Covalent Binding of Proteinases in their Reaction with α_2 -Macroglobulin

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Although it is known that most of the plasma proteinase inhibitors form complexes with proteinases that are not dissociated by SDS (sodium dodecyl sulphate), there has been disagreement as to whether this is true for α_2 M (α_2 -macroglobulin). We have examined the stability to SDS with reduction of complexes between α_2 M and several 125 I-labelled proteinases (trypsin, plasmin, leucocyte elastase, pancreatic elastase and papain) by gel electrophoresis. For each enzyme, some molecules were separated from the denatured α_2 M chains, but amounts ranging from 8.3% (papain) to 61.2% (trypsin) were bound with a stability indicative of a covalent link. Proteolytic activity was essential for the covalent binding to occur, and the proteinase molecules became attached to the larger of the two proteolytic derivatives (apparent mol.wt. 111 000) of the α_2 M subunit. We take this to mean that cleavage of the proteinase-susceptible site sometimes leads to covalent-bond formation between α_2 M and proteinase. Whatever the nature of this bond, it does not involve the active site of the proteinase, as bound serine-proteinase molecules retain the ability to react with the active-site-directed reagent [3 H]Dip-F (di-isopropyl phosphorofluoridate). Our conclusion is that the ability to form covalent links is not essential for the inhibitory capacity of α_2 M. It may, however, help to stabilize the complexes against dissociation or proteolysis.

α_2 M, a glycoprotein found in the plasma of vertebrates, has been shown to be a potent inhibitor of most proteinases of all four catalytic classes (Barrett & Starkey, 1973). α_2 M has the unique property of inhibiting proteinases in such a way that their active sites remain free to hydrolyse low-molecular-weight substrates and interact with low-molecular-weight inhibitors but are restricted from reactions with larger substrates and inhibitors. This property, among others, led Barrett & Starkey (1973) to propose a 'trap hypothesis' to explain the binding of proteinases by α_2 M.

The trap hypothesis suggested that, upon interaction with a proteinase and hydrolysis of one or more susceptible peptide bonds in the molecule (at the 'bait region'), α_2 M undergoes a change in conformation. The conformational change physically entraps the proteinase molecule within the body of the α_2 M molecule, thus sterically hindering reaction between the proteinase and other large molecules.

Most plasma proteinase inhibitors, and many other protein proteinase inhibitors, form complexes

with proteinases in which the enzyme lacks all catalytic activity, and that are not dissociated by complete reduction and denaturation, for example in SDS (Travis *et al.*, 1979). These characteristics suggest that one of the reactive groups in the catalytic site of the enzyme forms a covalent link to the inhibitor. In contrast, the trap hypothesis for the action of α_2 M suggested a mechanism whereby stable complexes might be formed without such covalent interaction with the active sites of proteinases.

Some reports have suggested that α_2 M-proteinase complexes are dissociable under denaturing conditions, as was to be expected from the trap hypothesis (Nagasawa *et al.*, 1970; Abe & Nagai, 1972; Birkedal-Hansen *et al.*, 1975), but other workers have indicated that the complexes are not dissociable (Harpel & Rosenberg, 1976; Steinbuch *et al.*, 1976; Harpel, 1977). The failure of the complexes to dissociate has been attributed to the formation of a covalent bond between α_2 M and the proteinase (Harpel, 1977). In the present paper we describe the dissociation of several α_2 M-proteinase complexes in the presence of SDS and consider the results in relation to the general mechanism of binding of proteinases by α_2 M.

Abbreviations used: SDS, sodium dodecyl sulphate; α_2 M, α_2 -macroglobulin; Dip-F, di-isopropyl phosphorofluoridate; PPO, 2,5-diphenyloxazole.

Experimental

Materials

Sources of chemicals and other materials were as follows: Brij 35 and sodium iodoacetate (special purity) were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.; Trizma base, Dip-F, papain (twice-crystallized), trypsin (bovine, twice-crystallized) and pancreatic elastase (porcine) were from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K.; *Staphylococcus aureus* V8 proteinase was from Miles Laboratories Ltd., Slough SL2 4LY, Berks., U.K.; Ultrogel AcA 34 was from LKB Instruments, South Croydon, Surrey CR2 8YD, U.K.; Sepharose 4B and Sephadex G-25 were from Pharmacia (G.B.) Ltd., Middx. TW3 1NE, U.K.; streptokinase (Kabikinase) was from Kabi Vitrum Ltd., Ealing, London W5 2TH, U.K. [it was further purified to remove added human serum albumin by passing it through a column (5 cm $\times$ 1 cm) of Cibacron Blue–Sepharose, prepared as described by Travis & Pannell (1973)]; Na¹²⁵I ('essentially carrier-free') and [³H]Dip-F (3 Ci/mmol), The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Aprotinin (Trasylol) was the gift of Dr. E. Philipp, Bayer A.G., D-5600 Wuppertal 1, Germany.

