Biochimica et Biophysica Acta, 568 (1979) 408—415

BBA 68758

INHIBITION OF TRYPSIN AND CHYMOTRYPSIN BY THIOLS

BIPHASIC KINETICS OF REACTIVATION AND INHIBITION INDUCED BY SODIUM PERIODATE ADDITION

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(Received December 5th, 1978)

Key words: Trypsin; Chymotrypsin; Thiol inhibition; Periodate; Reactivation kinetics

Summary

Biphasic kinetic data were obtained when trypsin (EC 3.4.21.4) which had previously been complexed with a thiol-containing inhibitor (present in Ehrlich ascites tumour cells) was incubated with incremental additions of periodate. At low concentrations of periodate the trypsin was re-activated whilst at higher concentrations of periodate the trypsin was irreversibly inhibited. This biphasic reactivation followed by inhibition was also demonstrated when trypsin was first inhibited by dithiothreitol and followed by incremental addition of periodate. Similar results were obtained with chymotrypsin (EC 3.4.21.1). Incremental additions of either dithiothreitol or periodate caused inhibition of both these enzymes. The biphasic kinetic data can be explained in terms of reduction and oxidation of a significant disulphide bond in both trypsin and chymotrypsin which can be cleaved by thiols in a disulphide exchange reaction [1]. This bond is thought to maintain the active centres of each of these enzymes in a conformation sterically favourable for enzymic cleavage of specific peptide bonds in the protein substrates (polymeric collagen fibrils and casein) employed in this study.

Introduction

Ehrlich ascites cells grown in mice have been shown to possess a cytoplasmic inhibitor of trypsin (EC 3.4.21.4) and a trypsin-like neutral protease obtained

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from the granule fraction of these cells [1,2]. It was demonstrated that the inhibitor possessed a reactive thiol which interacted with a significant disulphide bond in the trypsin resulting in inhibition [1,2]; this inhibition was reversed by addition of cystine [2]. We now wish to present evidence for the biphasic reactivation and subsequent inhibition of the enzymes, trypsin and chymotrypsin (EC 3.4.21.1) which had previously been inhibited by thiols, by the addition of sodium periodate. These data have been obtained by use of the incremental analysis technique [3] designed for the analysis of complex mixtures of enzymes and their inhibitors. The evidence presented in this study can be explained in terms of (a) an initial thiol-disulphide exchange reaction leading to the enzyme-inhibitor complex formation, (b) followed by the reversal of this process by low concentrations of periodate resulting in re-oxidation of the significant disulphide bond and regain of enzymic activity, and (c) further oxidation of the significant disulphide bond with consequent irreversible inhibition of the enzyme. The opposing effects of steps (b) and (c) above result in the observed biphasic kinetics. The data to be presented have been obtained initally with the Ehrlich ascites inhibitor fraction acting on both trypsin and neutral protease [1] in the presence of periodate and assayed with fluorescein-labelled polymeric collagen fibrils as substrate [4]. In order to simplify this system, both crystalline trypsin and chymotrypsin have been inhibited first with dithiothreitol [2] and subsequently treated with incremental additions of sodium periodate and the extent of proteolysis assayed with casein as substrate. This simplified system exhibited the same biphasic kinetic data as were found with the tumour system. The actions of dithiothreitol and sodium periodate on both trypsin and chymotrypsin were shown to be inhibitory, one being capable of reversible reductive cleavage of the significant disulphide and the other of irreversible oxidative cleavage of the significant disulphide.

Materials and Methods

The preparation of the post-granule supernatant fraction obtained from Ehrlich ascites cells has already been described [1]. This fraction contained the cytoplasmic inhibitor as well as a latent form of the trypsin-like neutral protease [1]. L-(Tosylamide-2-phenyl)ethylchloromethylketone-treated trypsin with 262 units [5]/mg was obtained from Worthington and three times crystalline chymotrypsin (45 units [5]/mg) was obtained from Sigma. Dithiothreitol was purchased from B.D.H., casein and sodium periodate (Analar reagent grade) from Fisons. 'Fluam' was obtained from Roche.

