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The Electrophoretically 'Slow' and 'Fast' Forms of the α_2-Macroglobulin Molecule

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α_2-Macroglobulin (α_2M) was isolated from human plasma by a four-step procedure: poly(ethylene glycol) fractionation, gel chromatography, euglobulin precipitation and immunoadsorption. No contaminants were detected in the final preparations by electrophoresis or immunoprecipitation. The protein ran as a single slow band in gel electrophoresis, and was designated 'S-α_2M'. S-α_2M bound about 2mol of trypsin/mol. Treatment of S-α_2M with a proteinase or ammonium salts produced a form of the molecule more mobile in electrophoresis, and lacking proteinase-binding activity (F-α_2M). The electrophoretic mobility of the F-α_2M resulting from reaction with NH_4^+ salts was identical with that of proteinase complexes. We attribute the change in electrophoretic mobility of the α_2M to a conformational change, but there was no evidence of a change in pI or Stokes radius. Electrophoresis of S-α_2M in the presence of sodium dodecyl sulphate gave results consistent with the view that the α_2M molecule is a tetramer of identical subunits, assembled as a non-covalent pair of disulphide-linked dimers. Some of the subunits seemed to be 'nicked' into two-thirds-length and one-third-length chains, however. This was not apparent with F-α_2M produced by ammonium salts. F-α_2M produced by trypsin showed two new bands attributable to cleavage of the subunit polypeptide chain near the middle. Immunoassays of F-α_2M gave 'rockets' 12–29% lower than those with S-α_2M. The nature of the interactions between subunits in S-α_2M and F-α_2M was investigated by treating each form with glutaraldehyde before electrophoresis in the presence of sodium dodecyl sulphate. A much greater degree of cross-linking was observed with the F-α_2M, indicating that the subunits interact most closely in this form of the molecule. Exposure of S-α_2M to 3M-urea or pH3 resulted in dissociation to the disulphide-bonded half-molecules; these did not show the proteinase-binding activity characteristic of the intact α_2M. F-α_2M was less easily dissociated than was S-α_2M. S-α_2M was readily dissociated to the quarter-subunits by mild reduction, with the formation of 3–4 new thiol groups per subunit. Intact reactive α_2M could then be regenerated in high yield by reoxidation of the subunits. F-α_2M formed by reaction with a proteinase or ammonium salts was not dissociated under the same conditions, although the interchain disulphide bonds were reduced. If the thiol groups of the quarter-subunits of S-α_2M were blocked by carboxymethylation, oxidative reassociation did not occur. Nevertheless treatment of these subunits with methylammonium salts or a proteinase caused the reassembly of half-molecules and intact (F-) tetramers. It is emphasized that F-α_2M does not have the properties of a denatured form of the protein. The suggestion is made that one attractive interpretation of the difference in properties between the S- and F-forms is that the S-form is a planar tetramer, whereas the F-form is pseudotetrahedral. The results are considered in relation to the 'trap' hypothesis for the reaction of α_2M with proteinases.

α_2-Macroglobulin (α_2M) is a plasma glycoprotein of about 725000 mol.wt. (for reviews see Laurell & Jeppsson, 1975; Harpel, 1976; Starkey & Barrett, 1977). α_2M has the unique property of binding and inhibiting the great majority of endopeptidases, regardless of their specificity or catalytic mechanism, and there is good reason to think that the protein is important in the physiological control of extracellular proteolytic activity. In pointing out the broad reactivity of α_2M with proteinases, Barrett & Starkey (1973) put forward the 'trap' hypothesis for

Abbreviations used: α_2M, α_2-macroglobulin; F-α_2M, the electrophoretically fast form of α_2M; S-α_2M, the electrophoretically slow form of α_2M; IgA, immunoglobulin A; IgM, immunoglobulin M; SDS, sodium dodecyl sulphate.

the reaction, It was argued that most proteinases have the capacity to attack a particularly vulnerable region in the macroglobulin molecule, and that this triggers a rapid conformational change in the macroglobulin molecule that results in the trapping of the enzyme molecule within it. The result is a characteristic steric inhibition of the reactivity of the bound proteinase with large substrate or inhibitor molecules, and also with antibodies (Geokas *et al.*, 1977).

Subsequently two new pieces of evidence were obtained in support of the 'trap' hypothesis. It was shown that reaction of α_2M with proteinases does indeed involve proteolytic cleavage (Harpel, 1973), and that this occurs at essentially the same point in the molecule despite widely differing proteinase specificities (Barrett *et al.*, 1974). It was also demonstrated that the electron-microscopic appearance of α_2M is consistent with a conformational change being associated with the reaction with proteinases (Barrett *et al.*, 1974).

Research on the interaction of α_2M with proteinases has received a stimulus from the suggestion that α_2M of people homozygous for the hereditary disease cystic fibrosis reacts abnormally (Shapira *et al.*, 1977; Wilson & Fudenberg, 1976). Clearly it is of interest to understand in detail the structure of the α_2M molecule, and to know how it is changed in course of reaction with a proteinase.

The α_2M molecule is a tetramer of identical subunits linked in pairs by disulphide bonds, and then assembled non-covalently (Hall & Roberts, 1978) (Fig. 1). Here we report that the native tetramers can exist in two forms that differ in electrophoretic mobility ('slow' or S-, and 'fast' or F-) as well as other properties and that the apparently irreversible transition of the S-form into the F-form is associated with the binding of a proteinase. The results are considered in relation to the structure of α_2M and its mechanism of action.

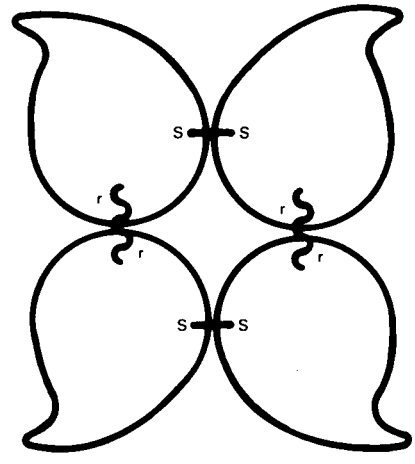


Fig. 1. *Diagrammatic representation of the a_2M molecule*
The identical quarter-subunits of the α_2M molecule are linked in pairs by disulphide bonds, and the resultant half-molecules are associated non-covalently (at the sites marked 'r'). The shapes indicated for the subunits and the whole molecule are not intended to resemble their actual shapes, which are far more complex.

Materials and Methods

Materials

Sources of chemicals and other materials were as follows: Brij 35, iodoacetic acid (special purity), methylammonium chloride, ethylammonium chloride, 1,2-diaminoethane, ethanolamine and poly(ethylene glycol) 6000, from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.; 1,3-diaminopropane from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K.; albumin (bovine serum, crystallized), carbonic anhydrase (bovine erythrocyte), catalase (bovine liver, 2×crystallized), α-chymotrypsin (bovine, 3×crystallized), cytochrome *c* (horse heart, type IIa), ferritin (horse spleen, type I), papain (2×crystallized), phenylmethanesulphonyl fluoride, phosphorylase *a* (rabbit muscle, 2×crystallized), thermolysin, transferrin (human), neuraminidase (*Clostridium perfringens*, type V), trypsin (bovine, 2×crystallized), trypsin inhibitor (soya-bean, type I-s), thyroglobulin (pig, type II), from Sigma (London) Chemical Co., Poole, Dorset BH17 17NH, U.K.

Trypsin preparations were standardized by active-site titration (Chase & Shaw, 1967), but weights of trypsin given in the text refer to the commercial solid (52% active enzyme).

Ultrogel AcA 22 and AcA 34, Ampholines and the preparative isoelectric-focusing apparatus were from LKB Instruments, South Croydon, Surrey CR2 8YD, U.K., and benzoyl-DL-arginine 4-nitroanilide hydrochloride and benzyloxycarbonyl-L-arginine 2-(4-methoxy)naphthylamide hydrochloride from Bachem Feinchemikalein, Liestal CH-4410, Switzerland.

