Binding of anhydrotrypsin to α_2 -macroglobulin

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Radiolabelled anhydrotrypsin was bound by α_2 M (α_2 -macroglobulin) sufficiently tightly to resist separation during gel electrophoresis; 2 mol of anhydrotrypsin were bound/mol of α_2 M, but the interaction differed in important respects from that between active proteinases and α_2 M. Anhydrotrypsin was bound by the electrophoretically 'fast' form of α_2 M, although much less effectively than by the 'slow' form. The inactive enzyme was displaced from α_2 M by trypsin inhibitors, the order of effectiveness being aprotinin > soya-bean trypsin inhibitor > benzamidine. Saturation of α_2 M with anhydrotrypsin did not prevent subsequent binding and inhibition of active trypsin by the α_2 M, and the anhydrotrypsin was not displaced during this reaction. Anhydrotrypsin bound by α_2 M retained its ability to react with antibodies against trypsin, whereas bound trypsin did not.

α_2 M is a plasma protein that binds and inhibits the great majority of proteinases. The complexes have little or no proteolytic activity, and are rapidly cleared from the circulation (reviewed by Starkey & Barrett, 1977). The recognition of proteinases by α_2 M seems to be highly specific for this group of enzymes, but does not depend upon the precise specificity of the enzymes or their catalytic mechanism.

The 'trap hypothesis' for the action of α_2 M (Barrett & Starkey, 1973) suggested that, in the first stage of reaction of α_2 M with a proteinase, the enzyme cleaves the inhibitor molecule in a sensitive region, and that this represents the recognition step in the specific interaction. The idea was supported by evidence that the inactive zymogens of various serine proteinases do not bind to α_2 M, and nor do enzymes inactivated with active-site-directed reagents such as Dip-F and Tos-Lys-CH₂Cl.

The trap hypothesis would become unacceptable, however, if there were any examples of a protein that reacted with α_2 M in the same way as a typical proteinase, but without causing a cleavage in the α_2 M molecule. For this reason, the recent report of Tsuru *et al.* (1978) that the binding of anhydrotrypsin to α_2 M resembles in some respects that of active trypsin requires careful consideration. In anhydrotrypsin, the serine residue essential for catalysis has been converted into inactive anhydro-

alanine, and yet Tsuru *et al.* (1978) found that the modified enzyme was tightly bound to α_2 M and decreased the capacity of the macroglobulin to inhibit active trypsin. We have therefore re-examined the binding of anhydrotrypsin to α_2 M.

Materials and methods

Bovine serum albumin, soya-bean trypsin inhibitor, phenylmethanesulphonyl fluoride, Tos-Lys-CH₂Cl, Dip-F and thermolysin were the products of Sigma (London) Chemicals Co., Poole, Dorset BH17 7NH, U.K. Tos-Phe-CH₂Cl was purchased from Bachem Feinchemikalien, Bubendorf CH-4416, Switzerland, benzamidine hydrochloride from Ralph N. Emanuel Ltd., Wembley, Middx. HA0 1PY, U.K., and aprotinin (Trasylol) was a gift of Dr. E. Philipp, Bayer A.G., D-56000 Wuppertal, Germany. Hammarsten casein and methylamine hydrochloride were obtained from BDH Chemicals Ltd., Poole, Dorset, BH12 4NN, U.K.; Sepharose 4B was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K., and bovine pancreatic trypsin was from Miles Laboratories Ltd. (Research Products), Slough SL2 4LY, Berks., U.K. Hide powder azure was from Calbiochem, Bishops Cleeve, Herts. CM23 3AL, U.K.

Rabbit anti-(human α_2 M) serum was prepared in this laboratory and rabbit anti-(bovine trypsin) serum was a gift from Dr. E. Shapira, The Children's Hospital, Chicago, IL, U.S.A. Ox haemoglobin was prepared as described by Barrett (1970), and human

Abbreviations used: α_2 M, α_2 -macroglobulin; Dip-F, di-isopropyl phosphorofluoridate; Tos-Lys-CH₂Cl, tosyl-lsylchloromethane; Tos-Phe-CH₂Cl, tosylphenylalanyl-chloromethane; Dip-trypsin, di-isopropyl phosphorotrypsin.

