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EVIDENCE FOR EXCHANGE OF INHIBITORS WHICH BIND TO THE ACTIVE SITE OF TRYPSIN

DISPLACEMENT OF ONE INHIBITOR WITH A COMPETITIVE INHIBITOR

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Two classes of inhibitors of trypsin (EC 3.4.21.4) have been studied, viz. active site-directed agents such as ovomucoid and active site titrants such as 4-methylumbelliferyl-4-guanidinobenzoate. The kinetics of β-naphthylamidase inhibition by an active site-directed agent were markedly different from simultaneous assays of the availability of the active site towards active site titrants in the presence of the active site-directed agents. Analysis of these data indicated an exchange of active site-directed agent by subsequent addition of active site titrant. One class of trypsin inhibitor could be displaced by another from the trypsin active centre. Competitive chase experiments were designed to measure this exchange in which the active site-directed agent was first equilibrated with trypsin, then partially displaced by incremental additions of an active site titrant; the degree of active sites occupied by these two agents was then determined by active site titration with a second reagent.

Introduction

Thiol compounds have previously been shown to reversibly inhibit trypsin (EC 3.4.21.4) [1] and collagenase [2] by a disulphide exchange mechanism involving a significant disulphide on the enzyme molecule required for the maintenance of the active centre in the correct conformation for the expression of biological activity. We wondered if it were possible to demonstrate exchange reactions involving specific peptide and protein inhibitors of trypsin which are known to bind directly to the active site, without employing changes in pH and ionic strength such as are routinely employed in affinity chromatography of these enzymes and inhibitor systems. Ohlsson [3] has previously demonstrated the in vivo exchange of the inhibitor in [^{125}I]trypsin-α_1-antitrypsin complex in dogs with α_2-macroglobulin but we know of no in vitro exchange studies of the type which we describe below.

Abbreviations: BANA, *N*-benzoyl-L-arginine β-naphthylamide; NPGB, *p*-nitrophenyl-4-guanidinobenzoate-HCl; MUGB, 4-methylumbelliferyl-4-guanidinobenzoate-HCl; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone.

Materials

Soybean trypsin inhibitor, ovomucoid, crystalline papain (EC 3.4.22.2), *N*-α-tosyl-L-lysine-chloromethyl ketone (TLCK), 4-methylumbelliferyl-4-guanidinobenzoate (MUGB), *p*-nitrophenyl-4-guanidinobenzoate (NPGB) and *N*-benzoyl-L-arginine-β-naphthylamide (BANA) were purchased from Sigma. Crystalline trypsin (EC 3.4.21.4) was purchased from Boehringer, Mannheim and aprotinin was kindly provided by Bayer as 'Trasylol' 10 000 KIU/ml. The aldehyde leupeptin was bought from Scientific Marketing Associates, London.

Methods

Soybean trypsin inhibitor derivatives. Fluorescein-labelled soybean trypsin inhibitor was obtained by

coupling soybean trypsin inhibitor to trypsin-Sepharose prior to reacting the bound soybean trypsin inhibitor with fluorescein-iso-thiocyanate. The fluorescein-labelled soybean trypsin inhibitor was eluted from the exhaustively washed soybean trypsin inhibitor-trypsin-Sepharose complex with 10^{-3} M HCl. The fluorescein-labelling carried out under these conditions resulted in a labelled 'protected' soybean trypsin inhibitor which was shown to inhibit free trypsin and bind to trypsin-Sepharose.

Papain digestion of soybean trypsin inhibitor was carried out with an enzyme: substrate ratio of 1 : 100 for 8 h at 37°C. The papain digest was dialysed against distilled water and the diffusible peptides freeze-dried prior to analysis.