Myosin from rabbit skeletal muscle was kindly given by Dr. A. Weeds, M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge, U.K.

Immune globulin preparations (rabbit) against human immunoglobulins, IgA, IgM, fibrinogen, plasminogen and haptoglobin were from Mercia Brocades, Weybridge, Surrey KT14 6RA, U.K. Horse anti-(human serum) antiserum was from Wellcome Reagents, Beckenham, Kent BR3 3BS, U.K. Antiserum (rabbit) against human α_2-pregnoglobulin (pregnancy-associated α-globulin) was a

gift from Dr. A. R. Bradwell, Immuno Diagnostic Research Laboratory, Department of Experimental Pathology, Birmingham B15 2TJ, U.K. Rabbit anti-(human α_2-macroglobulin) sera were a gift from Dr. N. Heimburger, Behringwerke A.G., Marburg, Germany. Standard human plasma and serum were from Behring Diagnostics, Hoechst Pharmaceuticals, Hounslow, Middx. TW4 6JH, U.K.

Electrophoresis

The systems of gel electrophoresis are distinguished as 'rate' electrophoresis of native proteins or SDS–protein complexes and as 'pore-limit' electrophoresis of native proteins run to pseudo-equilibrium in gradients of increasing polyacrylamide concentration.

Rate electrophoresis of native proteins. The buffer system was the discontinuous 2-amino-2-methylpropane-1-diol (Ammediol)/glycine/chloride system of Wyckoff *et al.* (1977), in which resolution occurs at pH9.58. Polyacrylamide slab gels (5% or 7% total acrylamide concentration, 2.6% of this as methylenebisacrylamide) were prepared as follows. Pairs of glass plates (81mm square, 1.2mm thick) were spaced with plastic strips (4mm wide, 2.8mm thick) and taped together at two opposite edges. Sets of ten such cassettes were placed with the taped sides vertical in a Perspex [poly(methyl methacrylate)] box. The box was filled to 1cm depth with aq. 25% (v/v) ethanol. The polyacrylamide monomer solution was then pumped into the bottom of the box, so that it gradually rose to fill the cassettes, overlayered with the aqueous ethanol. When the gel had polymerized, the slabs were removed from the box, sealed in polythene bags and stored at 4°C until required. For use, each slab was provided with an upper gel (3.2% total acrylamides, 6.25% as methylenebisacrylamide) in which six sample slots were formed by use of an acrylic comb (Pharmacia, code no. 91 482).

Samples containing about 1mg of protein/ml were mixed with an equal volume of double-strength upper-gel buffer containing 40% (v/v) glycerol and 0.01% Bromophenol Blue. A 20μl portion was applied to each slot.

Electrophoresis was in a Pharmacia GE-4 tank or a similar apparatus constructed in the laboratory. Samples were run into the gel at a current of 20mA (~100V), before the current was increased to 40mA (~200V) for the remainder of the run. The run was stopped when the Bromophenol Blue marker approached the lower edge of the gel (after about 60min).

Gels were stained with 0.1% Coomassie Brilliant Blue G in methanol/acetic acid/water (5:1:4, by vol.) during 1h at 55°C, before destaining in methanol/formic acid/water (30:1:69, by vol.) and eventual storage in 1% (v/v) formic acid.

Rate electrophoresis of SDS–protein complexes. The buffer system was as described for native proteins, except that the upper reservoir buffer contained 0.1% SDS, and the gels were of 7% total acrylamide concentration. Samples known to contain proteinases, e.g. trypsin–α_2M complex, were treated to inactivate the proteinase (with 1mM-phenylmethanesulphonyl fluoride, in this instance) before exposure to SDS. Samples (50μl containing about 50μg of protein) to be run reduced were mixed with an equal volume of sample buffer containing double-strength upper-gel buffer with 0.01% Bromophenol Blue, 40% (v/v) glycerol, 2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, and immediately heated at 100°C for 5min. Alternatively, some were incubated at 37°C for 45min, as indicated in the text. Portions (20μl) were placed in the sample slots of the upper gel.

It was found that boiling in SDS at pH8.37 (the pH of the upper-gel buffer) itself caused some cleavage of disulphide bonds. Samples to be run with disulphide bonds intact were therefore boiled with SDS (2%, w/v) and 2mM-sodium iodoacetate at pH6–7 for 5min before the upper-gel buffer, glycerol and Bromophenol Blue were introduced.

Electrophoresis, staining and storage of the gels were as described above for the non-SDS-containing gels. The standard proteins used for molecular-weight calibration of gels, and the molecular weights assumed for them, were as follows: rabbit muscle myosin heavy chain (mol.wt. 200000), *Escherichia coli* β-galactosidase (mol.wt. 130000), rabbit muscle phosphorylase *a* (mol.wt. 95000), human transferrin (mol.wt. 78000), bovine plasma albumin (mol.wt. 68000), sheep immunoglobulin G heavy chain (mol.wt. 50000) and bovine erythrocyte carbonic anhydrase (mol.wt. 29000).

Pore-limit electrophoresis of native proteins. Slab gels containing concave gradients of polyacrylamide concentration (4–26%) for pore-limit electrophoresis (Margolis, 1973) were prepared as described above, except that the acrylamide monomer gradient was produced by two Pharmacia P-3 pumps, used as described by Lakshman & Lieberman (1954); the two pumps were run at 162 and 180ml/h respectively. The buffer used in gel, reservoirs and samples was 50mM-Tris/7mM-disodium EDTA/2mM-boric acid, pH9.1 (Manwell, 1977). Gels were stored as described above, and when required were fitted with polypropylene sample applicators (Pharmacia, code no. 91 093).

Samples (50μl, containing 50μg of protein) were mixed with an equal volume of double-strength buffer containing 0.01% Bromophenol Blue and 40% (v/v) glycerol, and 10μl portions were applied to the slots in the applicator. Electrophoresis was at 125V,

in the Pharmacia GE-4 apparatus with buffer circulation and water-cooling, overnight. Staining and storage of gels were as described above.

Proteins used in an attempt to calibrate the pore-limit gels with regard to molecular weight were bovine thyroglobulin (mol.wt. 670000), horse spleen ferritin (mol.wt. 440000) and bovine liver catalase (mol.wt. 230000).

For some experiments gels were formed with a formic acid (50mM)/Tris (25mM) buffer, pH3.1, by use of an H_2O_2/thiourea catalyst system (Hurley, 1977), and samples were run in this buffer with Methyl Green as tracking dye.

Densitometry of electrophoresis gels. Coomassie Blue-stained gels were scanned at 592nm by use of the model 2520 accessory of a Gilford Instruments model 250 spectrophotometer, the absorbance being recorded and integrated with a Smiths Servoscribe RE 542.20 recorder.

Preparative isoelectric focusing

The LKB Instruments type 8101 column was used as described by the manufacturers, with a 9:1 (v/v) mixture of Ampholines pH4–6 and pH3–10.

Radiolabelling of proteinases

α-Chymotrypsin was labelled by treatment with [^{14}C]acetic anhydride, in aqueous solution, essentially as described by Barrett & Heath (1977) for haemoglobin, but without the precipitation step.

Trypsin was labelled by the method of Bolton & Hunter (1973), *N*-hydroxy-3-(*p*-hydroxyphenyl)propionylsuccinimide (Pierce and Warriner, Chester CH1 4EF, U.K.) being treated with [^{125}I]NaI in the presence of chloramine-T, and allowed to react with the protein.

Protein determination

Protein concentrations were determined essentially as described by Lowry *et al.* (1951), with bovine serum albumin as standard. The concentration of solutions of highly purified α_2M was determined by spectrophotometry, on the basis that $A_{280}^{1cm,1\%} = 9.1$ (Dunn & Spiro, 1967).