Methods

Purification of proteins. Human plasminogen was prepared from acid-citrate/dextrose-treated blood by the method of Deutsch & Mertz (1970), plasminogen types I and II being separated by 6-aminohexanoic acid gradient elution as described by Brockway & Castellino (1972). Activation of plasminogen was with streptokinase, at a streptokinase/plasminogen molar ratio of 1:100 for 15 min at 37°C in 0.1 M-Na₂HPO₄/NaH₂PO₄, pH 7.5. Human leucocyte elastase was prepared by a modification of the method of Baugh & Travis (1976) and was a gift of Dr. J. Saklatvala of this Institution. Human α_2 M was prepared in the active electrophoretically 'slow' form as described by Barrett *et al.* (1979).

Protein determination. Protein concentration was calculated on the basis of the following $A_{280}^{1\text{cm}, 1\%}$ values: α_2 M, 9.1 (Dunn & Spiro, 1967); pancreatic elastase, 20.2 (Hartley & Shotton, 1971); leucocyte elastase, 9.85 (Baugh & Travis, 1976); plasminogen 20.0; papain, 25.0; streptokinase, 9.5; trypsin, 15.6 (Kirschenbaum, 1970).

Gel electrophoresis. The buffer system used in the electrophoresis of SDS–protein complexes was as described by Barrett *et al.* (1979), a modification (A. F. Bury, unpublished work) of the 2-amino-2-methylpropane-1,3-diol/glycine/HCl discontinuous system of Wyckoff *et al.* (1977).

Samples to be run reduced (about 50 μ g in 50 μ l)

were mixed with an equal volume of double-strength upper-reservoir buffer containing 0.01% Bromophenol Blue, 40% (v/v) glycerol, 2% (w/v) SDS and 1% 2-mercaptoethanol and heated at 100°C for 5 min. Samples to be run without reduction were heated with 2% SDS and 2 mM-sodium iodoacetate at pH 6–7, to decrease disulphide-bond cleavage, and mixed with an equal volume of upper-reservoir buffer containing glycerol and Bromophenol Blue. Gels were 5, 7 or 15% (w/v) total acrylamide concentration (2.6% of this as methylenebisacrylamide). Gels containing 5 and 7% acrylamide were cast as described by Barrett *et al.* (1979), whereas 15% gels were longer (9 cm) with correspondingly longer stacking gels (4 cm) and deeper sample wells (1 cm).

Electrophoresis was stopped when the Bromophenol Blue dye front reached the bottom of the gel. Gels were stained in Coomassie Brilliant Blue G-250 and stored as described previously (Barrett *et al.*, 1979).

Molecular-weight calibration of SDS/polyacrylamide gels was done by using the following standard proteins (Barrett *et al.*, 1979): phosphorylase *a*, mol.wt. 94 000; transferrin, 78 000; bovine serum albumin, 68 000; immunoglobulin G heavy chain, 50 000; carbonic anhydrase, 29 000; immunoglobulin G light chain, 23 500; soya-bean trypsin inhibitor, 21 500; cytochrome *c*, 11 700; aprotinin, 6500; insulin B-chain, 3300.

Peptide 'mapping'. 'Mapping' by limited proteolysis of protein bands derived from native α_2 M and α_2 M–proteinase complexes was carried out much as described by Cleveland *et al.* (1977).

Protein (50–100 μ g/lane) was loaded on to a 5% gel, which was run with SDS and reduction in the normal way. To locate protein bands, the gel was stained in Coomassie Blue for 10 min at room temperature (22°C) and rinsed briefly in distilled water. Pieces of gel containing the bands were cut out with a scalpel and allowed to equilibrate with 1 ml of upper-reservoir buffer containing 0.1% SDS for 30 min. Sample wells in a 15% gel were filled with the same buffer, the gel slices were pushed to the bottom of the wells with forceps and 1 μ g of *S. aureus* V8 proteinase in 10 μ l of upper-reservoir buffer containing 20% glycerol, 0.1% SDS and 0.01% Bromophenol Blue was layered over them. Electrophoresis was carried out in the normal way, except that the current was interrupted for 30 min when the Bromophenol Blue dye front neared the bottom of the stacking gel.