α_2 M, in the electrophoretically 'slow' or S-form, as described by Barrett *et al.* (1979).

Affinity chromatography was carried out by using soya-bean trypsin inhibitor bound to Sepharose 4B that had been activated with CNBr (Axén & Ernback, 1971).

Measurement of proteolytic activity

Proteolytic activity against hide powder azure was determined as described by Barrett *et al.* (1979).

Gel electrophoresis

Polyacrylamide-gel electrophoresis was done in 5 or 7% polyacrylamide gels, in an Ammediol (2-amino-2-methylpropane-1,3-diol) buffer system, as described by Barrett *et al.* (1979).

Binding of trypsin to soya-bean trypsin inhibitor

Trypsin and anhydrotrypsin were mixed with an excess of soya-bean trypsin inhibitor and run in polyacrylamide gels. Binding was indicated by the appearance of a new zone of protein with lower anodal mobility than the inhibitor.

Preparation of anhydrotrypsin

Anhydrotrypsin was prepared by a method based on that of Ako *et al.* (1972) with some important modifications, and purified by affinity chromatography.

Bovine pancreatic trypsin (200mg) and phenylmethanesulphonyl fluoride (70mg), in 100ml of 0.05 M-Tris/HCl, pH 7.0, containing 0.02 M-CaCl₂, were incubated at 20°C for 30 min with occasional additions of 0.02 M-NaOH to maintain the pH at 7.0. The reaction was stopped by adjusting the pH to 3.0 with 1 M-HCl. After centrifugation for 12000 g-min, the supernatant was dialysed overnight against 3 litres of 1 mM-HCl, at 4°C. Dip-F was added to the dialysed solution to give a final concentration of 2 mM, and the solution was incubated at 4°C for 60 min. The solution of phenylmethanesulphonyl-trypsin thus obtained was made 0.05 M-KOH at 0°C for 12 min then adjusted to pH 5.0 with formic acid to stop the reaction. The mixture was adjusted to pH 8.0 by addition of 2 M-Tris/HCl, pH 9.0, and Dip-F was added to give a final concentration of 5 mM. After 60 min incubation at 4°C the mixture was applied to a column (2 cm × 13 cm; 40 cm³) of soya-bean trypsin inhibitor-Sepharose and the column washed with 100 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 60 mM-CaCl₂ and 50 mM-NaCl.

The bound material was eluted in two steps, with 100 ml of 100 mM-sodium formate buffer, pH 3.0, and 100 ml of HCl/KCl buffer, 100 mM, pH 2.0 respectively.

The protein-peak fractions obtained from each

step were examined for proteolytic activity and the ability to form complexes with soya-bean trypsin inhibitor.

Measurement of anhydrotrypsin concentration

Anhydrotrypsin concentration was measured by the protein determination of Lowry *et al.* (1951), trypsin being used as standard.

Radioiodination of proteins

Protein (0.1–0.5 mg) in 0.05 M-sodium phosphate buffer, pH 7.0, was mixed with 500 μ Ci of carrier-free ¹²⁵I (as iodide in NaOH; The Radiochemical Centre, and 100 μ g of chloramine-T (in 0.1 ml of the phosphate buffer) was added. After thorough mixing, the solution was left for 2–5 min at 0°C before the addition of 100 μ g of sodium metabisulphite (also in 0.1 ml of phosphate buffer) to stop the reaction.

The radiolabelled anhydrotrypsin was re-isolated by affinity chromatography on (soya-bean trypsin inhibitor)-Sepharose as described above, and other labelled proteins by passage through a column (1 cm × 13 cm; 10 cm³) of Sephadex G-25, in 0.05 M-phosphate buffer, pH 7.0.

Radioactivity was measured with a Packard BPGD Autogamma counter.