Immunochemical methods

All immunoplates were stained with Coomassie Brilliant Blue G (Barrett, 1974), and some also with Sudan Black for detection of β-lipoprotein. Double immunodiffusion and immunoelectrophoresis in agarose gels were performed as described by Barrett (1974). 'Rocket' immunoassay (Laurell, 1972) was used to determine the antigenic activity of α_2M; the gel contained 0.4% (v/v) rabbit anti-(human α_2M) serum, and standard human serum was used for standardization. Quantitative radial immunodiffusion (Mancini *et al.*, 1965) was used for determination of immunoglobulins, haptoglobin and fibrinogen. Plates contained 0.2% (v/v) of rabbit immune globulin preparations, and were standardized with standard serum or plasma.

Immunoadsorbents were prepared by coupling the rabbit immunoglobulins against human immunoglobulins, immunoglobulin A, α_2-pregnoglobulin, haptoglobin and fibrinogen to Sepharose 4B that had been activated with CNBr essentially as described by Cuatrecasas & Anfinsen (1971). Each gram of damp activated gel was allowed to react overnight at 20°C with 7.5–50μl of the commercial immune globulin solutions in 50mM-sodium phosphate buffer, pH6.5. The adsorbents were then treated with 1% (w/v) glycine in the same buffer for 1h, and washed with 100mM-sodium formate buffer, pH3.0, followed by 100mM-sodium phosphate, pH7.0, containing 0.1% NaN_3.

Assay of proteinase-inhibiting activity of α_2-M

Activity was determined as the inhibition of proteolytic activity of trypsin against a particulate substrate, hide powder azure (Calbiochem, London W1H, U.K.).

In each of the duplicate assay tubes, a 0.1ml sample containing 10–50μg of α_2M/ml was incubated for 10min at 37°C with 0.1ml of trypsin (2.0μg/ml in 0.10M-Tris/HCl buffer, pH8.1, containing 20mM-$CaCl_2$ and 0.1% Brij 35) in a total volume of 0.5ml made up with the same buffer/detergent solution. Then 0.5ml of water was added to the tube, followed by 0.8ml of substrate suspension, i.e. 12.5mg of hide powder azure/ml in 0.6M-sucrose/0.1% Brij 35/0.03% toluene. The tube was incubated in a roller rack (2 rev./min) at 37°C for 20min, and 1ml of water was added. After centrifugation (1200*g* for 5min), the A_{595} of the supernatant was determined.

A standard curve of 0–0.2μg of trypsin/tube was run in parallel, and results were calculated as the amount of trypsin inhibited per unit weight of α_2M, the trypsin itself being standardized by active-site titration (Chase & Shaw, 1967). A linear relationship between amount of α_2M and degree of inhibition was obtained in the range 0–80% inhibition, and this relationship was used to calculate the inhibitory capacity of the α_2M.

Inhibitory activity against thermolysin was measured in the same way, except that the buffer was 0.4M-Tris/HCl, pH7.5, containing 40mM-$CaCl_2$.

Preparation of α_2-M in the S-form

α_2M was purified by precipitation with poly(ethylene glycol), gel chromatography, euglobulin preci-

pitation and immunoadsorption. In detail, the procedure was as follows. Fresh human blood unsuitable for transfusion was provided by a local blood-transfusion service. In each bag 425ml of blood had been mixed with 75ml of ACD anticoagulant solution containing 0.60g of citric acid monohydrate, 1.65g of trisodium citrate dihydrate and 1.68g of anhydrous glucose. The blood had been stored at 4°C for 2–5 days after collection. Plasma of haptoglobin type 1–1 was used, having been identified by rate electrophoresis after saturation with haemoglobin (Halliday, 1976). The plasma was separated by centrifugation (10000*g* for 15min) in plastic bottles, and stored at −20°C until required.

Plasma (250ml) was mixed with 0.28vol. of aq. 25% (w/v) poly(ethylene glycol) 6000, pH6.5, and left at least 30min at 20°C. The precipitate was removed by centrifugation and discarded, and a further 0.72vol. (on the basis of the original volume) of the 25% (w/v) poly(ethylene glycol) added to the supernatant. After 30min the precipitate [formed at 5.5–12.5% (w/v) poly(ethylene glycol) (Giroux, 1975)] was centrifuged as before, dissolved in 0.1M-sodium citrate buffer, pH6.5, to an A_{280}^{1cm} of about 50, and run on a bed (5cm×80cm, 1570cm^3) of a 2:1 (v/v) mixture of Ultrogel AcA 34 and AcA 22, in the same buffer, at a flow rate of 20ml/h.

A peak of α_2M was detected (by gel electrophoresis or immunodiffusion) in fractions from the gel chromatography column early in the elution pattern, but not at the excluded volume. Fractions showing little contamination in polyacrylamide-gel electrophoresis were combined, concentrated by ultrafiltration over a Sartorius type 12133 membrane under pressure of N_2 (in a C-50 ultrafiltration cell; Chemlab Instruments Ltd., Hornchurch, Essex, U.K.) to about 10mg/ml and dialysed against 1% (w/v) glycine (two 100vol. portions) during 24–48h. A precipitate (largely composed of immunoglobulins) was removed by centrifugation (26000*g* for 15min) and discarded.

The final step of purification was immunoadsorption. The partially purified α_2M (about 100mg in 10ml of 1% glycine) was passed through a bed (20mm×24cm) containing a mixture of adsorbents for total immunoglobulins (30ml), IgA (20ml), α_2-pregnoglobulin (5ml), haptoglobin (20ml) and fibrinogen (20ml). The unadsorbed protein was washed through with 100mM-sodium phosphate buffer, pH7.0, and reconcentrated to A_{280} about 10 by ultrafiltration. The immunoadsorbent column was regenerated for re-use by washing with 0.1M-sodium formate buffer, pH3.0, containing 0.5M-NaCl.

The immunoadsorption process was repeated as necessary until no immunoprecipitation reaction could be obtained for any of the original contaminants. Sometimes small (20ml) columns of adsorbents for individual contaminants, IgA or α_2-pregnoglobulin, were also used.

Finally, the solution of α_2M was twice dialysed for 24h against 100vol. of 20mM-sodium citrate buffer, pH6.5, and stored at 4°C, sometimes after freeze-drying.

Results and Discussion

We have found that native α_2M can exist in two forms readily distinguishable by mobility in gel electrophoresis and many other properties. The form found in plasma, and capable of binding proteinases, is the electrophoretically 'slow' S-form. S-α_2M can be converted into F-α_2M, the electrophoretically 'fast' form, but we have not seen any evidence of the reverse change. An attempt has been made in Fig. 2 to summarize our main findings and conclusions on the structures of S-α_2M and F-α_2M.

Purification of S-α_2M

The purification procedure was developed with the aim of preparing α_2M free of detectable contamination, and also completely in the S-form. In the past, thorough purification has normally been achieved by procedures that have yielded a mixture of the S- and F-forms (e.g. Hall & Roberts, 1978).

In our procedure (see the Materials and Methods section) a typical yield of α_2M from 250ml of anticoagulated plasma was 200mg (40%) before immunoadsorption, and 160mg (32%) after.

Even before immunoadsorption, the purified α_2M showed only a single precipitin arc in immunoelectrophoresis against anti-(human serum proteins) serum, and a single line against this antiserum in double immunodiffusion. Nevertheless assays by single radial immunodiffusion at this stage typically revealed contamination by haptoglobins (0.4–1.4%), fibrinogen (0.4–1.2%), IgA (3.8–8.1%), IgM (0.52–2.2%) (these values being ranges for three preparations) and α_2-pregnoglobulin. After removal of these proteins by immunoadsorption, even sensitive tests by double diffusion against specific antisera revealed no plasminogen, haptoglobin, immunoglobulins, fibrinogen or α_2-pregnoglobulin. On one occasion α_2M was purified from plasma of haptoglobin type 2–1; 14% haptoglobin was then present before immunoadsorption, but this was successfully removed to yield α_2M at the normal standard of purity.

