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FURTHER INHIBITION STUDIES ON GUANIDINOBENZOATASE, A TRYPSIN-LIKE ENZYME ASSOCIATED WITH TUMOUR CELLS

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Guanidinobenzoatase is a proteolytic enzyme capable of degrading fibronectin and is a tumour associated enzyme. Guanidinobenzoatase has been shown to be an arginine selective protease and is distinct from trypsin, plasminogen activator, plasmin, thrombin and a newly described tumour associated enzyme specific for guanidino phenylalanine residues. These conclusions have been derived from inhibition studies employing 4-methyl-*p*-guanidinobenzoate as substrate. Three active site titrants for trypsin have been shown to be good substrates for guanidinobenzoatase. A new active site titrant for trypsin, rhodamine bisguanidinobenzoate, can also be used to assay guanidinobenzoatase in a stoichiometric manner. This active site titrant can be employed to label guanidinobenzoate on the surface of leukaemia cells.

KEY WORDS: Guanidinobenzoatase, competitive inhibition, tumour protease.

INTRODUCTION

Guanidinobenzoatase is a protease associated with cells capable of migration, in particular tumour cells. This enzyme can be obtained from the fluid surrounding tumour cells such as the Ehrlich ascites tumour cells cultivated in the peritoneal cavity of mice.[1] The enzyme is trypsin-like in cleaving arginyl peptide bonds but has no affinity for lysyl peptides[1,2] and has now been demonstrated to degrade fibronectin and the fibronectin peptide GlyArgGlyAsp.[3] The latter is thought to be the linking peptide[4] between fibronectin and the cell surface *in vivo*. The ability to degrade fibronectin may be the reason why cells capable of migration, such as invasive lymphocytes and the basal cell layers of epithelium in the cervix, also possess guanidinobenzoatase. In a recent report we examined the role of naturally occurring protein inhibitors of guanidinobenzoatase in the suppression of cell surface enzymic activity.[3] In this report we compare the actions of some competitive inhibitors and active site titrants on the behaviour of both trypsin and guanidinobenzoatase. These studies further defined the properties of guanidinobenzoatase and led to the discovery of an active site titrant for guanidinobenzoatase, rhodamine bisguanidinobenzoate. These

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studies on the inhibition of guanidinobenzoatase in free solution were linked to the use of fluorescent probes for the active centre of guanidinobenzoatase on the surface of rat T-cell leukaemia cells that had invaded the host animal's kidney.[5] The reasons for linking these studies are twofold. Firstly, cell surface proteases can exhibit entirely different inhibition kinetics to those observed in free solution,[6,7] it is therefore important to establish that information obtained from kinetic analysis of the soluble enzyme can be extended to cell surface enzyme. Secondly, we wish to design molecules that may have a cytotoxic effect on tumour cells and as the first step in this direction we have used the leukaemia cell surface guanidinobenzoatase to attract fluorescent and non-fluorescent molecules to these cells.[8] We can monitor these events with the leukaemic kidney sections.[8]

MATERIALS

The following reagents were obtained from Sigma Chemical Company, St Louis, Mo., USA:– 4-methylumbelliferyl-*p*-guanidinobenzoate, *p*-nitrophenyl-*p*-guanidinobenzoate, 9-aminoacridine, propidium iodide, ε-amino caproic acid, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, *m*-iodobenzylguanidine, *N*-tosyl-DL-lysine chloromethyl ketone, L-arginine-7-amino-4-methylcoumarin, benzamidine, *m*-aminobenzamidine, benzamine, mercuric chloride, plasminogen activator, plasmin, trypsin, rhodamine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and guanidinobenzoic acid.

Activated Sepharose-4B was purchased from Pharmacia, Uppsala, Sweden. PhePipArgNap was bought from Kabi, through Flow Laboratories Ltd, Woodcock Hill, Rickmansworth, Herts., UK.

The protease inhibitor Camostate or FOY-305 was kindly provided by Dr. Babst of Sanol, Schwarz-Monheim GmbH, Monheim, West Germany. Dr Walter Mangel very kindly provided 22 mg of each of the new active site titrants for trypsin, fluorescein monoguanidinobenzoate and rhodamine bisguanidinobenzoate. A sample of *m*-iodobenzylguanidine was kindly provided by Dr D. Ackery, Department of Nuclear Medicine, University of Southampton Medical School.

Preparation of crude rhodamine bisguanidinobenzoate

215 mg guanidinobenzoic acid was dissolved in 8 ml of ice cold pyridine/dimethyl formamide (1:1); 200 mg solid carbodiimide was added and the mixture stirred until all the carbodiimide had dissolved. 15 mg rhodamine was added in 1 ml of cold pyridine/dimethyl formamide and the reagents were gently stirred for 48 h at 4°C. Chromatographic analysis of the crude product indicated that it contained rhodamine bisguanidinobenzoate chromatographing with identical R_f value to the authentic sample supplied by Dr Mangel.