Two fluorimetric assays were employed to measure the proteolytic activity. Initially, the substrate employed was fluorescein-labelled polymeric collagen fibrils [1,2,4]: the casein assay system consisted of 3 ml of casein solution containing 12 mg casein adjusted to pH 7.8, plus 1 μg trypsin or chymotrypsin and incremental additions of dithiothreitol or sodium periodate. The reagents were mixed and the tubes incubated at 37° C for 2 or 3 h prior to adding 0.5 ml 25% (w/v) trichloroacetic acid to stop the reaction. After 18 h standing at room temperature to allow release of peptides from undigested substrate, the tubes were centrifuged at $300 \times g$ for 5 min and appropriate samples (50—100 μl) withdrawn by microsyringe from each of these, placed in 1.0 ml 2% (w/v) $NaHCO_3$

and 150 μg 'fluram' dissolved in 0.5 ml acetone added to each test sample. The solubilised peptides were estimated by fluorimetry with an excitation wavelength of 390 nm and emission wavelength of 490 nm. The proteolytic activity in each tube was then expressed as a percentage of the proteolytic activity observed in the internal control of either 1 μg trypsin or 1 μg chymotrypsin.

Results and Discussion

Trypsin

Inhibition of trypsin and neutral protease by cytoplasmic inhibitor followed by reactivation by $NaIO_4$. When a fixed quantity of trypsin was mixed with incremental additions of the crude cytoplasmic inhibitor both activation and inhibition of neutral protease as well as trypsin inhibition was observed [1]. The experimental conditions can be arranged so that when 0.5 μg trypsin and 30 μl post-granule supernatant are pre-mixed in each tube, the resultant enzymic activity in all tubes is equivalent to 36% of the initial trypsin activity (Fig. 1).

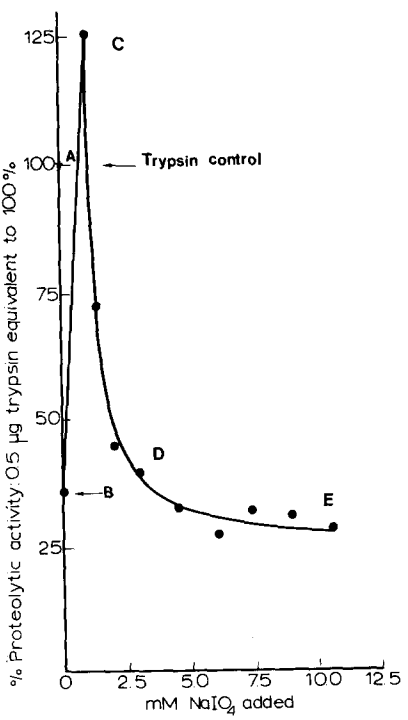
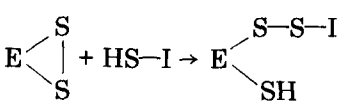


Fig. 1. Inhibition of trypsin by the inhibitor present in tumour cell post-granule supernatant, followed by biphasic reactivation and subsequent inhibition by incremental addition of $NaIO_4$. All tubes contained 0.5 μg trypsin preincubated with 30 μl post-granule supernatant plus 7.0 mg fluorescein-labelled polymeric collagen fibrils suspended in 5.0 ml (pH 7.6) buffer. $NaIO_4$ was added to give a final concentration in the range 0—12 mM and the tubes incubated at 37° C for 2 h prior to analysis of the solubilised fluorescein-labelled peptides. The results are expressed as a percentage of the initial trypsin activity exhibited by the internal control and indicated by the star at the arrow A. The arrow at B indicates the initial inhibition produced in all tubes by the addition of the 30 μl of post-granule supernatant. BC represents the reactivation of both trypsin and neutral protease, whilst the curve CDE represents their inhibition by incremental additions of $NaIO_4$.

