol-trypsin to the trypsin-tumour inhibitor complex (Fig. 5) demonstrated a time-dependent regain of trypsin and neutral protease activity over 5 h. It can be seen that the total proteolytic activity exceeds the 100% control level. This increase can be accounted for by trypsin activity of latent neutral protease present in the 50 μl post-granule supernatant. Thus, the trasylol-trypsin is still capable of providing the important disulphide bond required for thiol-disulphide linkage in a trasylol-trypsin-inhibitor complex.

The interaction of trasylol-trypsin with the inhibitor present in the post-granule supernatant fraction can be demonstrated by polyacrylamide gel electrophoresis; interaction of the thiol-containing inhibitor with trypsin is also prevented by metal ions, (Podrazký, V., unpublished data).

These data indicate that trasylol-trypsin displaces both trypsin and neutral protease from the tumour inhibitor, leading to a re-activation of these enzymes. The kinetic data indicate that this replacement is an equilibrium reaction, replacement being promoted by increasing quantities of trasylol-trypsin and lengthening the time of incubation. These results confirm and extend the evidence for a thiol-disulphide exchange [2,3] mechanism as the basis for the observed inhibition of trypsin and neutral protease by the tumour inhibitor and that the important disulphide bond is located at a site removed from the active centre of trypsin.

One further criticism might be made: these studies employed impure preparations of inhibitor and neutral protease in the form of the post-granule supernatant fraction. We have had great difficulty attempting to obtain a pure inhibitor fraction which retains its biological activity. Our main concern is the interaction of pure trypsin with the inhibitor present in the post-granule supernatant fraction and our interpretation rests on four scientific facts outlined below.

1. Pure trypsin has been shown to be inhibited by a number of pure thiol compounds [3].

2. Pure trypsin has been shown to be inactivated by an inhibitor present in the post-granule supernatant fraction [2,3] (see also Figs. 1—3 of this paper). This inhibition was released by the addition of the disulphide cystine to the test system [2] in a non-enzymic exchange re-activation step.

3. The tumour inhibitor can be selectively adsorbed from the post-granule granule supernatant fraction by passage through sepharose affinity columns containing either cystine or oxidised glutathione.

4. The complete inhibition of trypsin by the added post-granule supernatant fraction followed by the re-activation of trypsin (96%) recovery of theoretical maximum, Fig. 4) by incremental additions of the non-enzymic chymotrypsinogen or trasylol-trypsin (Fig. 5) can only be interpreted as a non-enzymic exchange in which the zymogen replaces trypsin in the inert trypsin-inhibitor complex. This result is of particular significance since neither chymotrypsinogen nor the derived chymotrypsin had any proteolytic activity on the fluorescein-labelled polymeric collagen fibrils when post-granule supernatant

was omitted from the test system. However, when the post-granule supernatant fraction was included in the test system, the newly re-activated enzyme was shown to be trypsin by the use of the specific soya bean trypsin inhibitor, (Fig. 4). Incremental additions of chymotrypsinogen (0—200 μg) to a fixed quantity of post-granule supernatant (50 μl) did not result in any detectable proteolytic activity after 1.5 h (Fig. 4(d)). This evidence would indicate that the latent form of the neutral protease in the post-granule supernatant is in the form of a zymogen-inhibitor complex and that the function of trypsin in our experimental system is to activate the released zymogen.

We do not feel that the lack of purity of the tumour inhibitor invalidates the evidence presented in this paper. This is especially so as the total biological system of the tumour cell is more closely represented by the post-granule supernatant than by a pure inhibitor which we know to be highly unstable. The composite plots presented in this paper were reproducible over a number of hours and were quite simply analysed by a computer programme [5] to provide reproducible quantitative data of the concentration of all the enzymes and inhibitor components described in this study. Thus the impurity of the components of the post-granule supernatant is not a hindrance to that quantitation.

We have used chymotrypsinogen extensively in this study, having first established that neither chymotrypsinogen nor any derived chymotrypsinogen played an enzymic role in these experiments. We have managed to illustrate the phenomenon of re-activation of trypsin by means of a suitably oriented disulphide on carrier protein molecules similar in shape to trypsin, in this instance the inactive zymogen chymotrypsinogen and trasylol-trypsin.

The re-activation described above does not require the cleavage of a peptide bond but merely a thiol-disulphide exchange. Since the spreading of tumour cells is claimed to be halted by the presence of an inhibitor for trypsin-like enzymes [8], it may well be of interest to be able to control such enzymes by means of reducing agents rather than having to isolate the specific proteinase inhibitors which are known to be highly unstable.

Acknowledgements

We wish to thank the Arthritis and Rheumatism Research Council for generously financing the study leave of VP. We also wish to thank Mrs. J. Watkins for her technical help.

References

1 Steven, F.S., Podrazký, V. and Itzhaki, S. (1977) Biochim. Biophys. Acta 483, 211—214
2 Steven, F.S. and Podrazký, V. (1978) Eur. J. Biochem. 83, 155—161
3 Steven, F.S. and Podrazký, V. and Itzhaki, S. (1978) Biochim. Biophys. Acta 524, 170—182
4 Kopitar, M. and Lebez, D. (1975) Eur. J. Biochem. 56, 571—581
5 Steven, F.S., Podrazký, V. and Foster, R.W. (1978) Anal. Biochem. 90, 183—192
6 Weigele, M., DeBernardo, S.L., Tengi, J.P. and Leimgruber, S. (1972) J. Am. Chem. Soc. 94, 5927—5928
7 Steven, F.S. (1976) Biochem. J. 155, 391—400
8 Kuettner, K.E., Hiri, J., Eisenstein, R. and Harper, E. (1976) Biochem. Biophys. Res. Commun. 72, 40—46
9 Hummel, B.C.W. (1959) Can. J. Biochem. 37, 1393—99
10 Steven, F.S., Torre-Blanco, A. and Hunter, J.A.A. (1975) Biochim. Biophys. Acta 405, 188—200
11 Mandl, I. (1961) Adv. Enzymol. 23, 391—400
12 Steven, F.S. and Itzhaki, S. (1977) Biochim. Biophys. Acta 496, 241—246