Radioactive labelling of proteins. Trypsin, plasminogen, papain, pancreatic elastase and leucocyte elastase were each labelled with ¹²⁵I by a direct chloramine-T method based on that of Hunter & Greenwood (1962). ¹²⁵I (0.5 mCi in 5 μ l) was added with mixing to 0.1 ml of 0.05 M-

$\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.0, containing 0.1 mg of protein. To this was added 0.1 mg of chloramine-T in 0.1 ml of the same buffer, followed 1 min later by 0.1 mg of sodium metabisulphite in 0.1 ml of the same buffer. The procedure was carried out at 4°C. The labelled protein was separated from free iodide on a 10 ml-capacity column of Sephadex G-25 equilibrated with 0.05 M-Tris/HCl buffer, pH 7.5. Fractions (0.3 ml) were collected and counted for radioactivity in a Packard Auto-Gamma counter, model BPGD. Fractions comprising the first radioactive (protein) peak were combined, divided into 0.1 ml portions and stored at -20°C until required.

Alternatively, free or $\alpha_2\text{M}$ -bound serine proteinases (trypsin, plasmin, pancreatic elastase and leucocyte elastase) were incubated in the presence of 0.1 mM-[^3H]Dip-F in 0.05 M-Tris/HCl, pH 7.5, for 30 min before analysis by polyacrylamide-gel electrophoresis and fluorography.

Preparation of ^{125}I -labelled proteinase- $\alpha_2\text{M}$ complexes. $\alpha_2\text{M}$ (1 mg in 0.5 ml of 0.05 M-Tris/HCl buffer, pH 7.5) was incubated at 37°C for 15 min with a 0.25–0.5 molar ratio of labelled proteinase in an equal volume of the same buffer (plus 2 mM-cysteine for papain). The mixture was made 1 mM with respect to Dip-F, or 3 mM with respect to sodium iodoacetate for papain, to eliminate proteolysis of $\alpha_2\text{M}$ in the presence of SDS, and held at 37°C for 30 min before application to a column [25 cm $\times$ 1.5 cm (45 ml)] of Ultrogel AcA 34 equilibrated with the pH 7.5 buffer. The column was run at a flow rate of 10 ml/h and 1 ml fractions were collected. Two radioactive peaks were identified, and the first, comprising labelled proteinase- $\alpha_2\text{M}$ complexes, was concentrated by dialysis against poly(ethylene glycol) to 1 mg of protein/ml and stored at 4°C until required.

Radioautography and fluorography. Gels containing ^{125}I -labelled protein bands were dried down on to dialysis membrane and exposed to Fuji Rx-medical film for 12–48 h. Gels containing [^3H]Dip-F-labelled bands were impregnated with PPO as described by

Laskey & Mills (1975) and exposed to the same film for 72 h. Films were developed with Kodak DX-80 developed according to the manufacturer's instructions.

Densitometry of electrophoresis gels, radioautographs and fluorographs. Protein bands in dried gels stained with Coomassie Blue were quantified in a Gilford 250 spectrophotometer at 592 nm with a gel scanner (model 2520) connected to a Smith's Servoscribe RE 541 flat-bed recorder with integrator. Radioautographed and fluorographed films were scanned in a similar manner at 600 nm.

Assay of inhibition of proteolytic activity by $\alpha_2\text{M}$. Assay of the proteolytic activity of ^{125}I -labelled proteinases and $\alpha_2\text{M}/^{125}\text{I}$ -labelled-proteinase mixtures was with hide powder azure (Calbiochem, London W1H 1AS, U.K.) as described for trypsin by Barrett *et al.* (1979). Samples of $\alpha_2\text{M}/^{125}\text{I}$ -labelled-proteinase mixtures were taken for assay before the addition of Dip-F, or sodium iodoacetate, and gel filtration on Ultrogel AcA 44. Results are expressed as percentage inhibition of proteolytic activity by $\alpha_2\text{M}$.

Results and Discussion

SDS-stable binding of proteinases to $\alpha_2\text{M}$

Complexes formed by allowing several ^{125}I -labelled proteinases (trypsin, plasmin, pancreatic elastase, leucocyte elastase and papain) to react with a 2–4-fold molar excess of $\alpha_2\text{M}$ were assayed for activity, purified by gel filtration (see Methods section), and were subjected to SDS/polyacrylamide-gel electrophoresis with reduction. The gels and radioautographs of the gels were scanned as described in the Methods section and the tracings are presented in Fig. 1. The free enzymes were run similarly as controls.