The objective of this preliminary step is to ensure that all the tubes contained a known proportion of the added trypsin in the form of trypsin-inhibitor complex. At this stage, incremental addition of $NaIO_4$ plus 5 ml buffer were added together with 7.0 mg fluorescein-labelled polymeric collagen fibrils. The test system was incubated and assayed for the solubilisation of fluorescein-labelled peptides after 2 h. The results were calculated as a percentage of the activity of the added trypsin and are presented in Fig. 1. It can be seen that the cytoplasmic inhibitor (30 μl) initially inactivated 64% of the added trypsin, i.e. the drop from A to B in each tube. On incremental additions of $NaIO_4$ to 1 mM final concentration, the total proteolytic activity of the test system rises steeply to 125% (at C, Fig. 1) of the original trypsin activity. The rise to a value greater than 100% is to be expected, since the 30 μl of post-granule supernatant fraction added to each tube contains latent neutral protease activated by trypsin [1—3], capable of assay by the substrate. This steep rise (Fig. 1, BC) in proteolytic activity demonstrates the reactivation of both trypsin and neutral protease which had previously been complexed with the inhibitor through a thiol-disulphide exchange reaction. It is suggested that each enzyme is reactivated by dissociation from the enzyme-inhibitor complex due to mild oxidation, leading to a reformation of the significant disulphide bonds in trypsin and neutral protease. At higher concentrations of $NaIO_4$, there is an equally sudden fall in total proteolytic activity along the curve C, D and E in Fig. 1. We interpret this fall to be caused by the irreversible oxidation [7] of the significant disulphide bonds of both trypsin and neutral protease with consequent loss in enzymic activity.

The mechanisms involved in these reactions can be summarised as follows assuming an inhibitor (ISH), with a single binding site for trypsin or neutral protease:

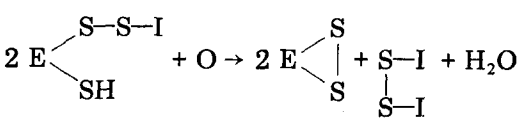
Region A—B: inhibition of trypsin



(1)

Active enzyme Inhibitor Inactive complex

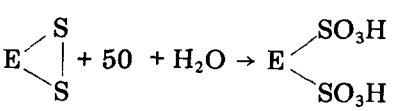
Region (B—C): re-activation of trypsin and neutral protease.



(2)

Active enzyme

Region CDE: inhibition of both neutral protease and trypsin



(3)

Inactive enzyme

Inhibition of trypsin by dithiothreitol followed by reactivation by $NaIO_4$. It should be possible to simulate the events in Fig. 1 with a simpler system. In this experiment the substrate was soluble casein and the initial enzyme inhibition achieved with dithiothreitol [2]. Each of a series of tubes contained 1 μg trypsin plus sufficient dithiothreitol to cause 71% inhibition of the trypsin, i.e. a final concentration of dithiothreitol of 3.3 mM. At this stage, incremental additions of $NaIO_4$ were made plus 3.0 ml casein, the mixtures were shaken at 37°C for 3 h prior to trichloroacetic acid addition and fluorimetric analysis of the proteolytic digestion in each tube. The results from these analyses are presented in Fig. 2 and expressed as a percentage of the proteolytic activity of 1 μg trypsin acting as an internal control.

The general shape of the graph in Fig. 2 corresponds to that in Fig. 1 for the tumour enzyme-inhibitor system. It is worth noting that far more dithiothreitol (3.3 mM) is required than 30 μl of impure cytosol inhibitor (M_r approx. 100 000; Podrazký, V., personal communication) to produce an equivalent degree of trypsin inhibition (i.e. 71%). Addition of periodate results in a corresponding increase in enzymic activity due to the reactivation of trypsin followed by a subsequent fall in enzymic activity due to further oxidation by increasing concentrations of periodate as described for the data in Fig. 1.

Inhibition of trypsin by dithiothreitol independently. It was important to establish the inhibition of 1 μg of trypsin by incremental addition of dithio-