METHODS

The inhibition kinetics were essentially those already described for guanidinobenzoatase in solution.[1,2] We employed either a fixed amount of enzyme and substrate but varied the concentration of potential inhibitor, or alternatively we used a fixed amount of enzyme pre-incubated with inhibitor with incremental additions of sub-

strate leading to Lineweaver-Burk type plots. In studies on the inhibition of the insoluble enzyme bound to the surface of tumour cells we employed kidney sections obtained from leukaemic rats[8] or bovine pancreas sections.[3] Inhibition was demonstrated by the failure of the guanidinobenzoatase on the surface of the cells to stain with 9-aminoacridine[2] in competition with the potential inhibitor.[8]

RESULTS AND DISCUSSION

Inhibition of guanidinobenzoatase with PhePipArgNap or Kabi substrate S-2238 for thrombin

This substrate is used for the fluorimetric assay of thrombin; since guanidinobenzoatase has specificity for arginyl peptide bonds it was necessary to define whether our enzyme was really thrombin. The thrombin substrate PhePipArgNap inhibited guanidinobenzoatase (Figure 1), furthermore, when the α-amino group of PhePipArgNap

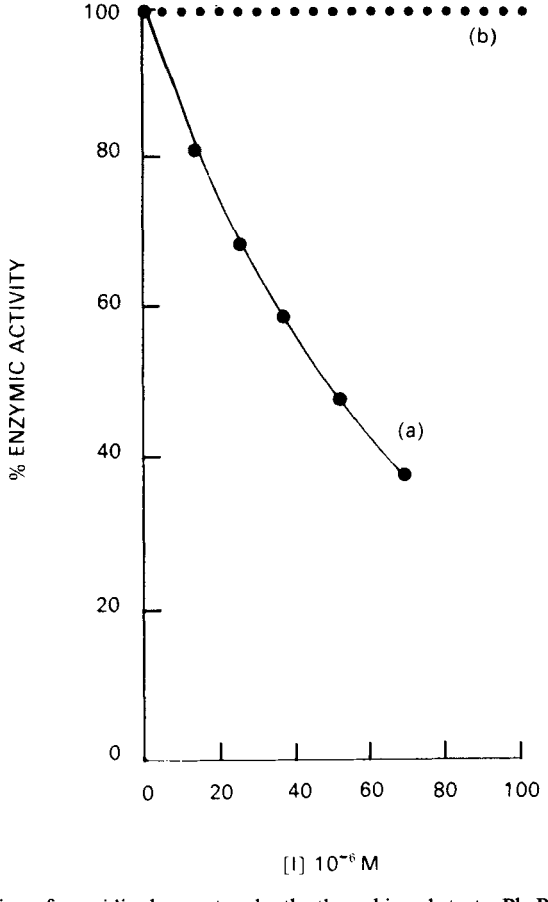


FIGURE 1 Inhibition of guanidinobenzoatase by the thrombin substrate, PhePipArgNap, assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. Curve (a) with PhePipArgNap showing inhibition. Line (b) with ε-aminocaproic acid, showing no inhibitory action.

was linked to activated CH-Sepharose 4B, this liganded Sepharose bound guanidinobenozatase from solution and could be used to purify the enzyme (data not presented, but similar to that described for agmatine-Sepharose-4B).[2] Since PhePipArgNap inhibited guanidinobenzoatase in solution we explored the possibility that this peptide could prevent the staining of leukaemia cells[8] by the fluorescent competitive inhibitor of guanidinobenzoatase, 9-aminoacridine.[2] It was observed that when an aqueous solution contained both 9-aminoacridine and PhePipArgNap (each 10^{-3} M), no staining took place with 9-aminoacridine. In the absence of PhePipArgNap, excellent staining of the leukaemia cells took place (Figure 1 following paper). This can be explained only if the Phe Pip Arg Nap complexed for the same binding site as 9-aminoacridine, in fact this must be the active centre of guanidinobenzoatase on the surface of the leukaemia cells. These observations confirm the kinetic studies (Figure 1, this paper) that PhePipArgNap is a competitive inhibitor of guanidinobenzoatase both in solution and on the surface of leukaemia cells. Independent studies with guanidinobenzoatase purified by affinity chromatography[2] demonstrated that this enzyme did not activate fibrinogen or degrade polymerised fibrin but did degrade fibronectin.[3] The evidence presented above indicates that guanidinobenzoatase is a trypsin-like protease which is distinct from both thrombin and plasmin.

Failure of ε-amino caproic acid to inhibit guanidinobenzoatase

ε-Amino caproic acid is frequently used as a general inhibitor of trypsin-like enzymes, especially in tissue extraction procedures. This agent failed to inhibit guanidinobenzoatase assayed with 4-methyl-*p*-guanidinobenzoate[1] when used in concentrations up to 10^{-2} M (Figure 1). Since ε-amino caproic acid has been used effectively as an inhibitor of plasminogen activator,[9] the observed lack of inhibition of guanidinobenzoatase suggests that our enzyme is distinct from plasminogen activator in its inhibition behaviour. This was confirmed when we attempted to activate plasminogen with affinity column-purified guanidinobenzoatase; no plasmin formation could be detected on plates of fibrin or on polyacrylamide gels when the reaction products of fibrin plus guanidinobenzoatase were examined (data not presented here). Plasminogen activator may be assayed with synthetic arginyl substrates,[10] e.g. L-Arg-7-amido-4-methyl coumarin. Neither the crude guanidinobenzoatase present in the Ehrlich ascites tumour fluid nor the affinity purified enzyme cleaved this substrate with the release of a fluorescent product. This substrate for plasminogen activator was found to be an inhibitor of guanidinobenzoatase when assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate (Figure 2). The evidence presented above would suggest that although the activity of plasminogen activator is known to increase on the surface of tumour cells[11] and transformed cells[12] and may be found in similar situations to guanidinobenzoatase, these two enzymes are distinct in their substrate and inhibition characteristics.