The profiles of stained protein bands were interpreted on the basis of the known forms of fragmentation of the $\alpha_2\text{M}$ subunit, which can be split under alkaline conditions at high temperature in SDS at a point approximately one-third of the way

Table 1. Inhibition of proteolytic activity, and percentage of enzyme forming SDS-stable links in the reaction of various proteinases with $\alpha_2\text{M}$

Complexes of ^{125}I -labelled proteinases with $\alpha_2\text{M}$ were assayed for proteolytic activity, purified as described under 'Methods', and subjected to SDS/polyacrylamide-gel electrophoresis with reduction. The gels were dried and radioautographed, and radioactive bands were cut out and counted for radioactivity in a Packard gamma counter. The means $\pm$ S.D. were calculated from six analyses, three on each of two reaction mixtures. Abbreviation used: n.d., not determined.

	Proteinase ...	Trypsin	Plasmin	Pancreatic elastase	Leukocyte elastase	Papain
Inhibition by $\alpha_2\text{M}$ (% of control)		95	n.d.	91	95	96
$\alpha_2\text{M}$ -bound radioactivity (% of total $\pm$ S.D.; $n = 6$)		61.2 $\pm$ 10.8	54.1 $\pm$ 11.7	36.9 $\pm$ 2.8	17.0 $\pm$ 3.4	8.3 $\pm$ 1.1

Fig. 1. *SDS/polyacrylamide-gel-electrophoresis patterns of radioactively labelled proteinases free or bound to α_2M* Electrophoresis in the presence of SDS with reduction was in 7% (w/v) gels, which were stained, radioautographed and scanned. The lower tracings in each panel are α_2M + proteinase protein, the middle tracings are radioautographs of ^{125}I -labelled α_2M -proteinase, and the upper tracings are fluorographs of α_2M -proteinase mixtures labelled with $[^3H]$ Dip-F. α_2M -papain complexes did not label with $[^3H]$ Dip-F, so the tracing is omitted. The short horizontal bars correspond to the position of labelled proteinase material in adjacent gel lanes (not made to react with α_2M). The left-hand margin of each plate is the top (cathodal) end of the gel, whereas the right-hand margin is the Bromophenol Blue dye front. Material labelled 'Dimer' ran at the same rate as the non-reduced dimer of α_2M subunits. (a) Plasmin; (b) trypsin; (c) pancreatic elastase; (d) leucocyte elastase; (e) papain.

along the chain, to give polypeptides of apparent mol.wts. 139 000 (II) and 68 000 (III) (Barrett *et al.*, 1979; Harpel *et al.*, 1979). Alternatively, cleavage at the proteinase-susceptible site (the 'bait region')

gives two polypeptides of apparent mol.wts. 111 000 and 98 000 (now termed IVa and IVb respectively) (Barrett *et al.*, 1979).

The scans of the radioautographs show partial

release of ^{125}I -labelled enzymes from $\alpha_2\text{M}$, but also high-molecular-weight labelled polypeptides. The percentage of total radioactivity in the form of high-molecular-weight polypeptides (Table 1) varied from 8.3 for papain to 61.2 for trypsin and was not substantially altered by varying the pH of the reaction mixture within the limits 6.0–8.5, or by different buffer composition: 0.01–0.1 M-Tris/HCl, 0.05 M-Tris/HCl + 0.2 M-NaCl, 0.1 M- Na_2HPO_4 /NaH $_2$ PO $_4$ (all pH 7.5), or by addition of 0.1% Brij 35 detergent.

As shown in Fig. 1(a), plasmin, which is composed of two disulphide-linked polypeptide chains, gave two high-molecular-weight ^{125}I -labelled components, Brockway & Castellino (1972) isolated two plasminogens from human plasma with differing affinities for lysine-Sepharose, plasminogens I and II. Both forms were labelled with ^{125}I , converted into plasmin with streptokinase and made to react with $\alpha_2\text{M}$; they were found to be indistinguishable in the percentage of radioactivity in high-molecular-weight components, and the position of bands corresponding to these on the gels. Bovine trypsin, which exists in several forms in most preparations [intact and autolytically cleaved forms stabilized by disulphide links (Schroeder & Shaw, 1968)] gave four bands containing high-molecular-weight ^{125}I -labelled components (Fig. 1b). Leucocyte elastase, pancreatic elastase and papain, all of which are known to exist mainly as single polypeptide chains, each gave a single band of about 25 000 mol.wt. for the free enzyme, and only one band corresponding to high molecular weight (Figs. 1c, 1d and 1e). These bands were seen only when proteinases were made to react with $\alpha_2\text{M}$ and are concluded to be SDS-stable complexes in which proteinase molecules are firmly bound to $\alpha_2\text{M}$ polypeptide chains.