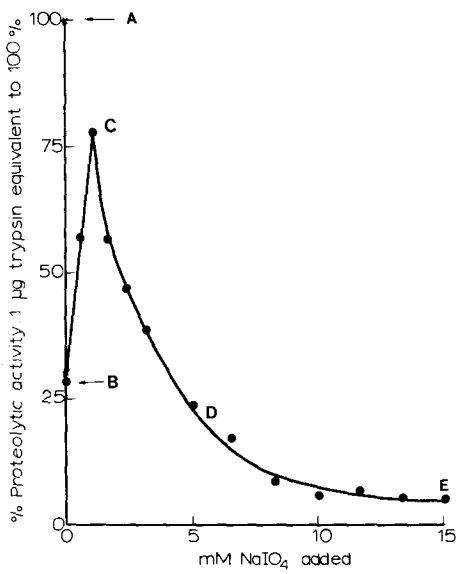


Fig. 2. Inhibition of trypsin by dithiothreitol followed by biphasic reactivation and subsequent inhibition by incremental additions of $NaIO_4$. All tubes contained 1 μg trypsin preincubated with 3.3 mM dithiothreitol followed by incremental additions of $NaIO_4$ over the range 0.4—15 mM and incubated with 12 mg casein in 3.0 ml with pH adjusted to 8.0. After 3 h at 37°C, the reaction was terminated by addition of 0.5 ml trichloroacetic acid (25%, w/v) and the proteolytic activity determined by 'fluram' analysis of the solubilised peptides. The results are expressed as a percentage of the initial trypsin activity indicated by the star at A. The arrow at B indicates the initital inhibition of trypsin in all tubes, caused by the dithiothreitol. The line BC represents the reactivation of trypsin and the curve CDE the subsequent inhibition of trypsin by incremental additions of $NaIO_4$.

threitol [2] employing the casein assay systems. Fig. 3 presents data on the dithiothreitol inhibition of trypsin after 3 h digestion. It can be seen that the thiol is an effective inhibitor of trypsin and that 3.3 mM produced a 75% inhibition corresponding closely to the 71% observed in the experimental data obtained for Fig. 2.

Inhibition of trypsin by $NaIO_4$ independently. Data obtained after 2 h digestion of casein by trypsin in the presence of incremental additions of $NaIO_4$ are presented in Fig. 4. Clearly $NaIO_4$ is a strong inhibitor of trypsin. It was shown independently, that once periodate inhibition of trypsin had been achieved, this process was not reversed by incremental additions of dithiothreitol, in contrast to the results presented in Figs. 1 and 2 in which periodate was used to reactivate the thiol-induced inhibition of trypsin. It could well be argued that since both dithiothreitol and periodate, acting independently, cause inhibition of trypsin it might be difficult to explain the biphasic reactivation and subsequent inhibition of trypsin shown in Figs. 1 and 2, produced when dithiothreitol treatment is followed by $NaIO_4$ additions. This can be explained on the basis of the cleavage of the significant disulphide bond in trypsin (a) by the reducing agent, and (b) by the oxidising agent, each causing inhibition by opposing chemical reactions. When the two reagents are combined a biphasic plot will result, due to the oxidising agent's ability to destroy the reducing agent, and also oxidise the enzyme-dithiothreitol complex with the release of the enzyme

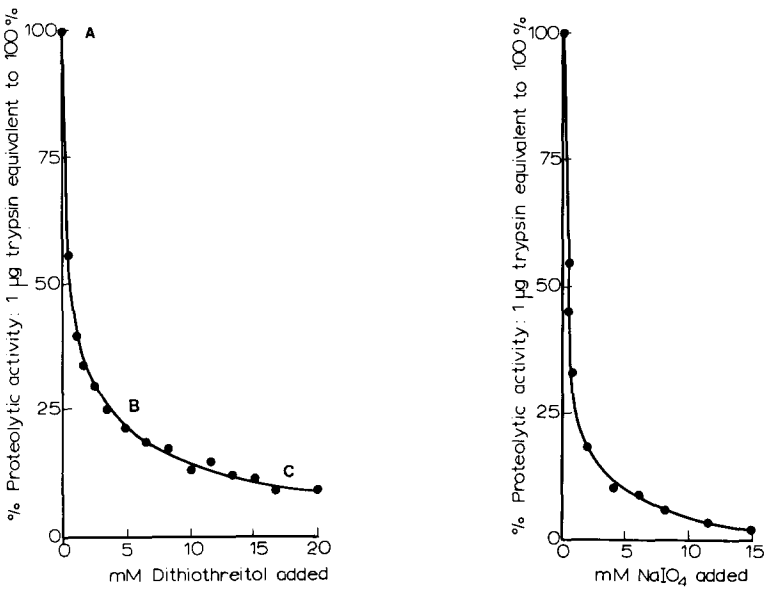


Fig. 3. Inhibition of trypsin by incremental additions of dithiothreitol. Each tube contained 1 µg trypsin plus a range of dithiothreitol concentrations (0—20 mM) and the proteolytic activity assayed with casein as in Fig. 2. The initial trypsin activity exhibited by 1 µg is represented by 100% and progressive inhibition of the trypsin by the curve ABC with increasing concentrations of dithiothreitol.