Simultaneous β-naphthylamidase assay and active site titration of trypsin in the presence of inhibitor. Active site titration requires much greater quantities of enzyme than are necessary for routine assays. We therefore used 200 μg trypsin/3 ml 0.1 M phosphate buffer, pH 6.0, as our stock solution in each of a series of 5 ml capped plastic tubes. Three tubes were retained as controls with no inhibitor added, the remaining tubes contained incremental additions of inhibitor (e.g., leupeptin or ovomucoid). The tubes were equilibrated at 37°C for 5 min, and then 100 μl aliquots from each tube transferred to 3 ml 0.15 M Tris/HCl pH 8.0 buffer for assay with 40 μl BANA solution (32 mg BANA/ml dimethyl sulphoxide). The β-naphthylamidase activity was determined fluorimetrically [4] after 1 h at 37°C and the enzyme activity in each tube calculated as a percentage of the control enzyme activity in which no inhibitor was added.

The remaining 2.9 ml stock solution containing 193 μg trypsin was immediately reacted with 10 μl 1 mM MUGB dissolved in dimethylformamide, to give a final concentration of 3.3 μM MUGB. The active site titration technique of Coleman et al. [5] was carried out immediately, the released 4-methylumbelliferone being determined fluorimetrically. The results obtained for each tube were calculated as a percentage of the active site titration yielded by the control tubes which contained no added inhibitor. A plot of the percentage active sites available to MUGB titration against the concentration of added inhibitor (e.g., leupeptin or ovomucoid) defined the distribution of active sites occupied by the added inhibitor in competition with the added MUGB.

Competitive chase experiments. Both MUGB [5] and NPGB [6] are active site titrants specifically designed for trypsin. If 200 μg trypsin is first exposed to an inhibitor (e.g., 200 μg ovomucoid, virtually all the β-naphthylamidase activity is inhibited) and subjected to incremental additions of NPGB (0–3.3 μM) some of this NPGB will fill active sites on the trypsin which were previously blocked by ovomucoid. In our experiments the product of NPGB reaction with the active site is nitrophenol and this has been ignored since artefactual nitrophenol forms after the initial burst analysis [6]. We can measure the remaining unblocked sites by a subsequent active site titration with 10 μl MUGB (as above) added to every tube. The MUGB is in excess of the total trypsin molarity and the product of MUGB titration is very stable, unlike the NPGB assay.

The procedure is as follows. A series of plastic tubes contained 3 ml 1 mM phosphate buffer and 200 μg trypsin. We then added a fixed quantity of inhibitor (e.g., 200 μg ovomucoid) to each tube, leaving three control tubes and one blank tube. The tubes were equilibrated at 37°C for 5 min, then incremental additions of 1 mM NPGB were added to yield a range of final concentrations of 0–3.3 μM. The contents of the tubes were again mixed and left at 37°C for 5 min. Finally 10 μl 1 mM MUGB was added, the contents of the tubes mixed and left at 37°C for 10 min prior to fluorimetric analysis [5] of the released 4-methylumbelliferone. The MUGB active site titration value was expressed as a percentage of the control tubes in which no inhibitor had been added. These results were then plotted against the NPGB molarity in each tube for each inhibitor studied.

Results and Discussion

Initial observations

Preliminary studies with trypsin-Sepharose mixed with a known quantity of soybean trypsin inhibitor enabled us to calculate the quantity of soybean trypsin inhibitor bound to the trypsin-Sepharose. The soybean trypsin inhibitor-trypsin-Sepharose was exhaustively washed with 1% NaCl/0.15 M Tris/HCl, pH 8.0, buffer and then equilibrated with a known quantity of trypsin; after 5 min the supernatant frac-

tion was removed from the soybean trypsin inhibitor-trypsin-Sepharose and assayed for trypsin activity with the β-naphthylamidase assay [4]. Partial inhibition of the added trypsin was observed resulting from an exchange of soybean trypsin inhibitor from the soybean trypsin inhibitor-trypsin-Sepharose to the added trypsin in free solution. We were able to confirm the exchange by adding fluorescein-labelled soybean trypsin inhibitor to trypsin-Sepharose and subsequently measuring the release of labelled soybean trypsin inhibitor in the presence of added trypsin in free solution. We concluded that soybean trypsin inhibitor bound to trypsin could be exchanged.