These results suggest that proteinases can form SDS-stable non-reducible links to $\alpha_2\text{M}$ and that each of the polypeptide chains of the enzyme has the possibility of becoming bound. The extent of SDS-stable binding varies greatly between proteinases. As expected, radioactively labelled proteinases that had been specifically inactivated with Dip-F formed no complexes with $\alpha_2\text{M}$.

Active-site labelling of proteinase- $\alpha_2\text{M}$ complexes

Serine proteinases were allowed to form complexes with a 2–4-fold molar excess of $\alpha_2\text{M}$ in the normal way and incubated with 0.1 mM- ^{3}H -Dip-F. Dip-F binds exclusively to the active-site serine residue of catalytically active molecules (Darzynkiewicz & Barnard, 1967), thus obviating the need to isolate complexes free of catalytically inactive proteinase material. Free proteinase and proteinase- $\alpha_2\text{M}$ complexes, both labelled with ^{3}H -Dip-F, were run in SDS/polyacrylamide gels with reduction. Scans of fluorographs of the gels (see

under 'Methods') showed identical patterns of radioactive bands between ^{3}H -Dip-F-labelled proteins and ^{125}I -labelled ones for the single-chain enzymes pancreatic elastase and leucocyte elastase (Figs. 1c and 1d). With trypsin and plasmin complexes, however, there were ^{125}I -labelled high-molecular-weight components bound to $\alpha_2\text{M}$ that did not label with ^{3}H -Dip-F. These most probably represent SDS-stable attachment to $\alpha_2\text{M}$ of proteinase chains that do not possess the active-site serine residue, i.e. plasmin heavy chain and the autolytically cleaved chains of trypsin lacking this residue.

If both heavy and light chains of a plasmin molecule form SDS-stable links with a single $\alpha_2\text{M}$ polypeptide chain, as suggested by Harpel (1977), we would expect to see two ^{3}H -Dip-F-labelled bands associated with $\alpha_2\text{M}$, i.e. one $\alpha_2\text{M}$ chain with light chain attached and one with both heavy and light chains attached. The lack of the second ^{3}H -Dip-F-labelled band indicates that both plasmin chains cannot be directly attached to a single $\alpha_2\text{M}$ chain, probably because each chain contains

Fig. 2. Peptide 'mapping' of native $\alpha_2\text{M}$ fragments and $\alpha_2\text{M}$ -proteinase-derived fragments

Electrophoresis in the presence of SDS and *S. aureus* V8 proteinase was in 15% (w/v) gels and was carried out on polypeptides derived from $\alpha_2\text{M}$, $\alpha_2\text{M}$ -plasmin and $\alpha_2\text{M}$ -pancreatic elastase as described under 'Methods'. The Figure is a composite drawing from several 'mapping' gels. The thickness of the lines indicates the relative intensities of the corresponding stained peptides. Polypeptides composed of $\alpha_2\text{M}$ chains linked by SDS-stable bonds to pancreatic elastase, plasmin light chain and plasmin heavy chain are labelled 'Elas.', 'Plas. light' and 'Plas. heavy' respectively.

Fig. 3. Structure of the α_2M subunit

A diagrammatic representation of the underlying structure of the α_2M quarter subunit as deduced from peptide 'mapping'. The diagram is to scale, the lengths of the various regions reflecting their apparent molecular weights as determined by SDS/polyacrylamide-gel electrophoresis (Barrett *et al.*, 1979). Although there is no intention to depict the actual conformation of the native chain, which is obviously more complex, we have attempted to show the relationship between the various fragments and the regions susceptible to attack by proteinases (bait region) and hot alkaline SDS solutions. Results indicate that the IVa fragment (the lower arm of the chain in the diagram) contains the site at which SDS-stable binding of proteinases occurs.

only a single binding site. This is consistent with the appearance of only two, rather than three, labelled bands with the iodinated plasmin.

Peptide 'mapping'

Samples (50–100 μ g) of free α_2M and α_2M as a complex with pancreatic elastase or plasmin, were run in first-dimension 'mapping' gels (see under 'Methods'), and α_2M bands I, II, III, IVa, IVb and α_2M chains with proteinase polypeptides attached were cut out and re-run in the second dimension.