Failure of N-α-*Benzoyl-DL-Arginine*-p-*nitroanilide to interact with guanidinobenzoatase*

N-α-Benzoyl-DL-arginine-*p*-nitroanilide was hydrolysed by trypsin but not by guanidinobenzoatase; a separate study showed that this reagent did not inhibit guanidinobenzoatase either (Figure 2). The evidence suggests that guanidinobenzoatase had no affinity for *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide and it must be concluded that

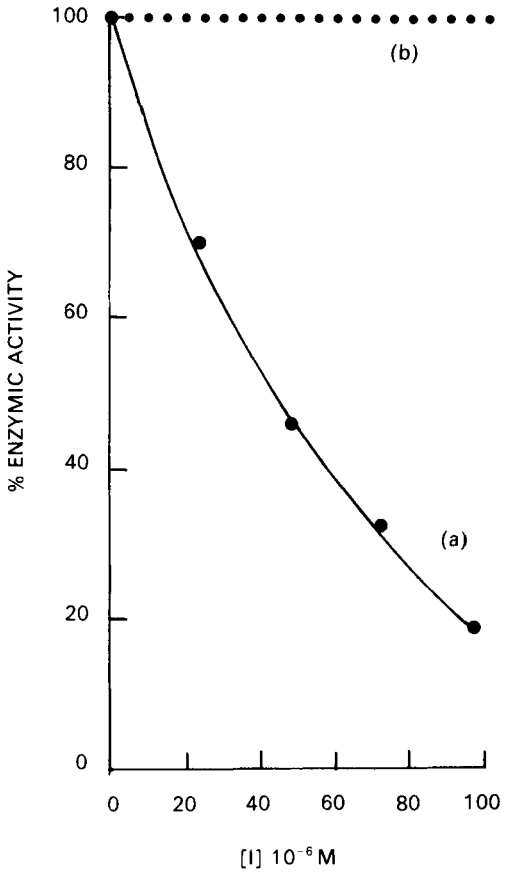


FIGURE 2 Inhibition of guanidinobenzoatase by L-arginine-7-amido-4 methyl coumarin. Curve (a) with L-arginine-7-amino-4 methyl coumarin showing inhibition. Line (b) with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide showing no inhibitory action.

not all arginyl substrates for trypsin-like enzymes are attracted to the active centre of guanidinobenzoatase.

Inhibition of guanidinobenzoatase with m-*iodobenzylguanidine*

The ability of *C*-dansyl glycocyamine and *C,C'*-bisdansyl-octopine to bind to guanidinobenzoatase on the surface of tumour cells[2] and act as competitive inhibitors of guanidinobenzoatase in solution[2] suggested that *m*-iodobenzylguanidine might also inhibit this enzyme in solution and on the surface of tumour cells. Kinetic analysis (Figure 3) shows this inhibition in solution. We demonstrated, in an independent study, that 9-aminoacridine in the presence of 10^{-2} M *m*-iodobenzylguanidine was unable to stain the leukaemia cells in resin sections (similar experiments to those presented in the following paper). The evidence suggests that *m*-iodobenzylguanidine is an inhibitor of guanidinobenzoatase and locates tumour cells through its affinity for the active centre of guanidinobenzoatase on the surface of these cells in our experi-

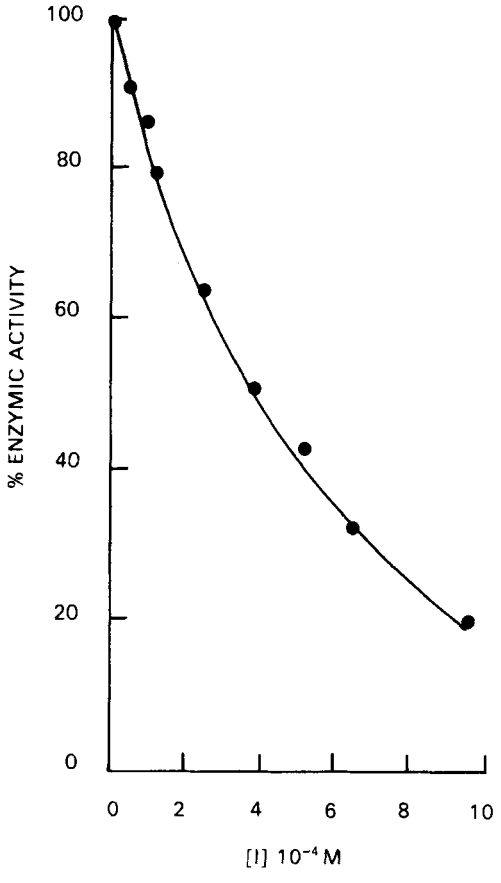


FIGURE 3 Inhibition of guanidinobenzoatase by *m*-iodobenzyl-guanidine assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate.

mental model. This evidence might provide an explanation for the successful use of radio labelled *m*-iodobenzylguanidine in the location of phaeochromocytomas.[13,14]