The method that we have developed avoids the disadvantages of working from serum (reviewed by Starkey & Barrett, 1977) and yields extremely highly purified α_2M in satisfactory yield with a minimum of steps. Fractionation with poly(ethylene glycol) is a mild procedure that removes the β-lipoprotein more conveniently than does preparative ultracentrifugation, and without the conversion of S-form into

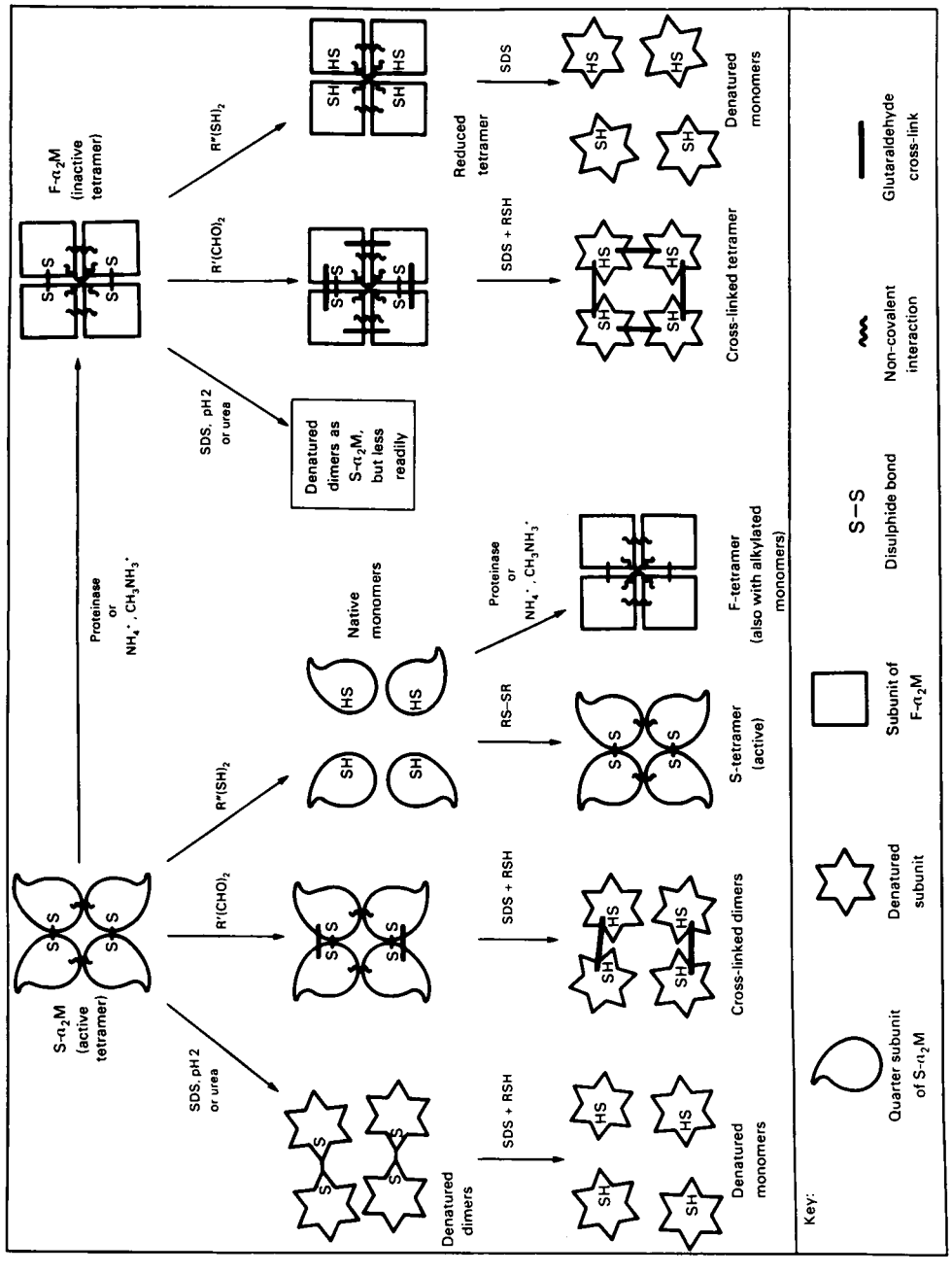


Fig. 2. *Diagrammatic representation of transitional possibilities of the α_2M molecule*

The shapes used for the subunit in its various states are purely diagrammatic. The tetramers are drawn as if planar, for clarity, although the F-form, at least, may well be tetrahedral, and additional subunit interactions in the F-tetramer are drawn as though it is tetrahedral (see the Conclusions section). Abbreviations used: RSH, 2-mercaptoethanol; RS–SR, 2-hydroxyethyl disulphide; R'$(CHO)_2$, glutaraldehyde; R''$(SH)_2$, dithiothreitol.

F-form risked by the use of $(NH_4)_2SO_4$ (see below). Gel chromatography has always been one of the most valuable steps in the purification of α_2M; we obtained much better results with columns containing the mixture of Ultrogel AcA 22 with AcA 34 than with others containing Bio-Gel A1.5m, Sepharose 6B or Sephadex G-200. After removal of euglobulins insoluble in 1% glycine, pH6.5, the α_2M contained only 6% of contaminants detectable by our methods, and these were removed by immunoadsorption. Attempts at final purification by preparative isoelectric focusing were unrewarding.

The freshly isolated α_2M ran as a single zone of low mobility in pore-limit electrophoresis (Fig. 3, track *a*) and in rate electrophoresis. There was no protein with the mobility of the 'fast' or F-form α_2M (see below), nor were there zones attributable to plasma proteins other than α_2M.

During storage at pH5.0–6.5 at −20°C or 4°C in various buffers, the S-α_2M gradually changed into the F-form, with the appearance of transitional forms of intermediate mobility. In the intermediate stages, the protein appeared either as a doublet similar to those described previously for preparations of individual rat macroglobulins (Gauthier & Mouray, 1976) and human α_2M (Zais & Roberts, 1977) or as a group of four or five bands similar to those studied by Saunders *et al.* (1971), according to loading. The change was accompanied by a partial or complete loss of inhibitory activity. The rate of this deterioration varied between preparations, but S-α_2M could normally be stored for several weeks in sodium citrate buffer, pH6.5, or in 20% sucrose, with little change. S-α_2M that had been dialysed into 20mM-sodium citrate buffer, pH6.5, and freeze-dried was reasonably stable during storage for several months at 4°C.

Formation of F-α_2M

S-α_2M can be converted into the electrophoretically more mobile F-α_2M by two distinct kinds of treatment: reaction with proteinases, or exposure to salts of ammonia or some closely related bases. We have studied these effects and examined the products.

Effect of proteinases. Solutions of S-α_2M (about 1mg/ml) in sodium phosphate or Tris/HCl buffers at pH values in the range 6–8 were treated with trypsin, papain or thermolysin (50μg of commercial enzyme/mg of α_2M, representing about 2mol of proteinase/mol) during 5min at 20°C, after which each enzyme was inactivated (with di-isopropyl phosphorofluoridate, iodoacetate or EDTA respectively, each at a concentration of 1mM).

