*Biochimica et Biophysica Acta*, 571 (1979) 369—373


BBA Report

BBA 61363

# BIPHASIC KINETICS OF METAL ION REACTIVATION OF TRYPSIN-THIOL COMPLEXES

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(Received July 9th, 1979)

*Key words: Trypsin-thiol complex, Protease; Metal ion activation; (Kinetics)*

## Summary

This report describes biphasic kinetic data obtained when trypsin was inhibited by a thiol-containing inhibitor present in Ehrlich ascites tumour cells and then subjected to addition of $Hg^{2+}$, $Cu^{2+}$ or $Ag^{+}$. This resulted in an initial re-activation of the trypsin, followed by inhibition of the enzyme with the addition of higher concentrations of these ions. The significance of these observations is 2-fold: (i) help to elucidate the mechanism of metal ion activation of latent enzymes, and (ii) also indicate that, in certain circumstances, the concentration of added metal ion determines whether the metal acts as an activator or an inhibitor of enzyme activity.

A thiol-containing inhibitor of both tumour-derived neutral protease and trypsin has been described [1—3], which is capable of forming an enzyme-inhibitor complex by means of a thiol-disulphide exchange reaction [3]. The enzyme-inhibitor complex was dissociated with mersalyl [1], an organo-mercurial compound, in a biphasic manner, dependent on the mersalyl concentration. Sodium periodate has recently been shown to exert a similar biphasic effect [4] on both trypsin-thiol and chymotrypsin-thiol complexes.

In contrast to these observations, Martinek et al. [5] reported that $AgNO_3$ exhibited a simple concentration-dependent inhibition of trypsin esterase activity. These authors concluded that $Ag^{+}$ bound to histidine located in the active centre of trypsin. We believe that metal ions also dissociate the disulphide bond linking the enzyme to the inhibitor in the enzyme-inhibitor complexes described in this study. This bond can also be dissociated by adding

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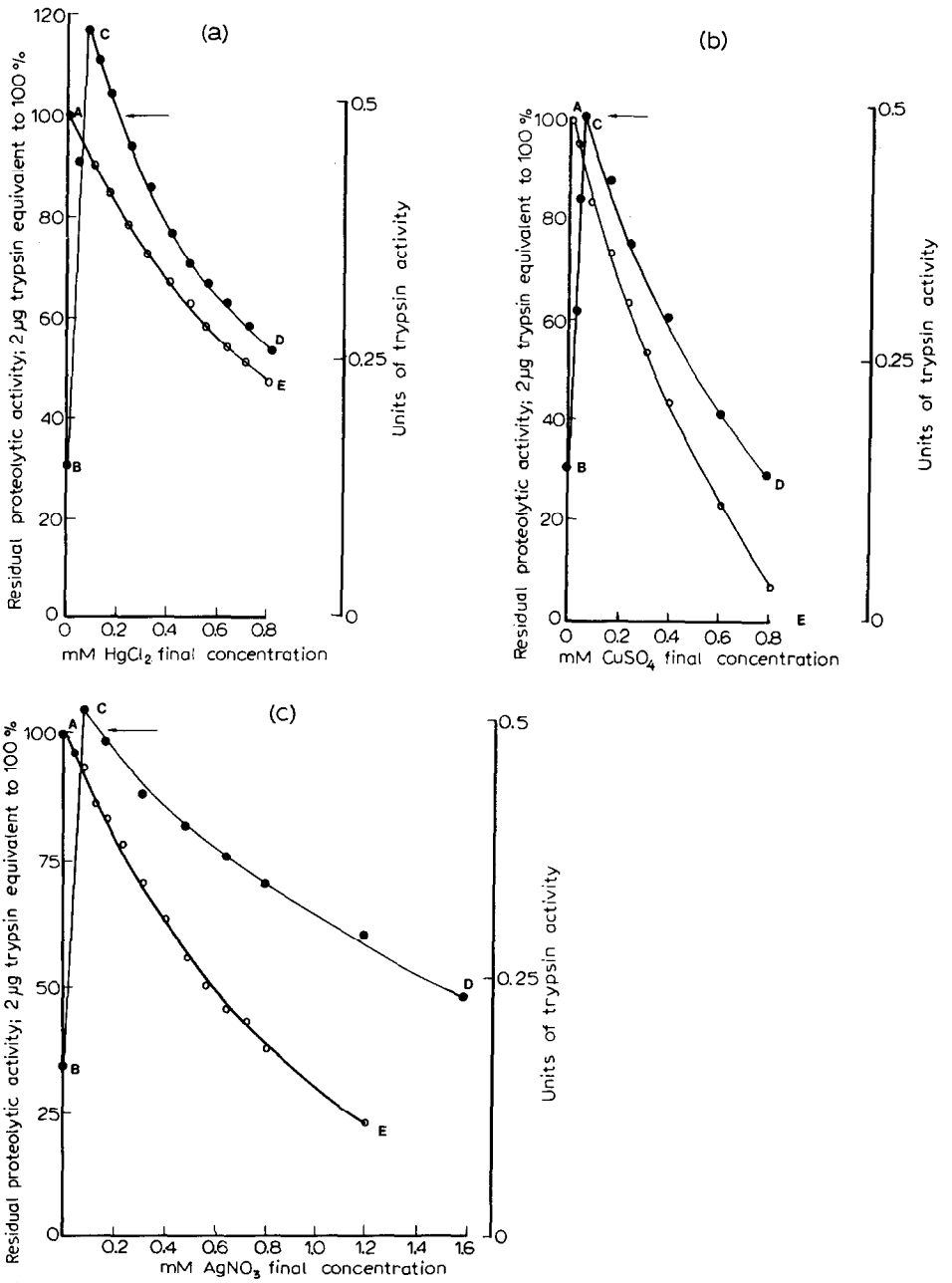