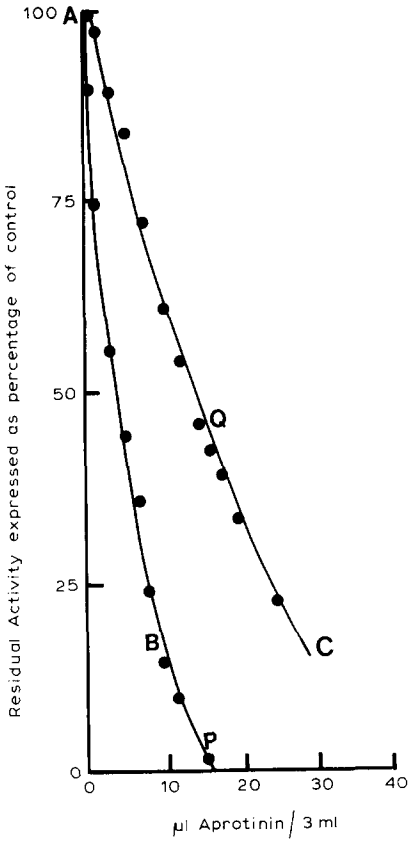
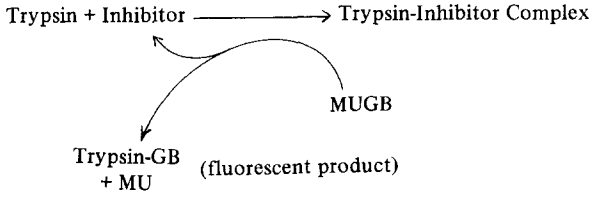


Fig. 1. Inhibition of trypsin by aprotinin. The control value (200 µg trypsin) is shown by A. Curve AB represents inhibition of β-naphthylamidase activity and curve AC represents the decline in active site titratability with MUGB.

Simultaneous β-naphthylamidase assay and active site titration of trypsin in the presence of inhibitor

Aprotinin (Trasylol) inhibition of trypsin assayed as β-naphthylamidase activity is presented in Fig. 1 (curve AB) whilst the availability of active sites in trypsin in the same stock solution of aprotinin is presented in Fig. 1 (curve AC). If there were no exchange reactions taking place, curves AB and AC should be identical and 15 µl aprotinin (150 KIU) would be expected to inhibit 200 µg trypsin β-naphthylamidase activity (point P) and also block all the active sites for MUGB binding. In fact 15 µl aprotinin resulted in 45% of the total number of active sites in trypsin being available for MUGB titration (point Q). The results would suggest MUGB displaces aprotinin from the active centre of aprotinin-inhibited trypsin molecules resulting in cleavage of MUGB and release of methylumbelliferone which is detected fluorimetrically (curve AC). The area between these two curves represents exchange of aprotinin with MUGB.

We observed a similar displacement of β-naphthylamidase and MUGB titration curves for inhibition with soybean trypsin inhibitor, ovomucoid and the aldehydic peptide leupeptin (Figs. 2, 3 and 4) as well as TLCK and human serum (data not shown). We interpret these curves to mean that trypsin, previously complexed with soybean trypsin inhibitor, leupeptin, TLCK and aprotinin will exchange part of the bound inhibitor for the active site titrant MUGB as indicated below.



Competitive chase experiments

The data obtained with leupeptin (Fig. 4) suggest that with 25 µg leupeptin a marked inhibition of β-naphthylamidase activity took place, but that the addition of MUGB displaced all the leupeptin from the active centre of trypsin molecules (curve AC).

