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Further characterization of the covalent linking reaction of α_2-macroglobulin

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It is shown that non-proteolytic proteins can become covalently linked to α_2M (α_2-macroglobulin) during its reaction with proteinases, and that this probably occurs by the mechanism that leads to the covalent linking of proteinases described previously [Salvesen & Barrett (1980) *Biochem. J.* **187**, 695–701]. The covalent linking of trypsin was at least partly dependent on the presence of unblocked lysine side chains on the protein. The covalent linking of proteinases was inhibited by nucleophiles of low M_r, and these compounds were themselves linked to α_2M in a molar ratio approaching one per quarter subunit. Peptide 'mapping' indicated that the site of proteinase-mediated incorporation of the amines was the same as that at which methylamine is incorporated in the absence of a proteinase. The nucleophile-reactive site revealed in α_2M after reaction with a proteinase was shown to decay with a $t_{1/2}$ of 112s, at pH 7.5. After the reaction with a proteinase or with methylamine, a free thiol group was detectable on each subunit of α_2M. We propose that the site for incorporation of methylamine in each subunit is a thiol ester, which in S-α_2M (the electrophoretically 'slow' form) is sterically shielded from reaction with large nucleophiles, but is revealed as a highly reactive group, free from steric hindrance, after the proteolytic cleavage. We have designated the activated species of the molecule 'α_2M*'.

α_2M is a tetrameric glycoprotein that inhibits proteinases of all four catalytic classes by a unique 'trap' mechanism (Barrett & Starkey, 1973). This is initiated by limited proteolysis at a 'bait' region near the middle of the primary sequence of one or more of the four identical subunits of the α_2M molecule, and takes the form of a conformational change that physically encloses the attacking proteinase molecule (Barrett *et al.*, 1979). In addition to the trapping reaction specific for proteinases, we now recognize two other binding reactions: one is the 'adherence' reaction seen with anhydrotrypsin and various other proteins, mostly cationic (Sayers & Barrett, 1980); the other is the covalent 'linking' reaction with which the present paper is concerned. Salvesen & Barrett (1980) showed that associated with the conformational change of the trapping reaction is the appearance of a chemical grouping in α_2M that covalently links some of the trapped proteinase molecule to the α_2M. We have now examined the covalent reaction in more detail, located the site on the molecule responsible for it, and shown that this is identical with the methylamine-reactive site described by Swenson & Howard (1979).

Abbreviations used: α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulphate; Dip-F, di-isopropyl phosphorofluoridate.

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Experimental

Materials

Sources of chemicals and other materials were as follows: sodium iodoacetate ('special purity') was from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.; Tris (Trizma base), Dip-F, trypsin (bovine, twice-crystallized) and chymotrypsin (bovine, three-times-crystallized) were from Sigma (London) Chemical Co., Poole, Dorset BH17 7NN, U.K.; *Staphylococcus aureus* V8 proteinase was from Miles Laboratories Ltd., Slough SL2 4LY, Berks, U.K.; Sephadex G-25 was from Pharmacia (G.B.) Ltd., Middx. TW3 1NE, U.K.; $Na^{125}I$ ('essentially carrier-free'), [^{3}H]glycine (16.0Ci/mmol, 62.5μM solution), [^{3}H]lysine (77.0Ci/mmol, 13μM solution, iodo[^{14}C]acetic acid (54mCi/mmol), iodo[^{3}H]-acetic acid (59mCi/mmol) and [^{14}C]methylamine (40.4mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Aprotinin (Trasylol) was a gift of Dr. E. Philipp, Bayer A.G., D-5600 Wuppertal 1, Germany.

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Pancreatic elastase (porcine) was the gift of Dr. J. Travis, University of Georgia, Athens, GA 30602, U.S.A. Acetylhydroxamic acid and benzylhydroxamic acid were gifts of Dr. T. Twose, Department of Biochemistry, ICI Ltd., Pharmaceuticals Division, Macclesfield, Cheshire SK10 4TG, U.K.

Methods

Purification of proteins. Human α_2M was prepared in the active, electrophoretically 'slow' form (S-α_2M) as described by Barrett *et al.* (1979). Anhydrotrypsin was prepared as described by Sayers & Barrett (1980).

Protein determination. Protein concentration was calculated on the basis of the following $A^{1\%}_{280}$ values: α_2M, 9.1 (Dunn & Spiro, 1967); pancreatic elastase, 20.2 (Hartley & Shotton, 1971); 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 of the 2-amino-2-methylpropane-1,3-diol (Ammediol)/glycine/HCl discontinuous system of Wyckoff *et al.* (1977). Samples were run reduced with 2-mercaptoethanol or unreduced as described below. Gels were of 7 or 15% (w/v) total acrylamide concentration. Molecular-weight calibration was done as described by Barrett *et al.* (1979).

Peptide 'mapping'. 'Mapping' by limited proteolysis of α_2M was performed as described by Salvesen & Barrett (1980), a modification of the two-dimensional method described by Cleveland *et al.* (1977), but with omission of the first-dimension run. α_2M samples were treated with SDS and mercaptoethanol (Barrett *et al.*, 1979) and loaded on to a 15% (w/v) gel. *S. aureus* V8 proteinase [1μg in 10μl of upper-reservoir buffer containing 20% (v/v) glycerol, 0.1% SDS and 0.01% Bromophenol Blue] was layered over the α_2M and electrophoresis was performed in the normal way except that the current was interrupted for 30min when the dye front neared the bottom of the stacking gel.