Inhibition of guanidinobenzoatase by Camostate or FOY-305

Camostate or FOY-305 is a derivative of guanidinobenzoate that is claimed to have a wide specificity for the inhibition of seryl proteases.[15] The structure of FOY-305[16] (see Scheme 1), suggests that it could act as an active site titrant for trypsin-like enzymes in the manner of *p*-nitrophenyl-*p*-guanidinobenzoate[17] and 4-methylumbelliferyl-*p*-guanidinobenzoate[18] but does not produce a chromophore in this inhibition. FOY-305 inhibited guanidinobenzoatase (Figure 4). In view of the ability of guanidinobenzoatase to cleave the other active site titrants for trypsin as true substrates,[1,2] we considered the possibility that the inhibition shown in Figure 4 might be an apparent inhibition due to FOY-305 acting as a competitive substrate (similar to studies published previously[1]). Electrophoretic analysis of the incubation products of

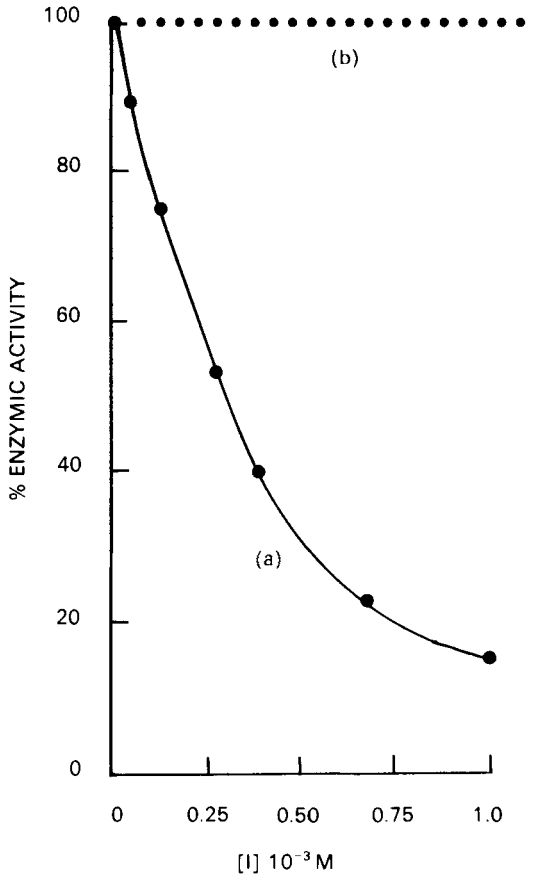
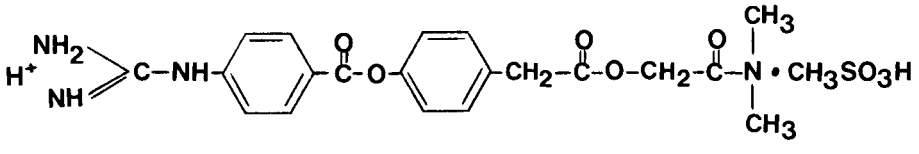


FIGURE 4 Inhibition of guanidinobenzoatase by FOY-305 assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. Curve (1) with FOY-305 showing inhibition. Line (b) with *N*-tosyl-DL-lysine chloromethyl ketone, showing no inhibition.

FOY-305 with guanidinobenzoatase demonstrated no cleavage products and only the presence of the original FOY-305. The data suggests that FOY-305 inhibits guanidinobenzoatase in solution and is not a substrate in the conventional sense of the word.

10^{-3} M FOY-305 in the presence of 10^{-3} M 9-aminoacridine prevented the binding of 9-aminoacridine to the active centre of guanidinobenzoatase on leukaemia cells (see following paper for this technique[8]) and to guanidinobenzoatase on the surface of formalin treated pancreatic cells.[3] These results confirm the inhibition of guanidinobenzoatase in solution and attached to cell surfaces by FOY-305. By contrast,



SCHEME 1 Structure of FOY-305.

another widely used inhibitor of trypsin-like enzymes, *N*-tosyl-DL-lysine chloromethylketone (TLCK), has no inhibitory action[1] on guanidinobenzoatase (Figure 4). These results are consistent with the substrate specificity of guanidinobenzoatase[1] which indicated a requirement for guanidino-groups or arginyl peptides. In this regard it is worth noting that a tumour associated protease with a preference for guanidinophenylalanine has recently been reported.[19] The enzyme described by the Japanese group is not inhibited by 2 mM $HgCl_2$ whereas guanidinobenzoatase is strongly inhibited by $HgCl_2$ (Figure 5). It would seem that these two tumour associated enzymes are similar in their requirement for *N*-substituted guanidine derivatives but that they may be distinguished by inhibition kinetics in the presence of $HgCl_2$.