When 25 µg leupeptin was added to 200 µg trypsin followed by incremental additions of NPGB (0–3.3 µM) and then 10 µl MUGB (1 mM) to give a final

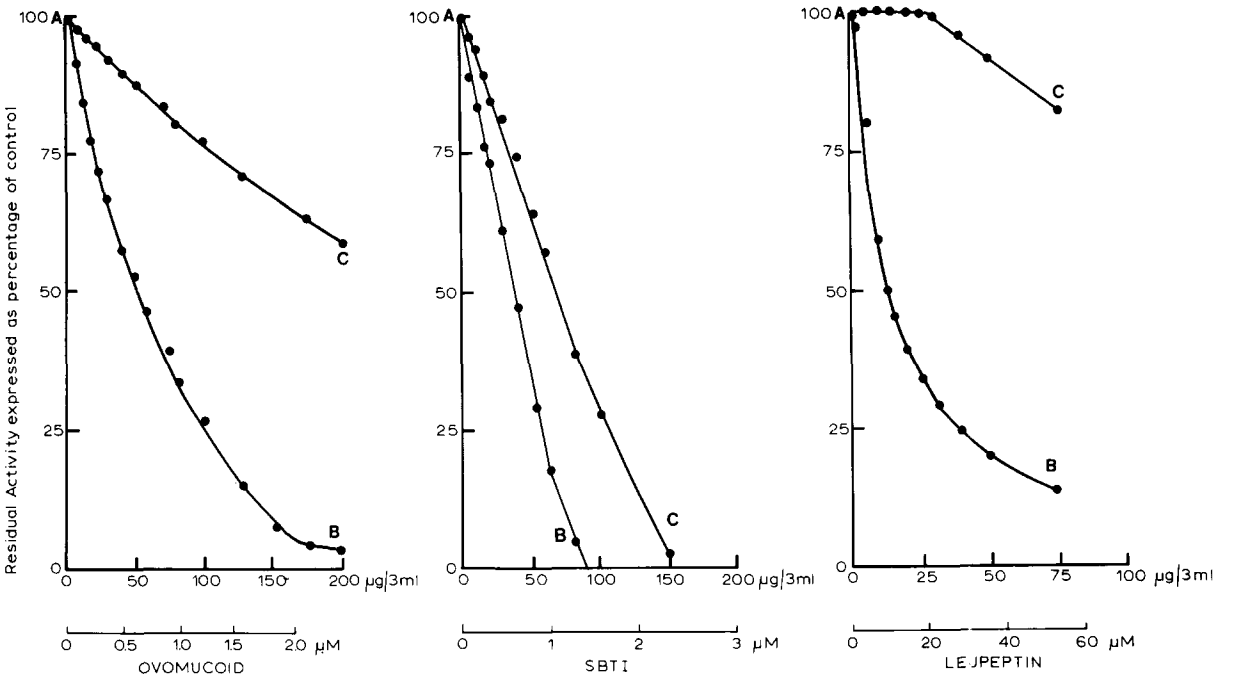


Fig. 2. Inhibition of trypsin with ovomucoid. Conditions as Fig. 1 (left).

Fig. 3. Inhibition of trypsin with soybean trypsin inhibitor. Conditions as Fig. 1 (middle).

Fig. 4. Inhibition of trypsin with leupeptin. Conditions as Fig. 1 (right).

concentration of 3.3 µM MUGB in a competitive chase experiment (Fig. 5) the data indicates that the MUGB displaced all the bound leupeptin and gave 100% of the expected yield of methylumbelliferone (point A) exactly as though no leupeptin had been added. On incremental addition of NPGB (which acts like MUGB but does not give a fluorescent product) the exchange of leupeptin took place along the curve ABC; but now for each molecule of NPGB replacing leupeptin on the trypsin, one less molecule of MUGB could react with the active centre since NPGB had already blocked this site. At C (3.3 µM NPGB) almost all the active sites of trypsin (approx. 2.8 µM) had been reacted with NPGB, as would be expected for an active site titrant.