Radioactive labelling of proteins. Trypsin, pancreatic elastase, lysozyme, anhydrotrypsin, aprotinin, soya-bean trypsin inhibitor and bovine serum albumin were each labelled with ^{125}I by the Iodogen method of Fraker & Speck (1978) as follows. A solution containing 1μg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Pierce and Warriner, Chester, Cheshire CH1 4EF, U.K.) in dichloromethane (10μl) was allowed to dry on to the inner surface of a polypropylene tube. A mixture of 0.5mCi (240pmol) of ^{125}I, 24nmol of KI and 0.1mg of the protein in 0.1ml of 0.05M-Tris/HCl, pH7.5, was added to the coated tube at 4°C. After 5min the reaction was stopped by decanting the solution into a fresh tube. (Iodogen, being insoluble, remains absorbed to the tube). ^{125}I-labelled proteins were divided into 10μl portions and stored at −20°C until required.

Radioautography and fluorography. Gels containing ^{125}I-labelled protein bands were dried down on to filter paper and exposed to Fuji-Rx medical film for 12–24h in a Kodak X-Omatic C-1 cassette with intensifying screens. Gels containing bands labelled with ^{3}H or ^{14}C were impregnated with sodium salicylate as described by Chamberlain (1979) and exposed to the same film for 72h. Films were developed with Kodak DX-80 developer according to the manufacturer's instructions.

Quantification of radioactivity. The radioactivity in ^{3}H- and ^{14}C-labelled compounds was measured in a Packard liquid-scintillation counter, model BPLD. Toluene scintillant and Picofluor scintillant (both from Packard) were mixed in equal volumes and 100vol. were added to 1vol. of aqueous sample containing the radioisotope. The samples were left for 2h and counted for radioactivity at room temperature with the appropriate energy window. Samples containing ^{125}I were counted in a Packard Auto-Gamma counter, model BPGD.

Standardization of α_2M and proteinase preparations. α_2M preparations showing no inactive electrophoretically 'fast' component, as judged by pore-limit electrophoresis (Barrett *et al.*, 1979), were used. α_2M was titrated with pancreatic elastase, trypsin or thermolysin to determine the amount of each proteinase that would saturate the inhibitory capacity, hide powder azure (Calbiochem, London W1H 1AS, U.K.) being used as substrate (Barrett *et al.*, 1979). A 'sub-saturating weight' of proteinase (used in several of the experimental procedures) was defined as the weight of proteinase required to saturate 90–95% of the inhibitory capacity of the given weight of α_2M.

Measurement of the incorporation of radiolabelled compounds into α_2M. ^{3}H- and ^{14}C-labelled compounds were incorporated into α_2M (0.7mg in 1.0ml of buffer) as described in the text. These radiolabelled compounds were used undiluted at the specific radioactivity stated under 'Materials' or diluted with the unlabelled compound. Protein was precipitated with an equal volume of 20% (w/v) trichloroacetic acid and centrifuged at 2000**g** for 5min. The supernatant containing trichloroacetic acid-soluble radioactivity was discarded, the pellet was resuspended in 1.0ml of water and centrifuged again at 2000**g** for 5min. The procedure was repeated three times, the final wash containing only trace amounts of radioactivity (less than 5% of the total incorporated). The final pellet was dissolved in 50% (v/v) formic acid for radioactivity counting.

Acetylation of trypsin. Trypsin was acetylated as described by Riordan & Vallee (1972). Seven 1.0μl portions of acetic anhydride were added at 15min

intervals to a stirred solution of 10mg of trypsin in 1.0ml of half-saturated sodium acetate at 4°C. Samples (50μl) of the protein solution were removed before addition of each portion of the anhydride and 5μl of this was taken for assay of amino groups (Spadaro *et al.*, 1979). Amino groups were found to be 18, 35, 52, 61, 71 and 76% decreased with respect to the unacetylated enzyme.

Results

Covalent linking of non-proteolytic proteins to α_2M

^{125}I-Labelled bovine serum albumin, soya-bean trypsin inhibitor, aprotinin, lysozyme, anhydrotrypsin and Dip-trypsin (all of specific radioactivity in the range 500000–2000000c.p.m./μg) were tested for covalent linking to α_2M in the presence of thermolysin.

Each ^{125}I-labelled protein was mixed with a 2-fold molar excess of α_2M in 0.05M-Tris/HCl buffer, pH8.0, to give a protein concentration of about 2mg/ml. The mixture was incubated at 23°C for 30min and thermolysin was added in 1.5-fold molar excess over α_2M. The mixture then was made 10mM with respect to $CaCl_2$ and incubated for a further 15min at the same temperature, and made 10mM with respect to EDTA. As a control, $CaCl_2$ was replaced by EDTA to inhibit the proteolytic activity of thermolysin. Portions (20μl) of the reaction mixtures were subjected to electrophoresis in 7% (w/v)-polyacrylamide gels in the presence of SDS and reducing agent as described under 'Methods'.