Radioautography

Gels were dried on to filter paper (Whatman 3MM) and exposed to Fuji Rx-medical X-ray film for 24–48 h, which was then developed with Kodak DX-80 developer. The A_{600} of bands on radioautographs was determined with a Gilford model 2502 gel scanner linked to a Smiths Servoscribe flat-bed recorder with integrator.

Immunoelectrophoresis

Immunoelectrophoresis in agarose gels buffered with Tris/borate, pH 8.0, was performed as described by Barrett (1974).

Preparation of α_2 M in the S- and F-forms.

Electrophoretically 'slow' (S-) α_2 M was prepared as described by Barrett *et al.* (1979). It was converted into the electrophoretically 'fast' (F-) form by reaction with trypsin, thermolysin or methylamine hydrochloride as follows. Trypsin and thermolysin were incubated with α_2 M at a molar ratio of 2:1 for 5 min at 20°C in 100 mM-Tris/HCl/20 mM-CaCl₂, pH 8.0, then the enzymes were inhibited by making the solutions 5 mM with respect to Dip-F or EDTA, as appropriate, and by allowing the samples to stand at 20°C for a further 30 min. α_2 M in the same Tris buffer was made 200 mM with respect to methylamine hydrochloride and incubated at 20°C for 30 min before use.

Results and discussion

Preparation of anhydrotrypsin

The protein-peak fractions obtained by the two-step elution of the (soya-bean trypsin inhibitor)–Sephacrose column were examined for proteolytic activity against hide powder azure and for the ability to form complexes with soya-bean trypsin inhibitor. Such complexes are stable and can be detected by gel electrophoresis.

The material eluted in the first step was found to have proteolytic activity equivalent to 30%-active trypsin and also to form complexes with soya-bean trypsin inhibitor. Both of these activities were almost completely inhibited by 5 mM-Dip-F or -Tos-Lys-CH₂Cl.

The second eluted peak fraction, however, was shown to have proteolytic activity equivalent to less than 0.5%-active trypsin. Complexes with soya-bean trypsin inhibitor were again formed, and no inhibition of this was seen with Dip-F, Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl or phenylmethanesulphonyl fluoride.

We concluded that the material applied to the affinity-chromatography column contained appreciable amounts of active trypsin, but that the second peak was essentially pure anhydrotrypsin. The yield was 48 mg or 24%.

Binding of ¹²⁵I-labelled anhydrotrypsin to S- α_2 M

α_2 M (60 μ g) in 10 μ l of 100 mM Tris/HCl buffer, pH 8.0, was incubated with 1.3 μ g of labelled anhydrotrypsin (75 000 c.p.m.) (anhydrotrypsin/ α_2 M molar ratio 0.65) for 30 min at 20°C. The mixture was then run on a 5% polyacrylamide gel, together with other samples in which the α_2 M was replaced by bovine serum albumin, casein or haemoglobin. The gel was stained, dried and radioautographed. Pieces of the dried gel were also cut out and used for the measurement of radioactivity.

It was found (Fig. 1) that over 90% of the radioactivity in the S- α_2 M samples was associated with the single stained band of α_2 M, but there was no detectable binding to F- α_2 M or the other proteins.

In addition, a variety of other ¹²⁵I-labelled proteins (soya-bean trypsin inhibitor, bovine serum albumin, aprotinin, lysozyme and Dip-trypsin) were incubated with α_2 M as above. No binding of these proteins to α_2 M could be detected by the methods used above.

S- α_2 M was electrophoresed in 5% polyacrylamide gels before and after treatment with an excess (10:1 molar ratio) of anhydrotrypsin. No change in electrophoretic mobility was detected.

We concluded that the anhydrotrypsin was bound to the α_2 M sufficiently tightly not to be dissociated

Fig. 1. *Specificity of anhydrotrypsin binding to α_2 M* Mixtures of ¹²⁵I-labelled anhydrotrypsin with other proteins were run in electrophoresis in a 7% polyacrylamide gel. The gel was (a) stained for protein and (b) dried and radioautographed. The proteins were: (i) S- α_2 M; (ii) F- α_2 M; (iii) serum albumin; (iv) casein; (v) ox haemoglobin; (vi) anhydrotrypsin alone. Free anhydrotrypsin, being a very cationic protein, does not run into the gel in this system. See the text for details.

during gel electrophoresis, and with a high degree of specificity. The finding that no change in the electrophoretic mobility of the S- α_2 M was associated with this interaction distinguishes it from the binding of an active proteinase, which causes the formation of electrophoretically 'fast' F- α_2 M (Barrett *et al.*, 1979).