Benzamidine derivatives fail to inhibit guanidinobenzoatase

Benzamidine is a well known inhibitor of trypsin and trypsin-like enzymes[20] but has no inhibitory action on guanidinobenzoatase (Figure 6). The introduction of an extra

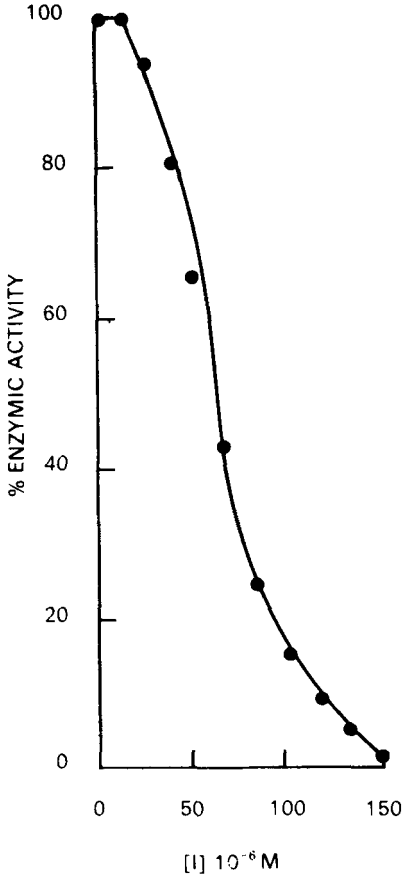


FIGURE 5 Inhibition of guanidinobenzoatase by mercuric chloride assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate.

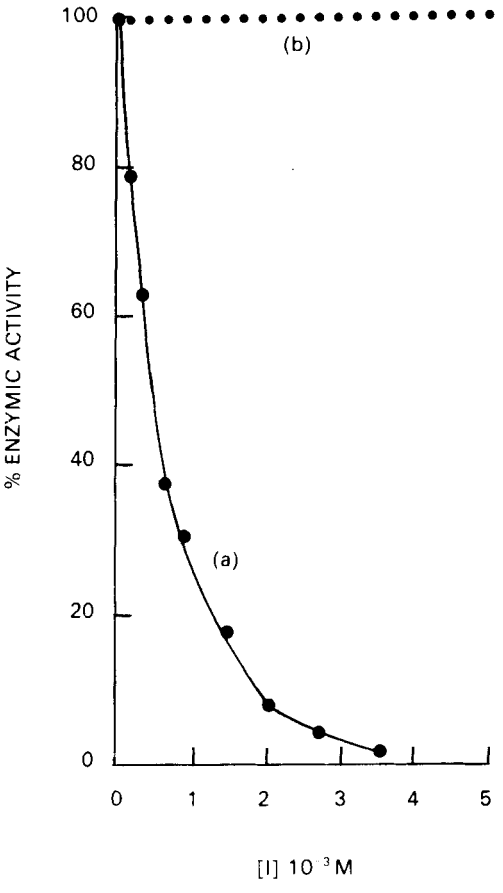


FIGURE 6 Apparent Inhibition of guanidinobenzoatase by *m*-aminobenzamidine assayed with 4-methyl-*p*-guanidinobenzoate as substrate. Curve (a) with *m*-aminobenzamidine showing apparent inhibition. (see text). Line (b) with either benzamidine or with benzamine, both of which failed to inhibit.

amino group, for example in *p*- and *m*-aminobenzamidine, leads to inhibition of trypsin but not guanidinobenzoatase. Similarly benzamine inhibits trypsin but not guanidinobenzoatase.

It is worth pointing out that incremental additions of *p*- and *m*-aminobenzamide to the assay of guanidinobenzoatase leads to an apparent inhibition (Figure 6). This apparent inhibition is due to the quenching of methylumbelliferone fluorescence by the added aminobenzamide giving the misleading impression of enzymic inhibition. No such inhibition was observed when using *p*- nitophenyl-*p*-guanidinobenzoate as substrate.

Fluorescein monoguanidinobenzoate acts as a substrate for guanidinobenzoatase

A series of papers[21–23] have recently been published on fluorescein and rhodamine substituted guanidino-derivatives. Some of these acted as substrates and others as active site titrants for trypsin. We employed fluorescein monoguanidinobenzoate (an

active site titrant for trypsin) to determine whether this compound could be used as an active site titrant for guanidinobenzoatase. It was observed that fluorescein monoguanidinobenzoate did not inhibit guanidinobenzoatase but was progressively hydrolysed as a substrate (Figure 7) rather than exhibiting the expected burst analysis of an active site titrant.[17]

The continuous cleavage of the guanidinobenzoate moiety from this substrate, as well as from nitrophenyl-*p*-guanidinobenzoate and 4-methylumbelliferyl-*p*-guanidinobenzoate,[1,2] suggests that guanidinobenzoate present at the active centre of our enzyme has no inhibitory action. Since this is the product of enzymic cleavage there can be no question about the neighbouring carboxyl group in guanidinobenzoate preventing this molecule reaching the active centre of guanidinobenzoatase. Independent kinetic studies with up to 10^{-2} M guanidinobenzoate indicated that this molecule