The radioautographs of the dried gels showed SDS-stable complexes of the proteins with α_2M that ran more slowly than the free proteins. Inspection of the gels showed that complexes were formed between α_2M and lysozyme, anhydrotrypsin, Dip-trypsin and aprotinin, but were barely detectable with soya-bean trypsin inhibitor, and not at all with serum albumin. Pancreatic elastase similarly caused covalent linking of the labelled proteins to α_2M.

Mixtures incubated with EDTA-inactivated thermolysin, or Dip-elastase, showed no such covalent linking of proteins to α_2M. We conclude that non-proteolytic proteins can become covalently linked to α_2M, but only in the presence of an active proteinase, and thus probably by the chemical mechanism that links proteinases. We postulate that proteolysis of α_2M generates a chemically reactive species of the protein, which we refer to as 'α_2M*'.

Inhibition of the covalent linking reaction

We tested various compounds for the capacity to inhibit the linking of ^{125}I-labelled proteinases to α_2M (Table 1).

It was found that several compounds inhibited the covalent linking, as shown by a decrease in the percentage of total radioactivity associated with α_2M; these were all compounds exhibiting significant nucleophilicity at pH8.0 (Table 1). However, none of the compounds tested decreased the capacity of α_2M to inhibit the proteolytic activity of the proteinases as measured with hide powder azure.

Table 1. *Inhibition by nucleophilic compounds of the covalent linking of proteinases to α_2M*

A 20μl portion of a 30mM solution of each compound in 0.1M-Tris/HCl buffer, pH8.0, was added to 20μl of a 2mg/ml solution of α_2M. To this was added 20μl of ^{125}I-labelled proteinase (elastase or trypsin, 25μg/ml in 5mM-HCl). After 30min at 23°C the mixture was made 2mM with respect to Dip-F. A 15μl portion of each mixture was run in a 7% (w/v) gel in SDS without reduction. Gels were stained and dried down, and radioactive bands identified by radioautography were cut out for gamma-radiation counting. Quantification of the covalent binding of proteinases by α_2M was done by determining the amount of radioactivity in high-M_r components co-migrating with the unreduced dimer of α_2M subunits as a percentage of the total radioactivity in each gel lane. There was no detectable inhibition by iodoacetate, EDTA, Tris, NaCl, glycerol or mannose at 100mM concentration.

Compound (10mM concentration in final reaction mixture)	Inhibition of covalent linking of proteinases to α_2M (% of control)	
	Elastase	Trypsin
Lysine	26	<5
Acetylhydroxamic acid	48	<5
Glycine	50	5
β-Aminoproprionitrile	72	13
Glycine methyl ester	76	16
Benzylhydroxamic acid	78	20

The 6-amino groups of lysine residues are among the most nucleophilic species in proteins and, since the covalent linking of pancreatic elastase (which contains three lysine residues) was more readily inhibited by nucleophiles than the linking of trypsin (which contains fourteen lysine residues), we reasoned that these groups might well be involved in the covalent linking of proteins to α_2M. Samples of trypsin were acetylated to block lysine residues to various extents, and labelled with ^{125}I (see under 'Methods'). Although the acetylated trypsin retained full proteolytic activity, the amount of covalent linking to α_2M was decreased (Fig. 1). Fig. 1 also shows (for each proteinase except papain) a correlation between the amount of covalent linking to α_2M, and lysine content.

The involvement of lysine residues in the covalent reaction is reminiscent of the action of transglutaminases, which catalyse the cross-linking of lysine to glutamine side chains in proteins, and which have been proposed to be responsible for the binding of α_2M during uptake by cultured fibro-

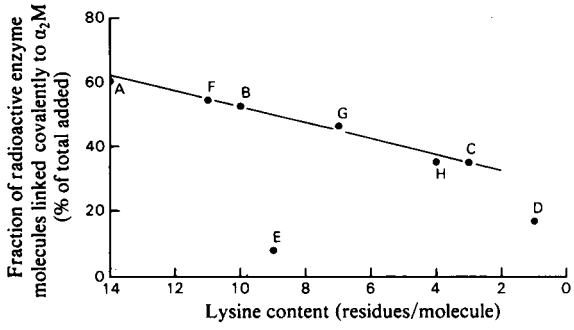

Fig. 1. *Extent of covalent linking of various proteinases to α_2M compared with their lysine content*

Trypsin was partially acetylated, and the number of lysine residues remaining unacetylated determined as described under 'Methods'. The extent of covalent linking of ^{125}I-labelled acetyltrypsin to α_2M was determined as described in Table 1. Data for the linking of trypsin (A), plasmin light chain (B), pancreatic elastase (C), leucocyte elastase (D) and papain (E) are taken from Salvesen & Barrett (1980). Lysine contents (residues/molecule) of proteinases were from: Dayhoff & Eck (1968) (A and E), Wallén (1978) (B), Shotton & Hartley (1970) (C), and Travis *et al.* (1978) (D). Acetyltrypsin samples (18, 52 and 71% acetylated) were calculated to contain 11 (F), 7 (G) and 4 (H) lysine residues/molecule, to the nearest integer.

blasts (Maxfield *et al.*, 1979). Such enzymes are inhibited by EDTA and iodoacetate, however (Folk & Finlayson, 1977), and these compounds had no effect on the linking of proteins to α_2M (see Table 1).