Displacement of bound anhydrotrypsin

An attempt was made to displace anhydrotrypsin from α_2 M by use of inhibitors of differing affinity for trypsin, aprotinin (K_i 6×10^{-14} ; Vincent & Lazdunski, 1972), soya-bean trypsin inhibitor (K_i 1.1×10^{-11} ; LeVillier *et al.*, 1974) and benzamidine (K_i 1.8×10^{-5} ; Mares-Guia & Shaw, 1965).

Labelled anhydrotrypsin was incubated with a

molar excess of $\alpha_2\text{M}$ as described above before the addition of aprotinin (25 μg), soya-bean trypsin inhibitor (100 μg) or benzamidine hydrochloride (to make 15 mM). The mixtures were incubated for a further 30 min at 20°C, and samples run on 5% polyacrylamide gels, which were radioautographed. Bound anhydrotrypsin was quantified as before, and also by scanning of the radioautograph (see the Materials and methods section).

Of the bound anhydrotrypsin, 60% was displaced by aprotinin, 30% by soya-bean trypsin inhibitor and none by benzamidine hydrochloride. The displacement of the bound anhydrotrypsin by soya-bean trypsin inhibitor was not increased by extending the incubation time to 24 h before gel electrophoresis, or by adding five times as much soya-bean trypsin inhibitor to the mixture. When ^{125}I -labelled active trypsin was used in place of anhydrotrypsin, there was no displacement of radioactivity with any of the inhibitors.

Similar experiments were carried out in which 100 μg of bovine serum albumin, casein or haemoglobin was added in place of the inhibitors.

No displacement of anhydrotrypsin was seen with any of the proteins. In addition, the presence of 0.5 M-Tris/HCl, pH 8.0, did not interfere with the binding of anhydrotrypsin to $\alpha_2\text{M}$.

In a further experiment, after labelled anhydrotrypsin and $\alpha_2\text{M}$ had been incubated together as usual, the $\alpha_2\text{M}$ was converted into the F-form by the addition of thermolysin in 2-fold molar ratio to $\alpha_2\text{M}$ (see the Materials and methods section). When samples were then allowed to react with the inhibitors described above, no displacement of bound anhydrotrypsin could be detected.

The fact that the bound anhydrotrypsin was partially displaced from the S- $\alpha_2\text{M}$ by the inhibitors, in the order of their affinity for trypsin, but not by 0.5 M-Tris/HCl, suggests that binding of anhydrotrypsin to $\alpha_2\text{M}$ is probably mediated through the specificity site and not simply an electrostatic interaction. Anhydrotrypsin bound by S- $\alpha_2\text{M}$

became much more firmly bound when the complex was treated with a proteinase. This phenomenon has been examined in detail (G. S. Salvesen, C. A. Sayers & A. J. Barrett, unpublished work).

Determination of binding ratio

Labelled anhydrotrypsin and $\alpha_2\text{M}$ in 100 mM-Tris/HCl, pH 8.0, were mixed together at molar ratios (anhydrotrypsin/ $\alpha_2\text{M}$) from 0.1 to 4.0 and incubated at 20°C for 30 min. Samples were run in 5% polyacrylamide gels and the percentage of anhydrotrypsin bound to the $\alpha_2\text{M}$ determined in the usual way. At molar ratios of anhydrotrypsin/ $\alpha_2\text{M}$ greater than 2.0, the percentage of anhydrotrypsin bound to $\alpha_2\text{M}$ decreased, indicating a molar binding ratio of 2:1 (Table 1).