**Fig. 1. Inhibition of trypsin by added tumour inhibitor followed by incremental additions of $HgCl_2$, $CuSO_4$ or $AgNO_3$ assayed with casein [4]. Each tube contained 2 μg trypsin plus 100 μl post granule supernatant fraction; the latter contained enough inhibitor to cause 70% inhibition of the trypsin. To each tube was added metal ions to give the final concentrations indicated in the figures, plus 3 ml $Cl^-$-free casein buffer (pH 8.0) containing 12 mg casein. The tubes were shaken for 2 h, the reaction terminated by adding 0.5 ml 25% (w/v) trichloroacetic acid and 100 μl solubilised peptides (analysed by fluram analysis [4]). The closed circles (●) following the pathway A, B, C, D represent the effect of metal ions on the latent form of the enzymes produced by forming the enzyme inhibitor complex (point B). The open circles (○) represent the effect of metal ions on the free enzyme and follow the curve A, E. The arrow at A represents the internal trypsin control. (a) $HgCl_2$; (b) $CuSO_4$; (c) $AgNO_3$.**

cystine with a consequent regain of enzyme activity [1]. The result of incremental additions of metal ions to the trypsin-inhibitor complex can therefore be explained as reactivation by dissociation, followed by inhibition at higher concentrations of metal ions.

In this study, 2 $\mu$g trypsin (Sigma) was partially inhibited by adding 100 $\mu$l of the naturally-occurring thiol inhibitor present in Ehrlich ascites cell sonicates. This latent trypsin was then incubated with incremental additions of $HgCl_2$, $CuSO_4$ or $AgNO_3$ for 5 min at 20°C followed by assay of the residual proteolytic activity with either casein and the "fluram" assay of solubilised peptides [4], or with fluorescein-labelled polymeric collagen fibrils as substrate [1—3]. The residual proteolytic activity was expressed as a percentage of the initial trypsin activity and also as units of trypsin activity, as defined by Hummel [6]. In each case, the initial trypsin activity is shown by the arrow at A (see figures). The addition of the thiol inhibitor resulted in 70% inhibition of the trypsin (shown by point B). Addition of metal ions resulted in the biphasic plot (B.C.D.) in each case. The plots presented here are typical of three such plots obtained for each system described in this study.

For each test system employing $Hg^{2+}$, $Cu^{2+}$ or $Ag^{+}$, a corresponding series of control analyses were carried out in which the post-granule supernatant fraction was omitted and the possible inhibitory action of the metal ion on free trypsin was studied. The resultant plots (labelled A, E in Figs 1 and 2) showed the effect of metal ion inhibition on free trypsin assayed in these test systems. It is worth recording that $Ag^{+}$ was a much more effective inhibitor of