It was found that the treatment with each proteinase slightly increased the mobility of the a_2M both in rate and pore-limit electrophoresis (Fig. 3, track *b*). In each system the mobilities of the 'fast'

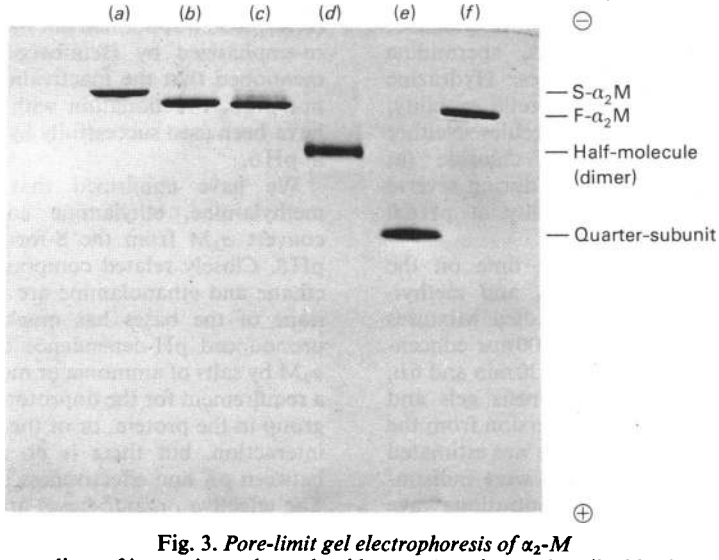


Fig. 3. *Pore-limit gel electrophoresis of α_2-M*

The samples, run in a gradient of increasing polyacrylamide concentration as described in the Materials and Methods section, were (*a*) S-α_2M, (*b*) F-α_2M formed by saturation with trypsin, (*c*) F-α_2M formed by treatment with methylammonium chloride, (*d*) subunit (dimeric) formed by exposure of S-α_2M to pH2.0, (*e*) subunit (monomeric) formed by treatment of S-α_2M with 1mM-dithiothreitol and (*f*) reduced tetramer formed by treating F-α_2M (trypsin-treated) with 1mM-dithiothreitol.

(F-) forms produced by reaction with the various proteinases were indistinguishable; a single fast zone was obtained even with some aged preparations that gave double zones before reaction.

In a further experiment the α_2M was titrated with increasing amounts of trypsin (5–40 μg/mg). Rate and pore-limit electrophoresis showed the formation of mixtures of S-α_2M and F-α_2M, with a progressive increase in the proportion of the F-form, and no detectable intermediate component.

Effects of trypsin and chymotrypsin on electrophoretic mobility have previously been described (Saunders *et al.*, 1971; Roberts *et al.*, 1974; Hall & Roberts, 1978), and we suspect that the effect will be seen with any proteinase with which α_2M has the capacity to bind. We suggest that the change is a result of the conformational change in α_2M postulated to occur in the reaction with proteinases.

Effect of NH_4^+ salts. Solutions of α_2M in 50mM-Tris/HCl buffer, pH8.2, were made 100mM with respect to $(NH_4)_2SO_4$ or 200mM with respect to methylammonium chloride for 30min at 20°C before being run in rate or pore-limit electrophoresis. Each of the ammonium salts caused an increase in mobility in both systems identical with that seen with the proteinases (Fig. 3, track *c*).

The effect on S-α_2M was restricted to the salts of low-molecular-weight ammonium analogues: it was obtained with $(NH_4)_2SO_4$, methylammonium chloride and ethylammonium chloride, but not under these conditions with hydrochlorides (at 200mM) of trimethylamine, triethylamine, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, glycine, β-alanine, 6-aminohexanoate, hydroxylamine, ethanolamine, triethanolamine, Tris, spermidine or a variety of other organic bases. Hydrazine produced the increase in electrophoretic mobility, and also released some free half-molecules. Neither $(NH_4)_2SO_4$ nor methylammonium chloride (at 200mM concentration of the anions during several hours) caused the change in mobility at pH6.0 (50mM-sodium citrate buffer).

The effects of concentration and time on the modification of α_2M by $(NH_4)_2SO_4$ and methylammonium chloride at pH8.0 were studied. Mixtures containing the anions at 10, 50 or 200mM concentration were incubated at 20°C. After 30min and 6h, samples were applied to electrophoresis gels and immediately run. The degree of conversion from the S-form into the F-form in each sample was estimated visually. The effects of the two salts were indistinguishable. At 30min the three concentrations gave about 0, 90 and 100% conversion respectively, whereas the values after 6h were estimated to be 70, 100 and 100%.

To determine whether the salts of amines structurally very closely related to ammonia and methylamine have the capacity to bring about the transition of the S-form into the F-form at all, although much more slowly, prolonged incubations were made with 1,3-diaminopropane and ethanolamine (each 300mM) in Tris/HCl buffer, pH8.2, at 4°C. With each amine, complete conversion of S-α_2M into the F-form required 5–6 days. Since the conversion is complete after 30min (and possibly much less) with $(NH_4)_2SO_4$ or methylammonium chloride, it can be said that the effect of the higher-molecular-weight amines was at least several hundred-fold slower (although at a lower temperature).

A sample of α_2M that had been completely converted into the F-form by 30min exposure to 200mM-$(NH_4)_2SO_4$ was dialysed against two changes of 100vol. of 50mM-Tris/HCl, pH8.1 during 48h. It was then re-examined by electrophoresis. No reversion into the S-form was detectable.

We do not know what kind of reaction occurs between α_2M and salts of ammonia or methylamine. Ratnoff *et al.* (1954) experimented with hydrazine because it was already known to inactivate complement components C3 and C4, and found that a serum inhibitor of plasmin was inactivated by this reagent and also by ammonia, methylamine and ethylamine, but not by several other amines. Norman & Hill (1958) selectively inactivated this 'immediate' inhibitor of plasmin with 0.2M-methylamine in assays of 'slow' inhibitor, and the inactivation of α_2M as an inhibitor of proteinases by methylamine has been reported explicitly by Steinbuch *et al.* (1968), Nagasawa *et al.* (1970), Saunders *et al.* (1971) and Ganea *et al.* (1974). The risk of inactivation of α_2M by ammonia during fractionation with $(NH_4)_2SO_4$ was pointed out by Mehl *et al.* (1964) and re-emphasized by Heimberger *et al.* (1971), who mentioned that the inactivation occurs at pH8 but not pH5. Fractionation with $(NH_4)_2SO_4$ seems to have been used successfully by Hamberg *et al.* (1973) at pH6.

We have confirmed that salts of ammonia, methylamine, ethylamine and hydrazine rapidly convert α_2M from the S-form into the F-form at pH8. Closely related compounds such as diaminoethane and ethanolamine are vastly less active, and none of the bases has much effect at pH6. The pronounced pH-dependence of the inactivation of α_2M by salts of ammonia or methylamine may reflect a requirement for the unprotonated form of a crucial group in the protein, or of the nitrogen base, for the interaction, but there is no apparent relationship between p*K* and effectiveness of the various amines. The effective organic bases are all primary amines, but other primary amines of larger molecular size have very little action on α_2M. It can be postulated that, at pH8, each subunit of α_2M contains a 'pocket' capable of accommodating NH_4^+ or NH_3 and binding it. Apparently the pocket can accommodate the extra bulk of a methyl or an ethyl group, but very

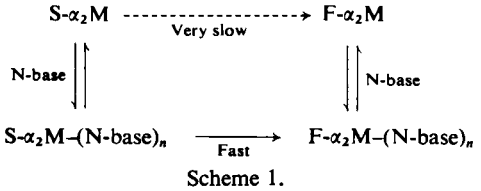
little more. It is unclear whether the nitrogen (N-bases) are effective in their protonated forms. If the cations react with α_2M, hydrogen-bonding is presumably involved, as inorganic ions of similar size are inactive (Barker, 1968). The alternative possibility that the free bases act as nucleophiles in a covalent reaction with some highly reactive group on the protein seems to be supported by a recent brief report (Swenson & Howard, 1979).

The requirement for a high concentration of N-base to produce the transition of S-form into F-form implies that S-α_2M has a low affinity for these compounds. Since dialysis does not reverse the transition, it seems improbable that the situation is a simple equilibrium in which F-α_2M is the S-α_2M–N-base complex. A system like that in Scheme 1 seems more probable. It is proposed that, whereas S-α_2M is very slowly transformed into F-α_2M in normal storage, the S-α_2M–N-base complex undergoes this transition very rapidly. We have no definite evidence that the reversal steps occur, however.

Properties of S-α_2M and F-α_2M

In this section various properties of S-α_2M and F-α_2M are considered, with a view to understanding more about the structure of the molecule in its two forms, and the relation of this to the biological activity of α_2M. The evidence relating to reductive dissociation of α_2M forms a separate section.