Proteinase-mediated incorporation of amines into α_2M

α_2M was made to react with elastase in the presence of [^{3}H]glycine and run on a column of Sephadex G-25 (Fig. 2*a*). The amino acid was incorporated into α_2M, but only in the presence of the active elastase. When the amine-radiolabelled α_2M was denatured, carboxymethylated and re-run on the same column, no second peak (corresponding to free radiolabelled amino acid) was detected, indicating that the glycine incorporated into α_2M in the presence of active proteinase was bound covalently. [^{3}H]Lysine and [^{3}H]glycine were both incorporated into the proteolytic derivative of α_2M designated IVa (Salvesen & Barrett, 1980) (see Fig. 2*b*). Thermolysin, trypsin and chymotrypsin also caused the covalent incorporation of [^{3}H]-glycine and [^{3}H]lysine into α_2M, whereas Dip-trypsin, Dip-chymotrypsin and EDTA-inactivated thermolysin caused no detectable incorporation.

The incorporation of ^{3}H-labelled amines into α_2M* was quantified with [^{3}H]glycine. α_2M (100μg in 50μl of the Tris buffer, pH 8.0, containing

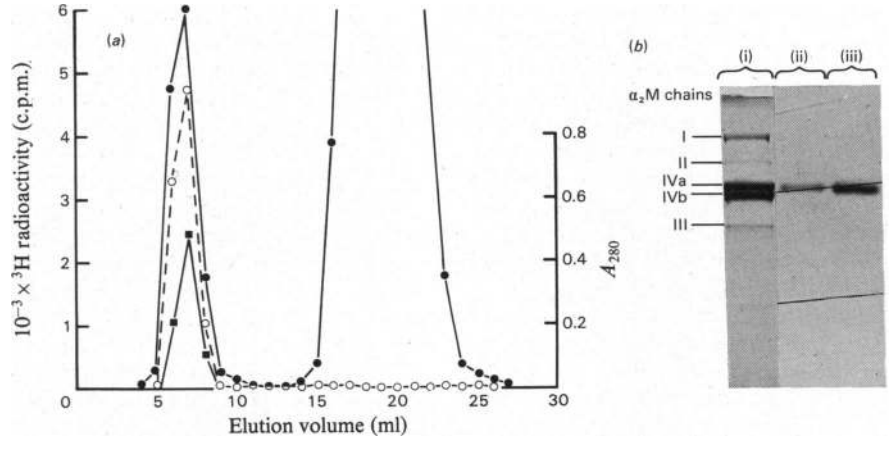

Fig. 2. *Proteinase-mediated incorporation of amines into α_2M*

(*a*) A sample (100μg) of α_2M (2mg/ml in 0.1M-Tris/HCl buffer, pH 8.0) was incubated with 5μmol of [^{3}H]glycine for 15min at 23°C. To this was added a sub-saturating weight (see under 'Methods') of elastase in 10μl of the same buffer. The mixture was incubated for a further 30min at 23°C, made 2mM with respect to Dip-F and run on a column (20ml; 1cm × 25cm) of Sephadex G-25 in pH 8.0 Tris buffer. Elution was at 50ml/h and fractions (0.5ml) were collected. The fractions were monitored for A_{280} (■) and for radioactivity (●). Fractions containing α_2M protein were combined, concentrated to 0.5ml by dialysis against poly(ethylene glycol), made 6M with respect to guanidinium chloride and 10mM with respect to dithiothreitol for 2h at 40°C and then carboxymethylated with 30mM-iodoacetic acid. The denatured sample was run on the same column in 5% (v/v) formic acid, and fractions were monitored for radioactivity (○). (*b*) A portion of the original mixture, as well as a portion of elastase made to react with α_2M in the presence of 5μmol of [^{3}H]lysine was run in a 7%-polyacrylamide/SDS gel with reduction. The gel was stained, dried and fluorographed as described under 'Methods'. Migration of (ii) [^{3}H]lysine and (iii) [^{3}H]glycine compared with (i) a photograph of the stained gel showing α_2M protein is shown.

0.3 M-[^{3}H]glycine) was made to react with elastase as described above. This concentration of glycine had been found to decrease the covalent linking of elastase to α_2M by more than 90%. α_2M-bound [^{3}H]glycine was separated from free glycine on Sephadex G-25 as described above, and it was found that 3.1 mol of [^{3}H]glycine were incorporated/mol of α_2M. Pre-mixing the α_2M with higher concentrations of [^{3}H]glycine did not increase the amount of amine incorporated, suggesting that the maximum incorporation of [^{3}H]glycine into α_2M mediated by a proteinase was one molecule per α_2M subunit.

We concluded from these results that α_2M* has one covalent reaction site per subunit, and that nucleophiles of low M_r are capable of reacting with these sites, and so decreasing the linking of proteins. There are indications that the reactive groups of α_2M* have some selectivity for amine nucleophiles in preference to hydroxamic acids, which would show much higher nucleophilic reactivity towards *p*-nitrophenyl acetate (Jencks & Carriuolo, 1960).