It should be re-emphasised that the initial addition of 25 µg leupeptin caused over 50% inhibition of β-naphthylamidase activity (Fig. 4), yet this was displaced by 3.3 µM MUGB (point A on Fig. 5) prior to adding the NPGB. Exactly the same curve was obtained when 200 µg trypsin in the absence of leupeptin were subjected to the competitive chase experiment with NPGB followed by MUGB (Fig. 5 triangles). The inverse of this graph (Fig. 5) is shown in Fig. 6 as the percentage substitution of active centres by NPGB, as measured by MUGB product formation when 200 µg trypsin was directly titrated with 0–3.6 µM NPGB and adding 3.3 µM MUGB. It must be concluded that leupeptin is displaced so easily from the active site of trypsin by both NPGB and MUGB that the presence of leupeptin plays an insignificant role in this competitive chase experiment.

The ease with which leupeptin is displaced from the active site of trypsin may be due to the small size of this peptide inhibitor. We obtained a papain digest of soybean trypsin inhibitor and employed the diffusible peptides in a similar assay of trypsin inhibition by β-naphthylamidase and MUGB titratability. The active peptides in the digest gave inhibition kinetic data (data not shown) similar to leupeptin (Fig. 4) as oppsed to the kinetic data obtained with intact

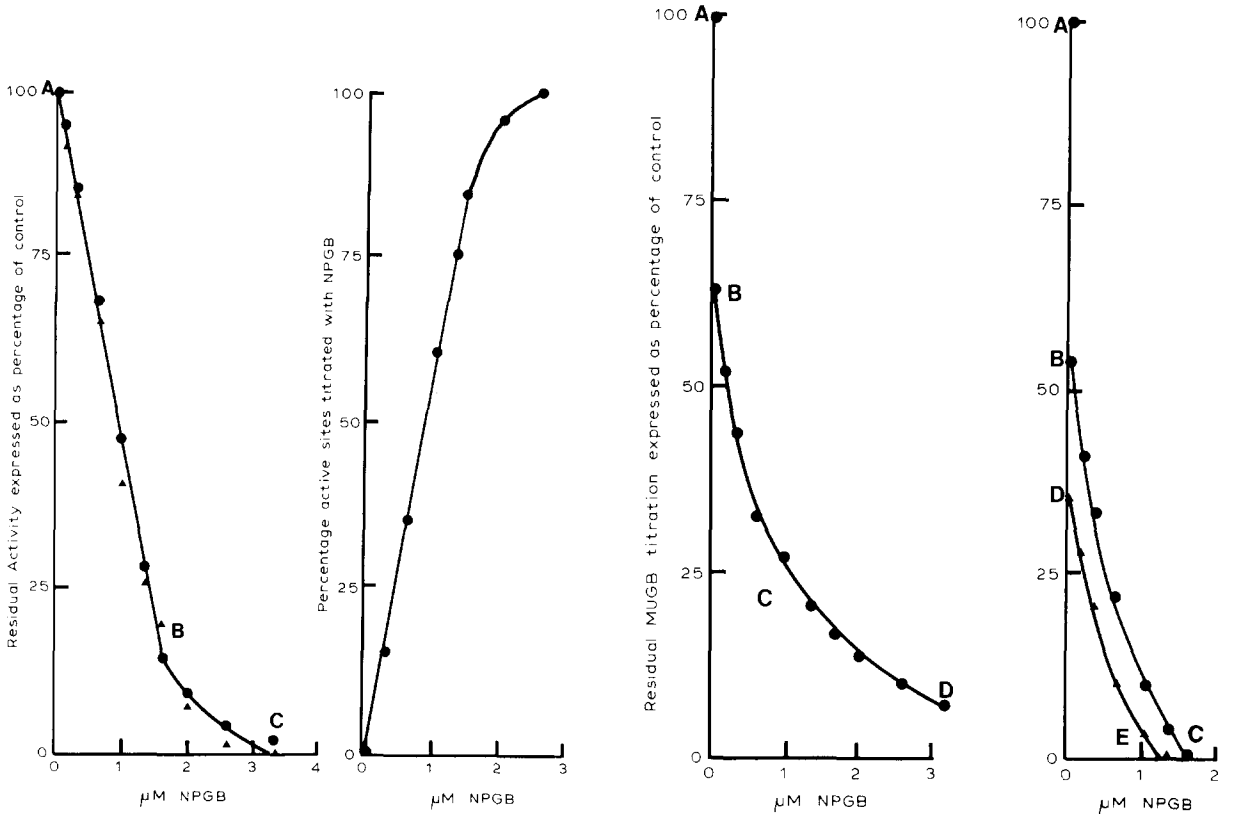


Fig. 5. Inhibition of trypsin by leupeptin, exhibited by competitive chase with NPGB and MUGB titration. (i) Curve ABC (●——●) MUGB assay. The control at A contained 200 μg trypsin and no leupeptin or NPGB. All other tubes contained 25 μg leupeptin, followed by 0–3.3 μM NPGB and finally assayed with 3.3 μM MUGB expressed as percentage on the vertical axis. (ii) Curve ABC (▲——▲). The same procedure as (i) but in the absence of leupeptin (left-hand).

Fig. 6. Active site titration of trypsin by NPGB measured by MUGB. Data derived from Fig. 5 (ii) (left centre).

Fig. 7. Inhibition of trypsin by ovomucoid, exhibited by competitive chase with NPGB and MUGB titration. The control at A contained 200 μg trypsin, all tubes then received 200 μg ovomucoid and NPGB was added over the range 0–3.3 μM to produce the curve BCD after MUGB active site titration (right centre).

Fig. 8. Inhibition of trypsin by aprotinin, exhibited by competitive chase with NPGB and MUGB titration. Similar to Fig. 7. Curve BC produced with 10 μl aprotinin per tube and curve DE produced with 15 μl aprotinin per tube (right).

soybean trypsin inhibitor (Fig. 3).