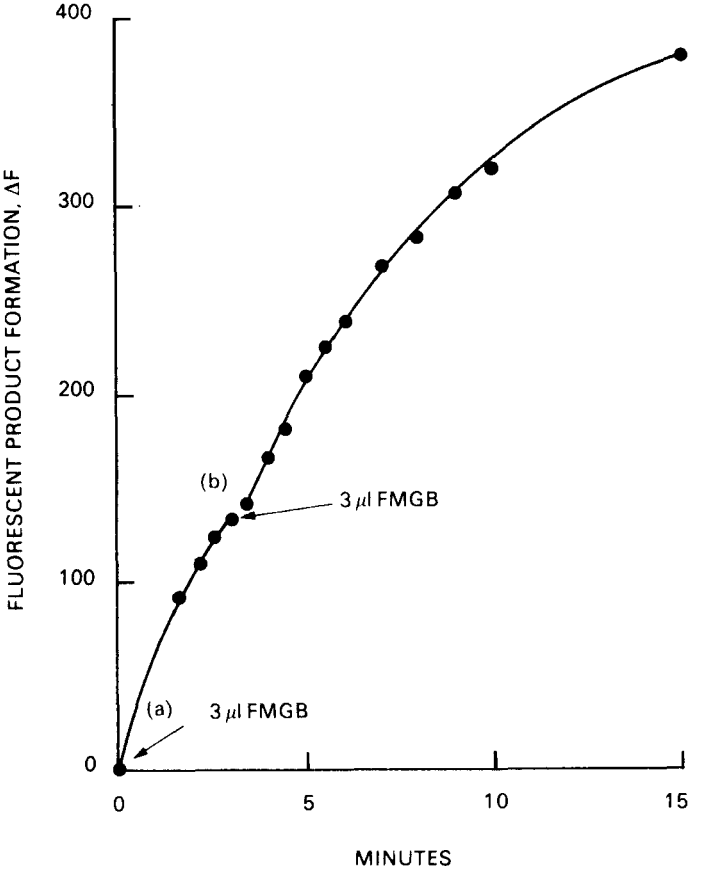


FIGURE 7 Continuous cleavage of fluorescein monoguanidinobenzoate by guanidinobenzoatase. The assay system contained 50 μl mouse ascitic plasma in 3 ml isotonic saline containing 0.1 M phosphate buffer, pH 7.2. The excitation was at 491 nm and the emission was at 514 nm: the fluorescent emission (ΔF) was recorded against time after two additions of 3 μl fluorescein-monoguanidinobenzoate (3.3×10^{-3} M) at (a) and (b). The final concentrations of substrate were 1×10^{-6} M and 2×10^{-6} M at (a) and (b) respectively.

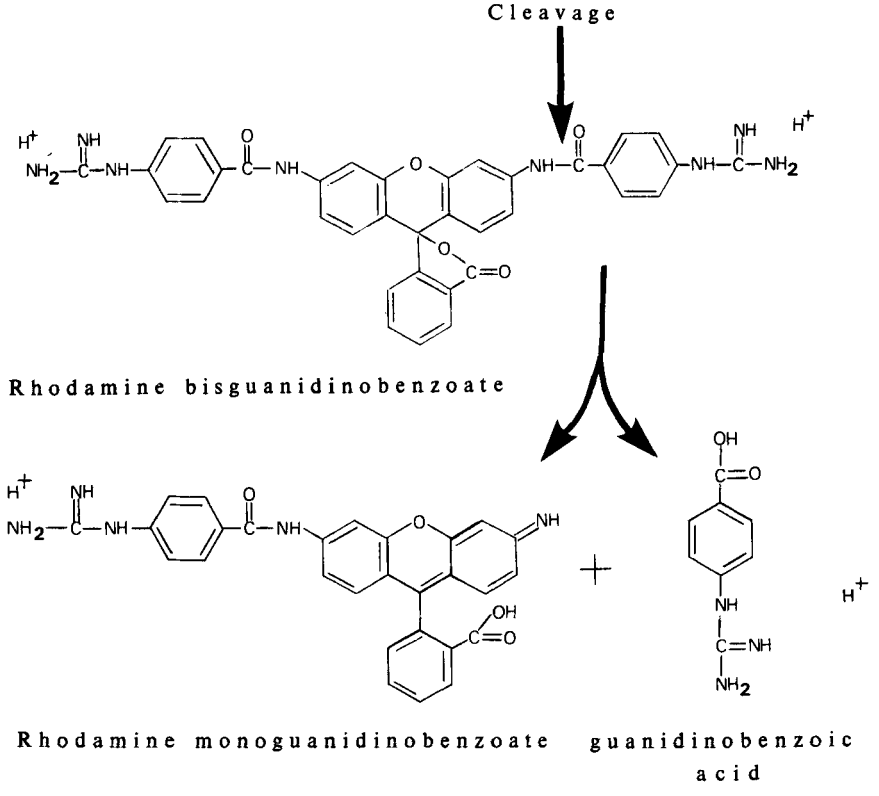
did not inhibit the cleavage of 4-methylumbelliferyl-*p*-guanidinobenzoate by our enzyme (data not presented here).

Fluorescein monoguanidinobenzoate did not compete with 9-aminoacridine for the binding site at the active centre of guanidinobenzoatase on leukaemia cells in the kidney sections (see following paper).[8] This result confirms that neither fluorescein monoguanidinobenzoate nor its cleavage products inhibit the cell surface-bound enzyme.