*Half-life of the nucleophile-reactive site in α_2M**

The rate of decay of the nucleophile-reactive site in α_2M* was determined as follows. α_2M (7.0mg, 10nmol) was made to react with a sub-saturating weight of trypsin or elastase in 10ml of 0.05 M-Tris/HCl buffer, pH 7.5. At specified time intervals, 10μl of [^{3}H]glycine or [^{3}H]lysine solution was added to portions of this solution and incubated for 1h at 23°C. A plot of log (covalent incorporation of the radiolabelled nucleophiles) against time (results not shown) showed that the decay was first-order in α_2M, with a rate constant of 6.2 ($\pm$0.5) $\times$ 10^{-3} s^{-1} at pH 7.5, equivalent to a half-life of 112 ($\pm$9)s, for α_2M* formed by both trypsin and elastase (two separate determinations with each enzyme). This estimate of the rate of decay of α_2M* is a maximal one, as no allowance was made for those sites that decay after the addition of labelled nucleophile, but is probably reasonably accurate, as both glycine and lysine, which have different reactivities towards α_2M*, gave essentially the same result. The observed decay of nucleophile-sensitive sites in α_2M is probably due to a combination of reaction with nucleophilic side chains on the proteinase and hydrolysis by water molecules and/or hydroxide ions.

Location of the covalent linking site

We used peptide 'mapping' to locate the reactive site in the α_2M chain, and to determine its relationship to the covalent attachment site for methylamine described by Swenson & Howard (1979).

Samples (50–100μg) of α_2M made to react with elastase in the presence of 0.1 M-[^{3}H]glycine or 0.2 M-[^{3}H]lysine, and of non-reacted α_2M incubated with 0.2 M-[^{14}C]methylamine, were run in 15% 'mapping' gels (see under 'Methods'). The gel was stained to detect protein and fluorographed to detect the radioactive-amine-labelled peptides (see Fig. 3). Radioactive peptides containing methylamine, glycine or lysine co-migrated with an apparent M_r of 33000, suggesting that a peptide of this M_r contains the methylamine and proteinase covalent attachment site(s).

To test the possibility that the site of reaction with nucleophiles in the proteinase-mediated linking might be the same as that which reacts with methylamine in the absence of proteinases, we asked whether methylamine was incorporated into proteinase-reacted α_2M and whether proteinases were covalently linked to methylamine-reacted α_2M.

α_2M (0.7mg, 1nmol) was incubated with a sub-saturating weight of elastase or trypsin in 1.0ml

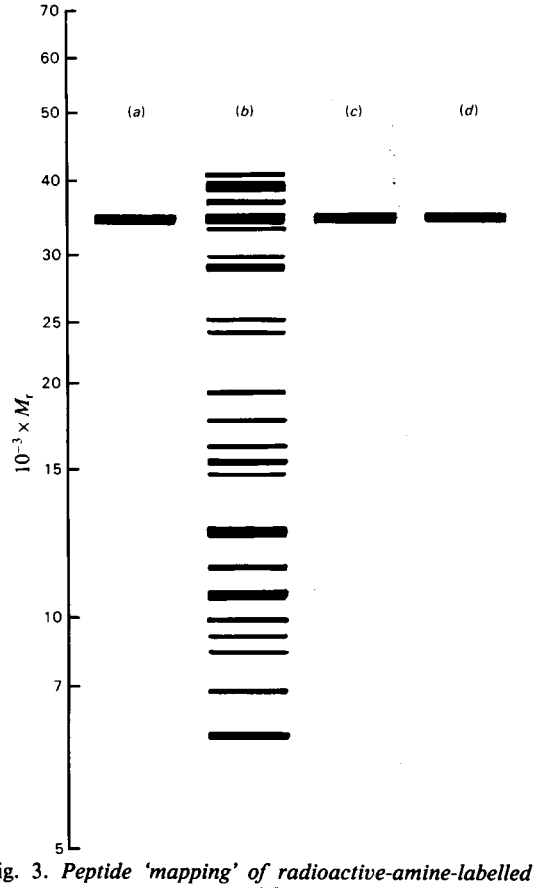

Fig. 3. *Peptide 'mapping' of radioactive-amine-labelled α_2M*

Samples electrophoresed in the presence of SDS and *Staphylococcus aureus* V8 proteinase were α_2M, which had incorporated [^{14}C]methylamine (*a*), and α_2M made to react with elastase in the presence of [^{3}H]glycine (*c*) or [^{3}H]lysine (*d*). α_2M peptides stained for protein are shown for comparison (*b*).

of 0.1 M-Tris/HCl buffer, pH 8.0, for 30 min at 23°C, during which time the nucleophile-sensitive sites in α_2M* had decayed (see above). The solution was made 2 mM with respect to Dip-F and 10 mM with respect to [^{14}C]methylamine and incubated at the same temperature for a further 40 h. Less than 0.2 nmol of [^{14}C]methylamine was incorporated into the trypsin-reacted and elastase-reacted α_2M. In a control experiment in which the proteinases were omitted from the reaction mixture, 3.4 nmol of [^{14}C]methylamine was incorporated into the α_2M. Consequently, we can deduce that methylamine cannot become incorporated into proteinase-reacted α_2M whose nucleophile-sensitive sites have decayed. Conversely, no covalent linking of ^{125}I-trypsin or ^{125}I-elastase was detected when these proteinases were made to react with methylamine-treated α_2M (0.2 M-methylamine for 2 h at 23°C in 0.1 M-Tris/HCl, pH 8.0). These findings suggest that the methylamine-reactive site is indeed the same as the proteinase-activated reactive site.