Fig. 4. Inhibition of trypsin by incremental additions of $NaIO_4$. The conditions were as described for Fig. 3, except that 0—15 mM $NaIO_4$ was employed to inhibit 1 µg of trypsin and the digestion terminated after 2 h.

with its significant disulphide bond reformed (as illustrated by region BC in Fig. 1 and Eqn. 2 above).

Chymotrypsin

Inhibition of chymotrypsin with dithiothreitol followed by re-activation by $NaIO_4$. Similar result were obtained with 1 μg chymotrypsin initially inhibited by 3 mM dithiothreitol treatment followed by incremental additions of $NaIO_4$ and assayed by the casein/'fluram' technique. The results are presented in Fig. 5. The data show an initial inhibition of 61% caused by 3 mM dithiothreitol. On incremental additions of $NaIO_4$, the chymotrypsin regains nearly all its original activity and this is subsequently inactivated for the second time by oxidation of the significant disulphide bond by the increasing concentration of periodate used.

Inhibition of chymotrypsin with dithiothreitol. When 1 μg chymotrypsin was incubated for 2 h with incremental additions of dithiothreitol, the effect was similar to that observed with trypsin (Fig. 3) and is consistent with the reductive cleavage of the significant disulphide bonds in each of these enzymes.

Inhibition of chymotrypsin with $NaIO_4$. The progressive inhibition of chymotrypsin by incremental additions of $NaIO_4$, assayed after 2 h, was similar to the action of $NaIO_4$ on trypsin (Fig. 4).

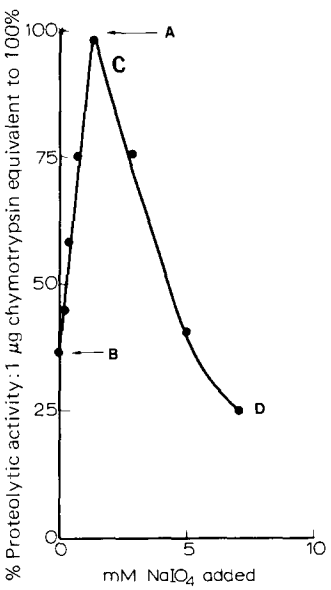


Fig. 5. Inhibition of chymotrypsin by dithiothreitol followed by biphasic reactivation and subsequent inhibition by incremental additions of $NaIO_4$. Each tube contained 1 μg chymotrypsin preincubated with 3.0 mM dithiothreitol followed by incremental additions of $NaIO_4$ (0—7 mM) made to the casein assay system. After 2 h inhibation at 37°C the digestion was terminated and the proteolytic activity determined by 'fluram' analysis. The proteolytic activity in each tube is expressed as a percentage of the control in which 1 μg of chymotrypsin was employed in the absence of $NaIO_4$ (star indicated by the arrow at A). All the tubes in the test system were initially inhibited by the dithiothreitol to the extent indicated by the arrow at B. The line BC represents the reactivation and the curve CD, subsequent inhibition of chymotrypsin by $NaIO_4$.

In conclusion, we would wish to draw attention to the fact that incremental analysis [3] has enabled a biphasic kinetic plot to be obtained in which reactivation and subsequent inhibition of both trypsin and chymotrypsin by a combination of thiol followed by an oxidising agent can be demonstrated. Such information could not have been obtained by conventional methods of analysis (e.g. autoanalysis) employing a single concentration of thiol or $NaIO_4$. The mechanism for such a biphasic plot has been discussed in terms of (a) reductive cleavage, (b) re-oxidation and disulphide bond reformation, and (c) irreversible oxidative cleavage of a significant disulphide bond in both trypsin and chymotrypsin [1,2] which is thought to be essential for the maintenance of the conformation of the active centres of these enzymes.

It is of interest to mention that the reactivation of trypsin- and neutral protease-inhibitor complex [2] by mersalyl also exhibits a biphasic plot, that is to say low concentrations of mersalyl promote reactivation, whilst higher concentrations result in irreversible deactivation of the newly reactivated enzymes.

Examination of the primary structure of trypsin suggests that the disulphide bond 191—220, also noted by Knights and Light [8], would be a likely candidate for the significant disulphide bond of trypsin suggested by these studies and earlier work [1,2].

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