In a further experiment, after the incubation of the anhydrotrypsin and $\alpha_2\text{M}$ at increasing molar ratios as before, soya-bean trypsin inhibitor was added in a large excess over the anhydrotrypsin and the mixture incubated at 20°C for a further 30 min. Gels were run and radioautographed as usual, then the amounts of anhydrotrypsin bound to the $\alpha_2\text{M}$ and to the soya-bean trypsin inhibitor were estimated both by cutting out the appropriate portions of the dried gel and measuring the amount of radioactivity, and by scanning the radioautograph.

It was found (Table 1) that the amount of anhydrotrypsin bound by the soya-bean trypsin inhibitor was 30% of the total radioactivity (c.p.m.) up to a molar ratio of 2.0 (consistent with the experiment in molar excess of $\alpha_2\text{M}$ described above), but at molar ratios greater than 2.0 the proportion of anhydrotrypsin bound to the soya-bean trypsin inhibitor increased as the molar ratio increased.

We concluded therefore that 2 mol of anhydrotrypsin were tightly bound/mol of $\alpha_2\text{M}$.

Possible competition between anhydrotrypsin and trypsin for binding to $\alpha_2\text{M}$

The possibility of competition between anhydro-

Table 1. *Effect of anhydrotrypsin/ $\alpha_2\text{M}$ molar ratio on the amount of anhydrotrypsin bound to $\alpha_2\text{M}$, and the amount bound to soya-bean trypsin inhibitor added subsequently*

Molar ratio (anhydrotrypsin/ $\alpha_2\text{M}$)	Anhydrotrypsin bound to $\alpha_2\text{M}$ (% of total radioactivity)	Anhydrotrypsin bound to soya-bean trypsin inhibitor (% of total radioactivity)
0.3	98	25
0.6	99	28
0.8	92	31
1.3	98	33
1.6	98	32
2.0	96	35
2.5	79	52
3.0	62	55
4.0	54	60

trypsin and trypsin was examined by determination of (a) trypsin-inhibiting activity of α_2 M pretreated with anhydrotrypsin, (b) binding of labelled trypsin to α_2 M pretreated with anhydrotrypsin, and (c) possible displacement of bound labelled anhydrotrypsin by subsequent treatment with trypsin.

The trypsin-inhibiting capacity of α_2 M that had been preincubated with anhydrotrypsin was measured with the hide-powder-azure assay as follows. Anhydrotrypsin and α_2 M, both in 100 mM-Tris/HCl, pH 8.0, were incubated at molar ratios of 2:1 and 10:1 (50 μ g of α_2 M and 3.5 or 17 μ g of anhydrotrypsin respectively) for 30 min at 20°C. The samples were diluted such that the α_2 M concentration was 30 μ g/ml in Tris/HCl buffer, pH 8.0, containing 20 mM-CaCl₂. Portions, 10–100 μ l, of each mixture were incubated with 0.1 μ g of active trypsin, in a total volume of 0.6 ml of the Tris/HCl/CaCl₂ buffer, at 37°C for 10 min. Trypsin activity against hide powder azure was then measured as described previously, and the inhibitory capacity of the anhydrotrypsin-treated α_2 M compared with that of untreated α_2 M.

No difference in inhibitory capacity was seen in any of the four experiments carried out in this way, in which several different preparations of anhydrotrypsin and α_2 M were used.

In a further experiment, unlabelled anhydrotrypsin was incubated with α_2 M in the usual Tris/HCl buffer, at a molar ratio of 10:1 (14 μ g of anhydrotrypsin and 40 μ g of α_2 M) for 30 min at 20°C. ¹²⁵I-labelled trypsin (2.5 μ g) was added and the samples left for a further 15 min at 20°C, then run in 5% polyacrylamide gels and radioautographed as usual.

A radioactive band was detected in a position coinciding with that of the stained α_2 M band. When the amount of radioactivity in the stained band was measured, it was found to be within 5% of that found when α_2 M and ¹²⁵I-labelled trypsin were made to react alone. We therefore concluded that saturation of the α_2 M with anhydrotrypsin did not prevent subsequent binding and inhibition of trypsin by the α_2 M.