Proteinase-inhibiting activity of S-α_2M. The trypsin-inhibiting activity of S-α_2M was determined as described in the Materials and Methods section. The results were then calculated in terms of mol of trypsin inhibited/mol of α_2M on the basis that α_2M has a molecular weight of 725000 (see the introduction). Several experiments were made with each of three preparations of S-α_2M, with the following results: preparation M57, 1.6, 1.6, 1.5; M58, 1.7, 1.6; M59, 2.1, 2.3, 1.8. These results point to an integer ratio of 2:1, which contrasts with our earlier finding of binding ratios only in the order of 1:1 (Barrett & Starkey, 1973). The α_2M isolated in the earlier study from serum showed both 'fast' and 'slow' components in electrophoresis (A. J. Barrett, unpublished work) and so cannot have been fully active (see below). Also, our previous results depended on the assumption that all trypsin molecules bound by α_2M are resistant to inhibition by soya-bean trypsin inhibitor. We have recently found that a certain proportion of the bound enzyme molecules do react with the soya-bean trypsin inhibitor, however (P. M. Starkey, C. A. Sayers & A. J. Barrett, unpublished work).



Scheme 1.

Lack of proteinase-inhibiting activity in F-α_2M. Samples of α_2M that had been converted into the F-form by treatment with $(NH_4)_2SO_4$ or methylammonium chloride at pH 8.0 were tested for the capacity to inhibit the action of trypsin and thermolysin on dyed hide powder (see the Materials and Methods section). No inhibitory action (less than 2% of untreated control) was detected. No recovery of inhibitory activity occurred when the F-α_2M was dialysed against two changes of 100 vol. of 50 mM-Tris/HCl buffer, pH 8.1.

Samples of the $(NH_4)_2SO_4$- or methylammonium chloride-treated α_2M were also treated with trypsin (50 μg/mg, for 5 min) and run in SDS/polyacrylamide-gel electrophoresis with reduction. All of the quarter-subunits were seen to have been cleaved, with the formation of fragments indistinguishable in mobility from those seen in experiments with trypsin-treated S-α_2M (Fig. 4, track *b*).

α_2M treated with 200 mM-diaminopropane hydrochloride for 3 days (which had resulted in a partial transition from the S-form into the F-form) showed only 20% of the trypsin-inhibiting activity of a control. After 5 days the electrophoretic change was complete, and no activity remained.

It thus seems that the specificity of cations for the inactivation of α_2M is the same as that for the modification of electrophoretic mobility, in consistency with the view that the two effects are aspects of the same phenomenon.

If, as we suggest, the F-form of α_2M produced by the amines is a 'closed' form of the molecule similar to the proteinase complex, it is not surprising that it lacks proteinase-binding activity. The finding that this F-α_2M was cleaved by trypsin in the same way as is S-α_2M suggests to us that the transition of S-form into F-form involves primarily a change of tertiary structure in the subunits, whereas the pattern of sensitivity to trypsin is dictated by secondary (as well as primary) structure. It is also important that the pattern of proteolysis of F-α_2M clearly distinguishes the molecule from denatured α_2M, which is cleaved quite differently from S-α_2M, presumably because the secondary structure has been disturbed (Harpel, 1973; Barrett *et al.*, 1974).

Effect of removal of sialic acid on electrophoretic mobilities. S-α_2M and the electrophoretically fast α_2M–trypsin complex were treated with clostridial neuraminidase (10 μg/ml) in 100 mM-sodium acetate buffer, pH 5.5, containing 5 mM-$CaCl_2$ at 37°C during 16 h. No attempt was made to quantify the liberation of sialic acid, but the electrophoretic mobility of both forms was markedly decreased, presumably owing to

the loss of sialic acid residues and lowering of net negative charge. Thus the difference in electrophoretic mobilities did not tend to be obscured, as would have been expected if sialic acid contributed more to the mobility of one form than the other.

Gel chromatography of α_2M forms. It seems likely that the greater mobility of F-α_2M in gel electrophoresis reflects a more compact molecular structure. An attempt was made to detect this by gel chromatography.

Mixtures of S-α_2M (untreated) and F-α_2M (methylammonium chloride-treated), in equal parts, were run on a column of Ultrogel AcA 34 in 100mM-sodium citrate buffer, pH 6.5. Electrophoretic analysis of the effluent fractions failed to reveal any separation of the two forms in several such experiments. Further experiments were made with mixtures of S-α_2M with α_2M complexed with [^{14}C]acetyl-chymotrypsin. Again no separation of the S-α_2M from proteinase complexes (identified as radioactivity) was obtained.

These results were taken to show that there is no large difference in Stokes radius between S- and F-forms of α_2M. Nevertheless the resolution of gel electrophoresis is undoubtedly greater than that of gel chromatography, and the possibility of a significant size difference is not excluded.

Isoelectric focusing of α_2M forms. Mixtures of equal quantities of control (S-)α_2M with F-α_2M produced by reaction with trypsin (50μg/mg; subsequently inactivated with phenylmethanesulphonyl fluoride) or with methylammonium chloride (as above) were subjected to isoelectric focusing in the LKB 8101 column. No separation of S- and F-forms was detected by electrophoresis of the fractions, suggesting that the forms do not differ appreciably in isoelectric point. Similarly, when complexes formed with [^{14}C]acetyl-chymotrypsin or ^{125}I-labelled trypsin were mixed with S-α_2M and focused, peaks of both radioactivity and protein (A_{280}) were coincident, in fractions of pH 5.2. We were thus unable to confirm the report by Ohlsson & Skude (1976) of a large change in pI of α_2M (from 5.0 to 6.0) associated with the complexing of a proteinase.

Antigenic activity of S-α_2M and F-α_2M. Triplicate samples of S-α_2M were treated with 200mM-methylammonium chloride (to produce F-α_2M) or 200mM-NaCl (control) for 30min at pH 8.1 at 22°C. Each sample was then run in triplicate in 'rocket' immunoelectrophoresis (see the Materials and Methods section) into agarose gel containing specific anti-(human α_2M) serum. The complete experiment was done with each of four separate antisera, and the results are given in Table 1.

It is apparent that the heights of 'rockets' for F-α_2M were 12.3–29.3% lower than those for S-α_2M, the exact value depending on the antiserum. The difference in height of the 'rockets' indicates that somewhat different sets of antigenic determinants are recognized on the two forms of α_2M by the antisera. In separate double-immunodiffusion experiments, only precipitating reactions of complete identity were obtained, so that the difference is likely to involve few antigenic determinants.

The commercial standard serum (Behringwerke batch no. 1001 R) used to standardize our immunoassays for serum proteins was found to contain α_2M only in the F-form. Clearly this factor would need to be taken into account, as well as the form of α_2M being determined and the discriminating tendency of the individual antiserum, if one were to make valid assays of α_2M by 'rocket' immunoelectrophoresis.

SDS/polyacrylamide-gel electrophoresis of S-α_2M. Samples of α_2M prepared without reduction (see the Materials and Methods section) ran in 7% gels as a single major band with molecular weight greater than that of myosin heavy chain or reduced thyroglobulin, and thus possibly in the region of 400000mol.wt. (Fig. 4, track *a*). This was consistent with the view that the minimal covalent subunit of α_2M is the half-molecule.

Samples prepared with reduction showed a major band (I) of mol.wt. 182000, undoubtedly the single quarter-subunit of the molecule of about 725000 mol.wt. [in confirmation of the conclusions of Harpel (1973) and Hall & Roberts (1978)]. When the sample preparation involved heating at 100°C, the gels showed, in addition to band I, a less intense band of 139000 mol.wt. (band II) and two further bands of 68000 and 64000 mol.wt. (bands IIIa and IIIb

Table 1. *Comparison of heights of 'rockets' in Laurell electrophoresis of S-α_2M and F-α_2M against four antisera*
The heights are expressed as percentages of the control (S-α_2M) mobility, and the statistical limits are standard errors ($n = 9$). The antisera nos. 4602, 4603 and 2743 were from Behringwerke, whereas no. RP-5 was raised in our laboratory.