Nature of the direct incorporation of methylamine into α_2M

Unlike the other nucleophiles (amines and hydroxamates) used in the present study, which are incorporated only into α_2M*, methylamine is incorporated into unchanged α_2M (see above). Since methylamine also produces the S-to-F transition in α_2M (Barrett *et al.*, 1979), it seemed possible that methylamine acts by converting α_2M and α_2M* in a similar manner to that brought about by proteinases. If so, it would be expected that glycine would inhibit the subsequent incorporation of methylamine. We tested this possibility by incubating methylamine and glycine with α_2M simultaneously.

α_2M (0.7 mg, 1.0 nmol) in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.2 M-glycine was made 1.0 mM with respect to [^{14}C]methylamine and incubated for 20 h at 23°C. In a parallel experiment, the α_2M was incubated under the same conditions, but in the presence of 0.2 M-[^{3}H]glycine, with 10 mM-methylamine. In contrast with its effect on the covalent linking of proteinases, glycine did not inhibit the incorporation of [^{14}C]methylamine into α_2M, nor was any [^{3}H]glycine incorporated during the reaction of methylamine with α_2M.

These data suggest that methylamine causes the conformational change by reacting directly with the electrophilic site of α_2M. This contrasts with the action of proteinases, in which the conformational change precedes reaction at the covalent binding site. The latter sequence of events is implied by the high rate of trapping of proteinases (a measure of the rate of conformational change), in contrast with the slow decay of the electrophilic site. Thus the $t_{1/2}$ for trapping of trypsin (0.046 s at the concentrations used in the present study, calculated from the data of Barrett & Salvesen, 1979) may be contrasted with a $t_{1/2}$ of 112 s for the decay of the electrophilic site.

pH-dependence of the covalent reactions

The pH-dependencies of the two separate covalent reactions (the direct methylamine reaction and the proteinase-mediated incorporation of glycine) were determined in the range pH 6.0–9.2. α_2M (0.7 mg) was incubated for 20 h with 0.2 M-[^{3}H]glycine in 1.0 ml of buffer (see Fig. 4 for details of buffers). A sub-saturating weight of elastase was added and samples were taken after 30 min for the quantification of radioactivity. No [^{3}H]glycine became incorporated into the α_2M in the presence of the Dip-elastase, whereas 0.75 mol was incorporated/mol of α_2M subunit in the presence of elastase. This proteinase-mediated incorporation of glycine showed no apparent pH-dependence over the range specified above, presumably because the covalent reaction had gone to completion before the samples were taken.

The direct incorporation of [^{14}C]methylamine into α_2M showed a definite dependence on pH (see Fig. 4). A plot of log (methylamine incorporated) against pH (Dixon, 1953) had a slope of 0.9 at 20 h and 0.85 at 40 h, suggesting the presence of one ionizing group responsible for the enhanced rate of incorporation with elevated pH. Since methylamine has a pK of 10.7, and only the unprotonated form is nucleophilic, it seems certain that the increase in rate of the methylamine reaction with increasing pH is simply the result of the deprotonation of the base.

Chemical nature of the nucleophile-reactive site

Swenson & Howard (1979) identified the site of incorporation of methylamine into α_2M as a

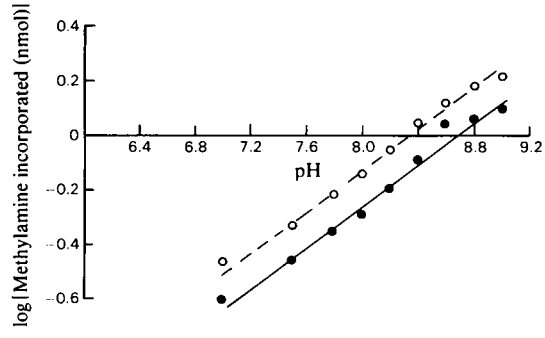

Fig. 4. *pH-dependence of the incorporation of [^{14}C]-methylamine into α_2M*

α_2M (0.7 mg) was incubated with 1.0 mM-[^{14}C]-methylamine for 20 or 40 h at 23°C in 1.0 ml of buffer. Buffers used were 0.1 M-Tris/maleic acid/NaOH for the range pH 6.0–7.2, and 0.1 M-Tris/HCl for the range pH 7.4–9.2. log ([^{14}C]methylamine incorporation) was plotted against pH after 20 (●) and 40 h (○) of incubation.

glutamic acid residue, which, they suggested, might exist in an activated f in the native molecule. If the electrophilic site in α_2M were a thiol ester, the leaving group after reaction with a nucleophile would be a thiolate anion, which should be detectable by reaction with iodoacetic acid.