Several further experiments were carried out in which ¹²⁵I-labelled anhydrotrypsin and α_2 M were incubated together in the usual way, and trypsin added in molar excess over the α_2 M. After 5 min the samples were made 5 mM with respect to Dip-F, in order to inhibit any free trypsin, and run in 5% polyacrylamide gels. Bound anhydrotrypsin was determined as usual and compared with samples to which no trypsin was added.

In no case was a decrease in bound anhydrotrypsin detected after trypsin had been added. It was evident that the trypsin had reacted with the α_2 M, since the characteristic 'S'-to-'F' transition of the α_2 M had taken place in those samples where trypsin

was present. We concluded that the trypsin was bound to the α_2 M without displacing the anhydrotrypsin already present.

Binding of ¹²⁵I-labelled anhydrotrypsin to F- α_2 M

F- α_2 M converted from the S-form by treatment with trypsin, thermolysin or methylamine hydrochloride (see the Materials and methods section) was incubated with 2-fold molar excess of labelled anhydrotrypsin.

Incubations were made in pH 8.0 buffer at 20°C for 30 min as usual. S- α_2 M and labelled anhydrotrypsin were incubated together in the same way as a control. Samples were run in 5% polyacrylamide gels and bound anhydrotrypsin quantified in the usual manner. Four such experiments were carried out, and in each case there was a marked decrease in the amount of anhydrotrypsin bound to the F- α_2 M compared with the S- α_2 M. Thus, when 'proteinase-F- α_2 M' was used, binding was decreased to 5–10% of the control value, whereas with 'methylamine-F- α_2 M' a less marked decrease to 40–50% was obtained.

We concluded that the conformation of F- α_2 M offers fewer, or weaker, sites for binding of anhydrotrypsin than does that of S- α_2 M.

Immunoelectrophoresis of anhydrotrypsin- α_2 M complexes

Molecules of active proteinases bound by α_2 M commonly lose their antigenic reactivity, presumably because of the steric hindrance inherent in the trapping mechanism (Starkey & Barrett, 1977). We decided to discover whether the same applied to bound anhydrotrypsin.

S- α_2 M (200 μ g) was incubated with 10 μ g of anhydrotrypsin, one-fortieth of which was ¹²⁵I-labelled under the usual conditions. A second identical sample of anhydrotrypsin bound to α_2 M was treated with 15 μ g of thermolysin to convert the α_2 M into the F-form. A third 200 μ g sample of S- α_2 M was treated with 10 μ g of unlabelled active trypsin. The final volume in each sample was 50 μ l. A 5 μ l volume of each mixture was applied to separate wells on an immunoelectrophoresis plate and subjected to electrophoresis at pH 8. The plate was developed with anti-(α_2 M) and anti-(bovine trypsin) sera for 24 h, washed thoroughly with 1% NaCl, dried, stained with Coomassie Brilliant Blue G to detect precipitin lines, and radioautographed. Samples were also diluted 1:10 and run in 5% polyacrylamide gels.

Free anhydrotrypsin formed precipitin arcs cathodal to the sample wells, but trypsin immunoreactivity associated with the α_2 M arcs showed the binding of anhydrotrypsin to the macroglobulin (Fig. 2).

Anhydrotrypsin remained bound to the α_2 M after

Fig. 2. Immunoelectrophoresis of anhydrotrypsin- α_2 M complexes

Samples placed in the wells were: (i) S- α_2 M; (ii) α_2 M with labelled anhydrotrypsin; (iii) α_2 M with labelled anhydrotrypsin and thermolysin; (iv) labelled anhydrotrypsin; (v) α_2 M with trypsin; (vi) S- α_2 M. The plate was run for 30 min at 20 mA current, and developed with (A) anti-(human α_2 M) serum, and (B) anti-(bovine trypsin) rabbit serum. The plate was then washed and dried before being stained for protein (a) and radioautographed (b).

treatment of the complex with thermolysin. The 5% gel confirmed that the α_2 M was in the S-form in the absence of thermolysin and the F-form when thermolysin was added.