		Mobility (% of control)			
Sample	Antiserum no. ...	4602	4603	2743	RP-5
S-α_2M		100±1.2	100±0.7	100±0.6	100±1.2
F-α_2M		83.6±0.8	70.7±0.7	87.7±0.9	74.4±1.0
Difference		16.4	29.3	12.3	25.6

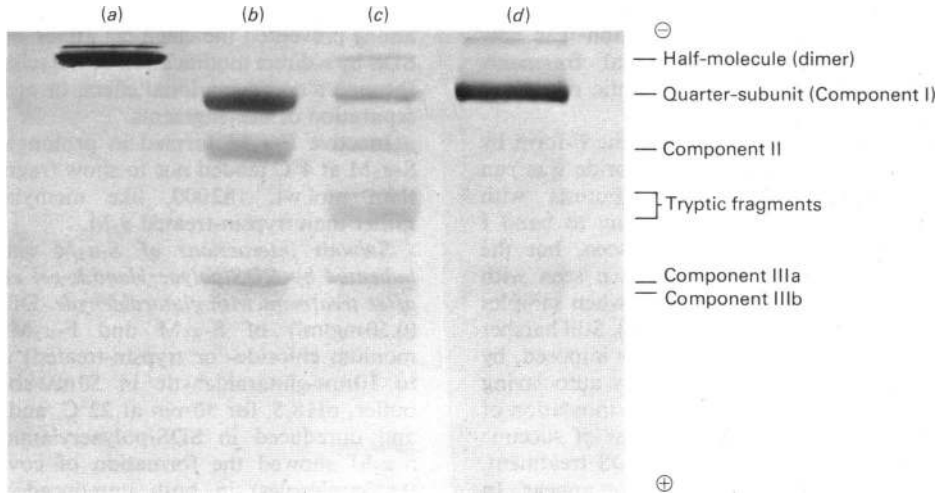


Fig. 4. *SDS/polyacrylamide-gel electrophoresis of α_2M*
Samples were prepared at 100°C and run in 7% gels as described in the Materials and Methods section. (*a*) S-α_2M, run without reduction, and (*b*) S-α_2M, (*c*) α_2M treated with 50μg of trypsin/mg and (*d*) F-α_2M (methylammonium chloride-treated), all run with reduction.

respectively) (Fig. 4, track *b*). In one experiment quantitative densitometry showed that the amount of stain in the band II and band III represented 24 and 16% respectively of the total. When native quarter-subunits were liberated by mild reduction (see below) and chromatographed on Ultrogel AcA 34, SDS/polyacrylamide-gel electrophoresis of the single protein peak again showed bands I, II, IIIa and IIIb. We have no doubt that our minor bands II, IIIa and IIIb (previously referred to as β, γ_1, and γ_2; Barrett & Salvesen, 1978) correspond to bands II and III described by Harpel (1978), which were shown by peptide 'mapping' to be fragments of the quarter-subunit. In consistency with the latter report, we found (not shown) that the minor bands were much weaker when samples were prepared by incubation at 37°C rather than 100°C.

It seems that components II and III arise from cleavage of a peptide bond about one-third of the way from one end of some of the chains (in contrast with the cleavage near the mid-point that triggers the trapping of a proteinase by α_2M), and we considered whether the bond cleavage occurs *in vivo*, during storage *in vitro* or during the treatment with SDS before electrophoresis. Comparison of some older preparations with new ones gave no indication that the quantity of components II and III increases during storage. It was also found that precipitation of samples with 10% (w/v) trichloroacetic acid before boiling with SDS, or the use of various proteinase inhibitors, did not decrease the quantity of components II and III, suggesting that enzymic activity was not involved at that stage. Like Harpel (1978), we consider that it is unlikely that proteolytic cleavage of the chains occurs at any stage *in vitro*, but we are not able to decide whether the cleavage occurs by limited proteolysis *in vivo* or chemical cleavage in the SDS at 100°C. It may be that some subunits are subject to this limited proteolysis *in vivo*, and that since this does not trigger the transformation of S-form into F-form, the molecules remain in the circulation. It would then be assumed that the association of the fragments is so tight in S-α_2M that high temperature is required to separate them in SDS. Alternatively it might be that the bond cleavage is due to chemical cleavage of an especially labile bond at high temperature. This point is considered further in connection with the results of SDS/polyacrylamide-gel electrophoresis of F-α_2M below.

SDS/polyacrylamide-gel electrophoresis of F-α_2M. SDS/polyacrylamide-gel electrophoresis of α_2M that had been converted into the F-form by treatment with proteinases or ammonium salts was done with and without reduction of the samples.

Samples of α_2M that had been treated with 2 molar equivalents of trypsin, pancreatic elastase or thermolysin, and run without reduction, showed the presence of half-molecules; no lower-molecular-weight material was detected, but sometimes traces of intact tetramer remained. It was concluded that the fragments of subunits produced in the reaction with proteinases are held together in the half-molecules by disulphide bonds. When run with reduction, the samples showed the expected frag-

mentation of the quarter-subunits (Barrett *et al.*, 1974), but the electrophoretic resolution was now sufficient to separate the individual fragments (mol.wts. 111000 and 98000, for tryptic digestion) (Fig. 4, track *c*).

α_2M that had been converted into the F-form by treatment with methylammonium chloride was run in SDS/polyacrylamide-gel electrophoresis with reduction. A single zone corresponding to band I (the intact polypeptide chain) was seen, but the minor bands II and III that had been seen with S-α_2M (see above) were absent, even when samples were prepared at 100°C (Fig. 4, track *d*). Still harsher conditions of sample preparation were imposed, by inclusion of 5M-urea in the sample, by autoclaving the sample (115°C for 5min) or by succinoylation of the F-α_2M with a 5-fold weight excess of succinic anhydride (Klotz, 1967) before the SDS treatment, but still the bands II and III did not appear. In contrast, the demonstration of the fragments of 111000 and 98000 mol.wt. was not interfered with when trypsin-treated α_2M was further treated with methylammonium chloride before SDS/polyacrylamide-gel electrophoresis.

The failure of the F-α_2M produced by methylammonium chloride to show bands II and III could be explained either in terms of the two-thirds/one-third cleavage of some quarter-subunits occurring *in vivo*, or in the preparation of samples for electrophoresis. In the first case one would conclude that the new conformation induced in the subunits by the amine was so rigid that the fragments were not released even under harsh conditions in SDS; in the second, one would have to assume that the reaction with the amine prevented the chemical attack in hot alkaline SDS by a direct modification of the scissile region, or through a conformational effect, or again prevented separation of the fragments.

Inactive F-α_2M formed in prolonged storage of S-α_2M at 4°C tended not to show fragments smaller than mol.wt. 182000, like methylamine-treated rather than trypsin-treated α_2M.

Subunit interactions of S-α_2M and F-α_2M as indicated by SDS/polyacrylamide-gel electrophoresis after treatment with glutaraldehyde. Dilute solutions (0.50mg/ml) of S-α_2M and F-α_2M (methylammonium chloride- or trypsin-treated) were exposed to 10mM-glutaraldehyde in 50mM-sodium borate buffer, pH8.5, for 30min at 22°C, and run reduced and unreduced in SDS/polyacrylamide gels. The S-α_2M showed the formation of covalent dimers (half-molecules) in both unreduced and reduced samples (Fig. 5, tracks *a* and *b*). F-α_2M produced with methylammonium chloride or trypsin yielded only tetramers (Fig. 5, tracks *c* and *d*). Reduced and alkylated quarter-subunits of α_2M did not show any tendency to form dimers or tetramers under the same conditions of treatment with glutaraldehyde (Fig. 5, track *e*).