α_2M (2.0mg in 1.0ml of 0.1M-Tris/HCl buffer, pH8.0) was incubated at 23°C for 1h with either 0.2M-methylamine or a sub-saturating weight of elastase. The solutions were made 2.0mM with respect to iodo[^{3}H]acetate and incubated for a further 1h. Portions (50μl) were taken for peptide 'mapping' and the remainder of each solution was counted for the incorporation of radiolabel as described under 'Methods'. About 3.5nmol of ido[^{3}H]acetate was found to be incorporated/nmol of methylamine-treated α_2M, and 3.1nmol was incorporated/nmol of elastase-treated α_2M, suggesting the presence of one iodoacetate-reactive site/α_2M subunit. Non-reacted α_2M incorporated less than 0.2mol of iodo[^{3}H]acetate/mol. Results of the peptide 'mapping' (results not shown) indicated that, in the case of methylamine-treated α_2M, iodo-[^{3}H]acetate was incorporated exclusively into the 33000-M_r peptide, which contains the nucleophile-reactive site. [^{3}H]Iodoacetate was incorporated into two peptides of elastase-reacted α_2M, the 33000-M_r peptide containing the nucleophile-reactive site and a peptide of 50000 M_r, which is probably composed of the 33000 M_r peptide linked to elastase.

To identify the amino acid residue responsible for iodoacetate incorporation, we ran the 24h acid hydrolysate of methylamine-treated α_2M that had been made to react with iodo[^{14}C]acetate on an amino acid analyser, mixed with [^{3}H]carboxymethylated cysteine for reference. A small peak of ^{3}H-containing material appeared after 34min, followed by a peak containing both ^{3}H and ^{14}C at 41min. The first ^{3}H peak was probably attributable to *NS*-dicarboxymethylcysteine, since the second, major, peak appeared at an elution time close to that of unlabelled *S*-carboxymethylcysteine run separately. About 88% of the ^{14}C from the α_2M hydrolysate (1.5×10^5c.p.m.) was recovered in the carboxymethylcysteine peak.

These results suggest that radiolabelled iodoacetate is incorporated into the thiol moiety of a cysteine residue in methylamine-reacted α_2M. As its extent of incorporation into elastase-reacted α_2M is the same as for methylamine-reacted α_2M, i.e. 1mol per mol of quarter subunit, it seems probable that iodoacetate reacts with the same cysteine residue in both. This strongly suggests that the nucleophile-sensitive site in α_2M is indeed a thiol ester formed from the thiolate moiety of a cysteine side chain and the γ-carbonyl carbon of a glutamic acid side chain. It is known that thiol esters are stable in neutral aqueous solutions and are very reactive towards nucleophilic attack by amines (Lynen, 1970). Indeed, a thiol ester has been reported to be responsible for the covalent binding site in complement component C3 (Law *et al.*, 1979). Tack *et al.* (1980) recently showed that the sequence of amino acids about the covalent binding site in C3 is identical with that reported for α_2M by Swenson & Howard (1979), and suggested that a cysteine residue forms a thiol ester with the glutamic acid residue at which covalent incorporation occurs in C3.

Discussion

*The electrophilic sites of S-α_2M and α_2M**

The properties of the electrophilic centre in S-α_2M suggest that it is located in a 'pocket' that shields it from reaction with any but the smallest nucleophiles, for NH_3 and ethylamine act in the same way as methylamine, but 1,3-diaminopropane does not (Barrett *et al.*, 1979). In contrast, the electrophilic centre in α_2M* has a very broad reactivity, encompassing amines generally, hydroxamates and proteins. This change in the properties of the reactive group presumably results from the S-form-to-F-form conformational change produced by proteinases, and seems to take the form of an opening of the 'pocket' that previously restricted reactivity. Possibly, the electrophilic group itself also becomes more reactive in the process.

The electrophilic centre in α_2M shares many properties with the covalent binding sites in complement components C3 and C4. These components, but not their cleaved forms (C3b and C4b), have been shown to undergo denaturation-dependent cleavage of a single peptide bond (Howard, 1980; Sim & Sim, 1981). In common with α_2M, both C3 and C4 are inactivated by low-M_r amines, and covalently incorporate them (Sim & Sim, 1981; Campbell *et al.*, 1980). The latter authors have shown that cleavage of C3 and C4 reveals on C3b and C4b covalently reacting groups of broad specificity, reactive with both amine and hydroxamate nucleophiles, similar to those on α_2M*.

Although the nature and reactivity of the electrophilic group in α_2M are now fairly well understood, its function remains a mystery. Salvesen & Barrett (1980) showed that the covalent reaction was not an essential requirement for the inhibition of proteinases by α_2M. Our present data endorse that finding, showing that nucleophiles which inhibit the covalent linking of proteinases to α_2M do not alter its proteinase-inhibitory capacity. The function of the C4 covalent binding site seems to be to attach C3 convertase to immunoglobulin-G–antigen aggregates, via an amide linkage (Campbell *et al.*, 1980), and the covalent binding site of C3 probably locates C5 convertase activity on cell surfaces (but via an

ester linkage to sugar hydroxy groups; Sim & Sim, 1981). It is conceivable that the α_2M electrophilic group performs a comparable function, i.e. links α_2M–proteinase complexes to surfaces of cells, bacteria, etc., when proteinases are present. This might have an opsonic effect, since macrophages and other cells rapidly bind and ingest α_2M in the reacted F-form (Debanne *et al.*, 1976).