Similar types of exchange could be shown with soybean trypsin inhibitor, ovomucoid and Trasylol bound to trypsin and followed by competitive chase experiments with NPGB followed by MUGB titrations. In these exchange reactions, some initial inhibitor remains bound (point B, Fig. 7) which is not readily displaced by MUGB. At B the 200 μg trypsin has 200 μg ovomucoid added with 95% inhibition of β-naphthylamidase activity, yet 63% of the active sites were available to MUGB. Incremental addition of NPGB along curve BCD resulted in titration with NPGB rather than MUGB, as would be the expected pattern from the analysis of Fig. 5.

The data in Fig. 1 suggest that aprotinin was not as readily displaced from trypsin by MUGB as was leupeptin (Fig. 4). This suggestion was confirmed by the competitive chase experiments presented in Fig. 8 in which trypsin was preincubated with aprotinin. Two parallel MUGB-NPGB titration curves are shown.

In the first case, 10 μl aprotinin were reacted with 200 μg trypsin (curve BC, Fig. 8) having the equivalent β-naphthylamidase activity of 15% (point B on Fig. 1) and having 54% of the active sites available to MUGB (point B on Fig. 8). In the second case, 15 μl aprotinin were reacted with 200 μg trypsin (curve DE, Fig. 8), as the equivalent β-naphthylamidase activity was zero (point P, Fig. 1) whilst 35% of the active sites were available to MUGB (point D, Fig. 8). These results can only be explained if the MUGB and NPGB are able to displace aprotinin (Fig. 8) from the active centre of trypsin even though the displacement is less effective than the displacement of leupeptin (Fig. 4).

We can conclude that the active site titrants which form irreversible complexes with the active site of trypsin displace naturally-occurring and some synthetic (e.g., TLCK) inhibitors of trypsin. These exchangeable inhibitors must therefore be involved in equilibrium reactions with the enzyme active centre. These equilibria may be shifted without changes in pH or ionic strength of the environment by the addition of a suitable competitive inhibitor. These exchange reactions, involving two or more inhibitors of trypsin, could not be followed by conventional kinetic analysis employing a single assay of a trypsin-cleavable substrate, since the presence of more than one inhibitor would just produce further inhibition but not discriminate between which inhibitor was bound to the enzyme. Competitive inhibition with selective active site titrants allows such an analysis to be performed readily.

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