Rhodamine bisguanidinobenzoate acts as an active site titrant for guanidinobenzoatase

Rhodamine bisguanidinobenzoate in HEPES buffer pH 7 does not exhibit fluorescence at 523 nm; when excited at 492 nm, however, the removal of one guanidinobenzoate moiety results in intense fluorescence (see diagram of chemistry involved, Scheme 2) under these conditions. This compound can be used to carry out active site titration of trypsin with a burst of product formation as initially described for *p*-nitrophenyl-*p*-guanidinobenzoate[17] and 4-methylumbelliferyl-*p*-guanidinobenzoate.[18]

Guanidinobenzoatase is inhibited by incremental additions of rhodamine bisguanidinobenzoate with the simultaneous production of a fluorescent product that is



SCHEME 2 Structures proposed for rhodamine derivatives after Leytus *et al.*[23]

believed to be rhodamine monoguanidinobenzoate (Figure 8), since neither the parent compound, rhodamine, nor the rhodamine bisguanidinobenzoate fluoresces under these conditions. This conclusion would be in accord with the cleavage of CbzArg from $(CbzArg)_2$-rhodamine described by Mangel and his colleages.[23] Typical burst analysis of an active site titration was obtained when four aliquots of crude guanidinobenzoatase were added to a fixed quantity of rhodamine bisguanidinobenzoate at 2 min intervals (Figure 9b) or when a fixed quantity of rhodamine bisguanidinobenzoate was added to incremental additions of enzyme (Figure 9a).

Rhodamine bisguanidinobenzoate, unlike the other three guanidinobenzoates used in this study, is a useful active site titrant for guanidinobenzoatase rather than a substrate for this enzyme. Guanidinobenzoatase is not present in serum as an active enzyme but a latent form of guanidinobenzoatase can be detected with rhodamine bisguanidinobenzoate by the burst of fluorescent product formed (similar to Figure

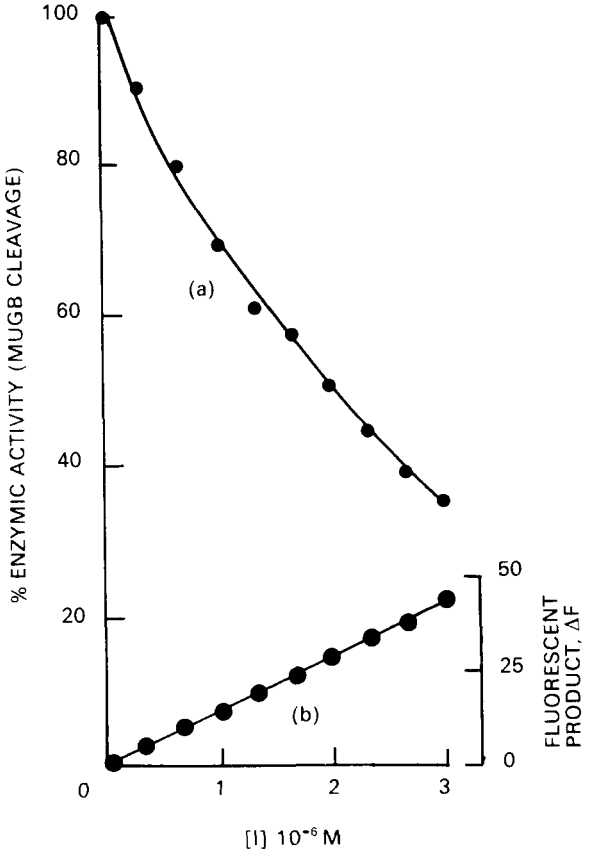


FIGURE 8 Inhibition of guanidinobenzoatase by rhodamine bisguanidinobenzoate assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate. Curve (a) with rhodamine bisguanidinobenzoate showing inhibition with 20 μl mouse ascitic plasma per 3 ml assay. Line (b), fluorescent product formed with rhodamine bisguanidinobenzoate, when excited at 492 nm and emitting at 523 nm. The analysis shown in curve (a) was performed first and the fluorescent spectrometer wavelengths then altered to obtain the data presented in line (b).