A possible explanation for the spontaneous peptide-bond-cleavage reaction in denatured S-α_2M

The peptide bond in denatured S-α_2M that is susceptible to cleavage, especially at alkaline pH values and high temperature (Barrett *et al.*, 1979; Harpel *et al.*, 1979), has been shown by Howard *et al.* (1980) to be on the *N*-terminal side of the covalently reacting glutamine residue in α_2M (see Fig. 5). If, as our data suggest, the electrophilic group in α_2M is a thiol ester, then it is possible that attack by the peptide-bond nitrogen atom on the electron-deficient carbonyl carbon of the ester occurs after deprotonation of the nitrogen atom under alkaline conditions, and leads to the formation of an internal imide (pyroglutamic acid residue). A similar reaction, the cleavage of β-benzyl esters of aspartic acid-containing peptides, has previously been shown to occur under mildly alkaline conditions, the rate of cleavage being 10^7-fold that of the competing hydrolysis because the attack by the nitrogen atom is intramolecular (Bernhard *et al.*, 1962). Similarly, the intramolecular N→S acyl migration in *S*-acetylmercaptoethylamine is faster than the hydrolysis of this thiol ester (Martin & Hendrick, 1962). We suggest that the attack on the thiol ester in α_2M is favoured, as compared with hydrolysis, because it, too, is an intramolecular process.

Peptides containing a pyroglutamic acid residue have been shown to undergo hydrolysis in alkali at one of the two sites designated *a* and *b* in Fig. 5 (Battersby & Reynolds, 1961). Howard *et al.* (1980) suggested that hydrolysis along pathway *a*, leading to peptide-bond cleavage, takes precedence in denatured S-α_2M, and that this pathway is particularly favoured at high temperatures. Although this scheme is consistent with the characteristics of the reaction, we doubt the existence of the preformed pyroglutamic acid residue because of the independent evidence for a thiol ester as the electrophilic group in α_2M, and the low reactivity of pyroglutamic acid with alkylamines (Lichtenstein, 1942). Sim & Sim (1981) showed that a similar autolytic cleavage of the peptide chain occurs in

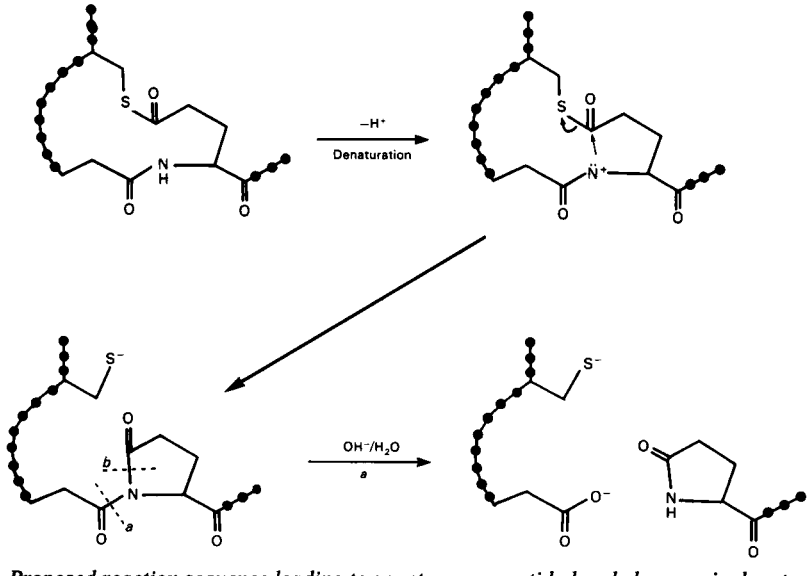

Fig. 5. *Proposed reaction sequence leading to spontaneous peptide-bond cleavage in denatured S-α_2M*
The diagram shows the thiol-ester structure proposed to be responsible for the nucleophilic reactivity of α_2M. The peptide backbone is shown by 'beads' and is expanded to depict the labile peptide bond. Positions of the labile bond and activated glutamic acid residue are taken from Howard *et al.* (1980). It is proposed that attack at the electrophilic centre occurs, under denaturing conditions above pH 7.0, by the peptide-bond nitrogen atom to give peptide-bond hydrolysis, with an internal pyroglutamic acid residue as an intermediate. Prior reaction of the electrophilic centre (in S-α_2M or α_2M*) with a nucleophile would eliminate the potential for peptide-bond cleavage by destroying the requirement for an activated centre. Hence, the cleavage is not seen in methylamine-treated α_2M (Barrett *et al.*, 1979) or in proteinase-reacted α_2M (Salvesen & Barrett, 1980).

complement components C3 and C4, and it is tempting to suggest that the complement components are cleaved by a mechanism similar to that proposed here for α_2M, especially in view of evidence for similarities in amino acid sequence at the corresponding points in the peptide chains (Tack *et al.*, 1980).

Note added in proof

The size-dependence of the rate of reaction of alkylamines with S-α_2M has been examined directly with the series methylamine, ethylamine, n-propylamine and n-butylamine. The rate of S-to-F conversion decreased through the series and was about 100-fold slower for butylamine as compared with methylamine.

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