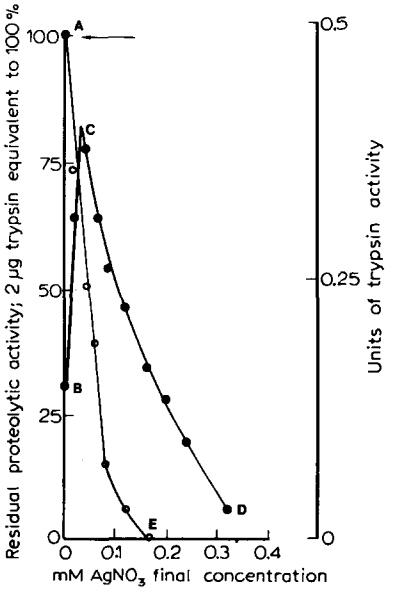
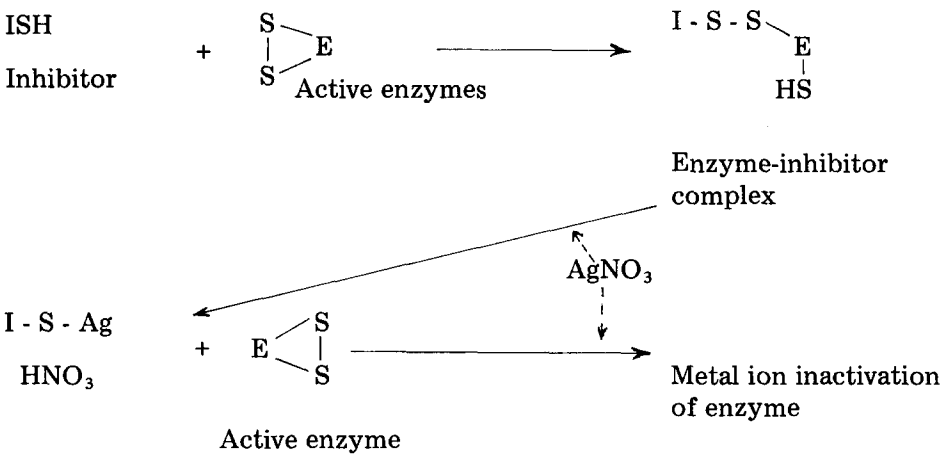



**Fig. 2. Inhibition of trypsin by adding tumour inhibitor followed by incremental additions of $AgNO_3$ with fluorescein-labelled polymeric collagen fibrils as substrate. Each tube contained 2 $\mu$g trypsin, 100 $\mu$l postgranule supernatant fraction, the latter contained enough inhibitor to cause 70% inhibition of the trypsin. To each tube was added incremental additions of metal ions in 5 ml 50 mM Tris-acetate (pH 7.5) buffer ($Cl^-$-free) plus 7 mg fluorescein-labelled polymeric collagen fibrils. After incubating for 30 min and 1 h, 100 $\mu$l samples were withdrawn for fluorimetric analysis [1—3] of the solubilised fluorescein-labelled telopeptides. A, B, C, D represents the biphasic kinetic plot and A, E represents the metal inhibition of free trypsin as in Fig. 1. The arrow at A represents the internal trypsin control.**

trypsin when assayed with fluorescein-labelled polymeric collagen fibrils, than with casein as substrate; hence, the nature of the substrate plays a role in the assay of this type of inhibition.

In Figs. 1 and 2, the reactivated enzyme activity exceeded the 100% value provided by the internal control of added trypsin (indicated by the arrow at A) due to the reactivation of latent neutral protease present in the added post-granule supernatant fraction [2,7]. A comparison of the biphasic plots (B,C,D) obtained with previously inhibited trypsin and the simple inhibition (A,E) produced by the metal acting on the free enzymes allows us to elucidate the molecular mechanisms of these experiments. In the first instance, the enzyme, trypsin, is inhibited by forming an enzyme-inhibitor complex (A,B) through an intermolecular thiol-disulphide exchange [3]. The addition of $Hg^{2+}$, $Cu^{2+}$ or $Ag^{+}$ causes a dissociation of the complex with regain of enzyme activity (B,C). As the metal ion concentration is increased the newly reactivated enzyme is inactivated by metal ions (C,D and A,E). These mechanisms are shown below for the $AgNO_3$-activation and inhibition exhibited with the preformed enzyme-inhibitor complex.




We have observed that the enzymes trypsin, chymotrypsin, fibrinolysin and the neutral protease present in tumour cells are all inhibited by a tumour-derived inhibitor and by synthetic thiols, with the consequent formation of latent forms of these enzymes. It is possible that other enzymes also possess these properties and, therefore, incremental additions of metal ions may reveal further examples of latency and biphasic kinetics. In this regard, it is of interest that one such activation has already been shown in the case of mammalian collagenase and a neutral protease employing organo-mercurial reagents as activators [5,9]. We wish to stress that the use of a number of different substrates for these studies is important, since metal ions have different degrees of inhibition on trypsin in the presence of different substrates.

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