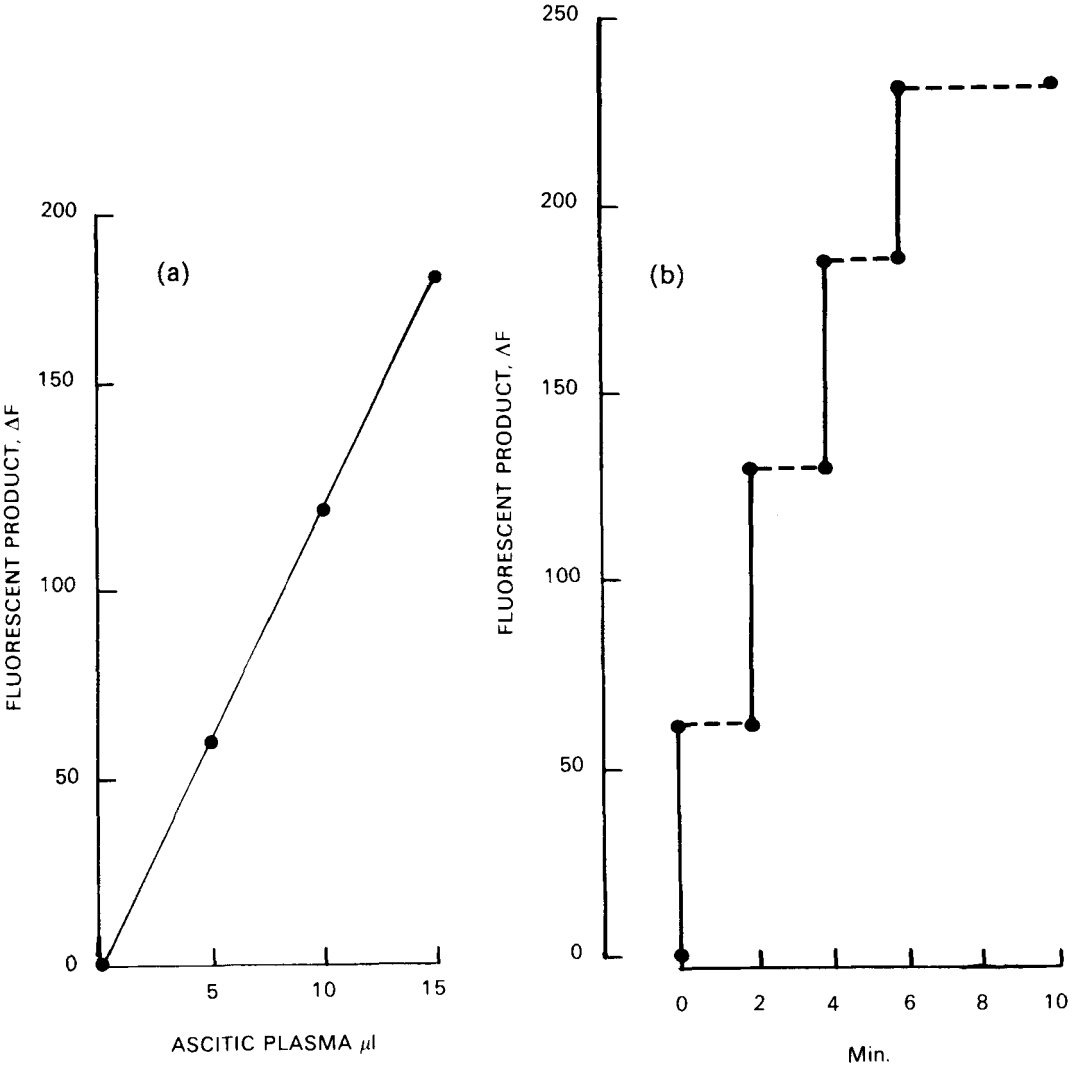


FIGURE 9 Active site titration of guanidinobenzoatase by rhodamine bisguanidinobenzoate. Instantaneous product formation with 3 ml of 0.025 M HEPES buffer, pH 7.4, containing 15% ethanol and 1.6×10^{-5} M rhodamine bisguanidinobenzoate were reacted with increments of mouse ascitic plasma containing guanidinobenzoatase. Product formation was measured as fluorescent emission at 523 nm when excited at 492 nm immediately after mixing the added enzyme. (a) Independent assays with 5, 10, 15 μl ascitic plasma in different cuvettes. (b) Consecutive assays with 5, 10, 15, 20 μl ascitic plasma added to the same cuvette in stepwise manner and followed over 10 min.

9a). It could be argued that this product could be formed from the action of rhodamine bisguanidinobenzoate on other trypsin-like enzymes or enzyme-inhibitor complexes.[24] This argument can be refuted by the fact that serum treated with excess 4-methylumbelliferyl-*p*-guanidinobenzoate (active site titrant for trypsin-like enzymes[18]) still gave the same burst of product formation when treated with rhodamine

bisguanidinobenzoate. The ability to titrate trypsin-inhibitor complexes with 4-methylumbelliferyl-*p*-guanidinobenzoate has already been established;[24] it seems highly probable that rhodamine bisguanidinobenzoate is capable of a similar titration of latent guanidinobenzoatase in serum. The fluorescent microscopy studies[8] with a crude preparation of rhodamine bisguanidinobenzoate indicate the strong affinity of the fluorescent product for the active centre of guanidinobenzoatase and this would indicate that the fluorescent product remains attached to guanidinobenzoatase as the result of the initial suicidal cleavage performed by guanidinobenzoatase. We have employed crude rhodamine bisguanidinobenzoate to displace guanidinobenzoatase bound to agmatine CH-Sepharose-4B, resulting in a protein band migrating in sodium dodecyl sulphate polyacrylamide gels with the characteristics of guanidinobenzoatase (data not presented here).

The data presented above indicate that rhodamine bisguanidinobenzoate can be used to displace guanidinobenzoatase from inhibitors and can therefore be employed to assay latent forms of the enzyme in biological fluids such as serum in a simple two-step reaction with 4-methylumbelliferyl-*p*-guanidinobenzoate. If guanidinobenzoatase in serum has any significant relationship to a patient's tumour loading it may be possible to assess this loading by means of a simple active site titration. This possibility will be explored in future when more of this new reagent becomes available. It is significant that this active site titrant is cleaved at a peptide bond rather than an ester bond (as is the case in the three guanidinobenzoates cleaved as substrates by our enzyme). This fact excludes the possibility of an esterase being assayed in serum by rhodamine bisguanidinobenzoate.

It has recently been established that the rhodamine bisguanidinobenzoate used in kinetic studies was impure. This means that the active site titrant for guanidinobenzoatase may not be the bisguanidinobenzoate although the generation of a cleavage product with the fluorescent properties of a rhodamine derivative in a typical burst analysis suggest that this is the case.

We conclude that our enzyme is distinct from trypsin, plasminogen activator, plasmin, thrombin and the newly described Japanese enzyme.[19] We have described competitive and non-competitive inhibitors that interact as the active centre of our enzyme, both in solution and on the surface of leukaemia cells in resin sections. These studies led to the development of a number of new fluorescent inhibitors which could be used to locate this enzyme on the surface of cells in resin sections.[8]

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