Fig. 2 is a composite drawing of the results obtained from several 'mapping' gels. The patterns of peptides produced by the *S. aureus* proteinase from α_2M fragments II and IVb have the majority of their peptides in common, as do fragments III and IVa. This information allowed us to construct a diagram of the α_2M subunit (Fig. 3).

The peptide 'maps' of α_2M polypeptides with pancreatic elastase, plasmin heavy chain or plasmin light chain attached are unlike those of bands II and IVb, but very similar to those of the III and IVa fragments. Major peptides of apparent mol.wt. 6000 and 8000 are present in proteinase-containing chains and fragment IVa, but absent from fragment III.

We conclude that pancreatic elastase, plasmin heavy chain and plasmin light chain become attached only to the fragment-IVa proteolytic derivative of α_2M . There are two peptides of

apparent mol.wt. 34 000 and 35 000 that are present in fragment IVa but absent from α_2M -proteinase-linked chains. It may well be that these peptides contain the attachment site or sites for proteinases. The presence of peptides of apparent mol.wt. 45 000–62 000 in the linked chains supports this idea, because these could well represent proteinase peptides bound to the missing α_2M peptides.

Conclusions

In its reaction with proteinases, α_2M forms links stable to SDS and reduction, but the extent of such binding varies greatly between different enzymes. The nature of the links is at present unclear, but they possess stability normally characteristic of covalent bonds, as was suggested by Harpel & Rosenberg (1976).

The simplest interpretation of our finding, that only one plasmin polypeptide (either the light chain or the heavy chain) can become covalently attached directly to any one α_2M chain, is that each α_2M chain has only a single covalent binding site. Since it seems that all four subunits of the α_2M molecule are identical (Jones *et al.*, 1972; Barrett *et al.*, 1979; Swenson & Howard, 1979), it is probable that each molecule has four potential covalent binding sites.

We found that a series of proteinases, all of which were inhibited 91–96% by α_2M , varied in the extent of covalent binding from 8 to 61%, and conclude that inhibition does not depend on the formation of covalent links.

The apparent molecular weights of the α_2M chains with attached proteinase molecules, on SDS/polyacrylamide-gel electrophoresis, tend to be greater than would be expected on the basis of our conclusions that each consists of the IVa fragment of α_2M and the proteinase chain. Such high apparent molecular weights have led workers previously to suggest that there is binding to intact quarter subunits (Harpel, 1977; Krebs *et al.*, 1978). The results of our peptide-'mapping' experiments preclude this. It seems probable that the α_2M -proteinase chains run anomalously on SDS/polyacrylamide-gel electrophoresis, perhaps because they are branched rather than linear.

As was shown by Barrett *et al.* (1979), the quarter subunit of α_2M can be cleaved in two distinct ways: at the bait region by a proteinase, to give the IVa and IVb fragments, or at another position by a hot alkaline SDS solution, to give the II and III fragments. [Fragments II and III may also be generated at high temperature in 6M-guanidine hydrochloride plus reducing agent (Harpel *et al.*, 1979) and in hot acidic reducing SDS solutions (G. S. Salvesen & A. J. Barrett, unpublished work).] If it were possible for both of these events to take place independently, we would expect to see a peptide of

apparent mol.wt. 43 000, corresponding to a portion of the chain between the two possible hydrolysis sites (see Fig. 3). The lack of such a peptide (see Fig. 1) leads us to suggest that proteolysis at the bait region stabilizes the alkali-labile bond. This stabilization may well result from the conformational change brought about by the interaction between α_2 M and a proteinase, because ammonium compounds, which bring about a similar conformational change, also stabilize the alkali-labile bond (Barrett *et al.*, 1979).

The reactivity with [3 H]Dip-F of proteinase molecules covalently bound to α_2 M shows that the link is not through the active site of the proteinase, in contrast with the SDS-stable links, which are formed by proteinases to other plasma proteinase inhibitors (Travis *et al.*, 1979).

Our conclusion from the present study is that the characteristics of the reaction of α_2 M with proteinases are entirely consistent with the trap hypothesis as formulated by Barrett & Starkey (1973), but that the proteolytic cleavage of the bait region is followed not only by the conformational change, which traps the proteinase molecules, but also by the formation of covalent binding sites, which react with a proportion of the trapped molecules. There may also be cross-linking within the α_2 M molecule, as SDS-stable units larger than the quarter subunit were detected in small quantities. The covalent reactions may help to stabilize the complexes, but are not essential for the inhibition of proteinases by α_2 M.

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