We concluded that the anhydrotrypsin was bound to α_2 M in such a way that it was immunologically reactive even when the α_2 M had been converted into the F-form. In contrast, when active trypsin was bound to α_2 M, it did not react immunologically. This distinguishes the mode of binding of anhydrotrypsin from the trapping of trypsin and other active proteinases.

Conclusions

Conversion of the catalytic serine residue of trypsin or chymotrypsin to anhydroalanine eliminates catalytic activity with minimal disturbance of the structure of the active site, so that the high affinity for many substrates and inhibitors is retained. This has clearly shown that, although peptide-bond cleavage commonly occurs in the reaction with inhibitors such as soya-bean trypsin inhibitor, it is not an essential part of the reaction (Ako *et al.*, 1972). If the binding of anhydrotrypsin to α_2 M described by Tsuru *et al.* (1978) had the same characteristics as that of active proteinases, it would have suggested that they too bind by a mechanism in which proteolytic activity is not essential.

Our results have shown that anhydrotrypsin does indeed bind to α_2 M quite tightly and with a high degree of specificity. The reaction differs in important respects, however, from that between active proteinases and α_2 M. The differences can be summarized as follows.

(a) Anhydrotrypsin can be displaced from α_2 M by aprotinin and soya-bean trypsin inhibitor, unlike active trypsin.

(b) Not only is α_2 M not proteolytically cleaved in the reaction with anhydrotrypsin (as shown by Tsuru *et al.*, 1978), but it does not undergo the characteristic change in electrophoretic mobility thought to represent the 'closing of the trap' (Barrett *et al.*, 1979).

(c) The bound anhydrotrypsin continues to react with antibodies, unlike bound trypsin.

(d) Contrary to the findings of Tsuru *et al.* (1978), we found no evidence that the binding of anhydrotrypsin to α_2 M interfered with the subsequent binding and inhibition of trypsin by the α_2 M (as does the binding of an active proteinase), even though the anhydrotrypsin was not displaced by the trypsin.

This latter discrepancy can be attributed to the different methods used for preparation of anhydrotrypsin. The alkaline conditions used for the β -elimination reaction in the formation of anhydrotrypsin regenerate an appreciable amount of active trypsin. In our hands it is extremely difficult to inactivate this quantitatively by further use of inhibitors, and it tends to contaminate anhydrotrypsin eluted from an affinity column with a single step of acid (as was used by Tsuru *et al.*, 1978), rather than the two-step elution that we used. An anhydrotrypsin preparation contaminated with trypsin would obviously be expected to saturate the trypsin-binding capacity of α_2 M as described by Tsuru *et al.* (1978). We conclude, therefore, that the trapping mechanism whereby α_2 M binds active proteinases still seems to be entirely specific for such proteins.

The actual mechanism of binding of anhydrotrypsin to α_2 M is much more difficult to define. The specificity of reaction for anhydrotrypsin (rather than Dip trypsin) and α_2 M (rather than other proteins tested) suggests some analogy with the trypsin- α_2 M reaction, and this is supported by the finding that the binding ratio, 2:1, is the same as that for active trypsin (Barrett *et al.*, 1979). The idea that binding is through the specificity site of the enzyme, rather than being electrostatic, for example, is consistent with the disruption of the complex by the powerful inhibitors aprotinin and soya-bean trypsin inhibitor, but not by 0.5 M-Tris/HCl, but active trypsin was not found to displace the inactive enzyme.

α_2 M has affinity for many types of molecule quite separately from its reactivity with active proteinases (Starkey & Barrett, 1977), and, in particular, there are previous reports of binding of certain basic proteins with a specificity suggestive of something more than an electrostatic interaction; these include cationic aspartate aminotransferase (Boyde & Pryme, 1968), carboxypeptidase A (Barrett & Starkey, 1973), myelin basic protein (McPherson *et al.* 1970) and histone H4 (Stoller & Rezukey, 1978). Possibly the interaction with anhydrotrypsin has something in common with these.

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