Since the separated subunits were not linked under the conditions used, it seemed clear that the cross-linking detected with the intact molecules was attributable to the way in which the subunits associate in the tetramers. In the S-α_2M, cross-linking occurred between subunits normally linked by disulphide bonds, but not between those associated

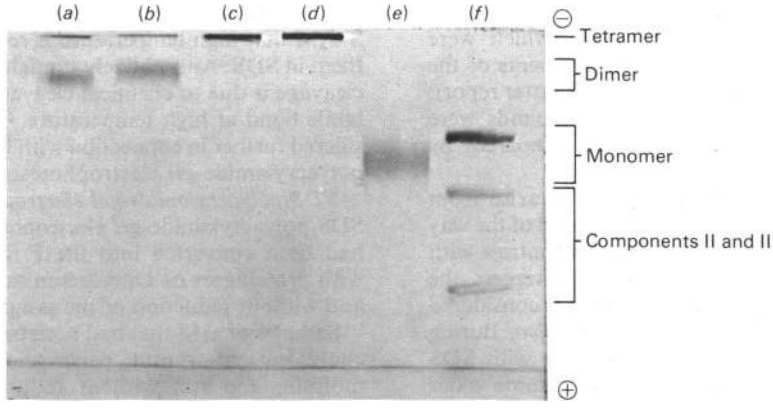


Fig. 5. *Effect of glutaraldehyde cross-linking on S-α_2M and F-α_2M seen in SDS/polyacrylamide-gel electrophoresis*
The samples run in a 5% gel were (*a*) S-α_2M treated with glutaraldehyde (reduced), (*b*) as (*a*), but unreduced, (*c*) F-α_2M (trypsin-treated) treated with glutaraldehyde (reduced), (*d*) as (*c*), but with F-α_2M (methylammonium chloride-treated), (*e*) alkylated (carboxymethylated) quarter-subunits treated with glutaraldehyde (reduced), and (*f*) S-α_2M untreated (reduced). S-α_2M was mainly cross-linked in dimeric half-molecules that separated without reduction. Both forms of F-α_2M were completely cross-linked as tetramers. No oligomers were produced in the control with isolated subunits, and the untreated S-α_2M sample was included merely for reference. Further experimental details are given in the text.

non-covalently. In contrast, the contacts between subunits in F-α_2M permitted stabilization of tetramers by linking of each subunit with at least two others. In view of other indications in the present paper, we consider it very likely that in fact there are larger areas of close contact between subunits in F-α_2M than in S-α_2M.

S-α_2M that had been treated with 5mM-glutaraldehyde for 30min had no detectable trypsin-binding activity, nor was electrophoretic mobility increased after exposure of the treated protein to the enzyme.

Separation of half-molecules of a_2M in denaturing conditions. Previously it has been reported that exposure of α_2M to low pH, urea, sodium thiocyanate or SDS without reduction causes the protein to dissociate into the minimal covalent subunit half-molecules (Jones *et al.*, 1972; Harpel, 1973; McConnell & Loeb, 1974; Starkey & Barrett, 1977).

Samples of S-α_2M, and of F-α_2M produced with trypsin or methylammonium chloride as usual, were kept in 5mM-sodium phosphate buffer, pH7.0, containing 4.0M-urea, for 4h at 22°C, and then run in pore-limit electrophoresis. It was seen (Fig. 6) that the S-α_2M had dissociated almost completely, whereas the F-α_2M samples still contained mainly tetramer. Results with KI or NaSCN as dissociating agent, in place of urea but under the same conditions, were very similar. Pore-limit electrophoresis of S-α_2M and F-α_2M at pH3.1 showed complete dissociation of the S-α_2M, much less dissociation of the methylammonium chloride-produced F-α_2M tetramer, and less still for the trypsin-produced F-α_2M molecule.

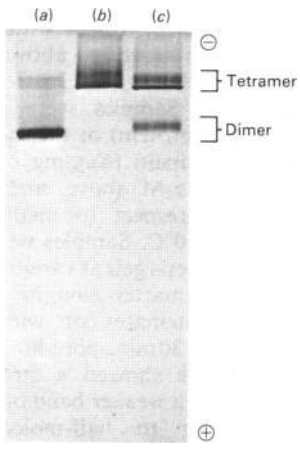


Fig. 6. *Dissociation of half-molecules of a_2M in urea*
The samples run on a pore-limit electrophoresis gel as described in the Materials and Methods section were (*a*) S-α_2M, (*b*) F-α_2M (trypsin-treated) and (*c*) F-α_2M (methylammonium chloride-treated), each incubated in 4M-urea for 4h at 22°C before the run. Dissociation of the tetramer was much more complete for S-α_2M than for either form of F-α_2M.

The above results again point to the strength of subunit interactions in F-α_2M. Krebs *et al.* (1978) similarly concluded from their work with the ultracentrifuge that α_2M complexed with trypsin is more resistant to dissociation at low pH than is unmodified α_2M.

Lack of inhibitory activity of half-molecules. S-α_2M almost completely converted into half molecules during 40h in 3M-urea was dialysed into 0.15M-NaCl/10mM-sodium phosphate/0.9% glycine, pH7.9, as described by McConnell & Loeb (1974), and tested for inhibitory activity against trypsin and thermolysin. No activity was detectable. This finding contrasted with that of McConnell & Loeb (1974) for plasma kallikrein.

Treatment of the half-molecules with 50μg of trypsin/mg at pH8 caused fragmentation different from, and more extensive than, that seen with 'fast' or 'slow' tetramers, or native quarter-subunits (see above), in that degradation was apparent on SDS/polyacrylamide gels even without reduction, and the pattern of bands produced with reduction was quite different from that described above for the native protein. Our conclusion was that half-molecules dissociated in urea behave as if denatured. Jones *et al.* (1972) similarly concluded that there was extensive unfolding of half-molecules dissociated in urea, on the basis of ultracentrifugation experiments.

Effect of mild reduction on forms of α_2M

Reductive dissociation of S-α_2M. S-α_2M, at a concentration of about 1mg/ml in 50mM-Tris/HCl buffer, pH8.1, was treated with 1mM-dithiothreitol for 30min at 22°C. The solution was then made 3mM with respect to sodium iodoacetate, and samples were run in electrophoresis. Electrophoresis in 5% polyacrylamide gels showed the complete disappearance of the normal α_2M band after dithiothreitol, and the formation of a new component that ran as a more-diffuse faster zone. In pore-limit electrophoresis the new component ran as a sharp zone at much higher gel concentration (slightly behind catalase, 230000 mol.wt.) (Fig. 3, track *e*). In SDS/polyacrylamide-gel electrophoresis the single zone of mol.wt. 182000 attributed to the quarter-subunit chains was seen even when samples were prepared without reduction in the SDS/polyacrylamide-gel electrophoresis.

When the experiment was repeated with exposure to dithiothreitol in 50mM-sodium citrate buffer, pH6.0, rather than the pH8 buffer, electrophoresis showed dithiothreitol to have had very little effect.

It was concluded that 1mM-dithiothreitol, pH8, reduces the intersubunit disulphide bonds in α_2M, and that the quarter-subunits then dissociate. The lack of effect at pH6 was attributable to the low

concentration of the reactive thiol anion of dithiothreitol at this value, and is typical of thiol–disulphide reactions. Jones *et al.* (1972) have previously detected dissociation of α_2M after exposure to 10mM-*N*-acetylcysteine, pH8.0, by ultracentrifugation. The alkylated quarter-subunits could be stored in solution (1mg/ml) in the Tris/HCl buffer, pH8.1, at 4°C for several weeks without appreciable precipitation.

Thiol content of separated quarter-subunits. S-α_2M (2mg/ml in 50mM-Tris/HCl buffer, pH8.0) was treated with 1mM-sodium iodoacetate for 20min to block any free thiol groups before treatment (30min) with dithiothreitol (2mM, 1.5mM excess over iodoacetate) to cause dissociation, as described above.

The reduced protein was freed from dithiothreitol by passage through a column (13cm×1cm) of Sephadex G-25 in 50mM-sodium acetate buffer, pH4.7, containing 2mM-disodium EDTA, and bubbled with N_2 before use.

Fractions comprising the protein peak from the Sephadex column were combined, and the material was examined by pore-limit electrophoresis and assayed for protein (Lowry *et al.*, 1951) and thiol groups (Ellman, 1959).

Pore-limit electrophoresis showed that reductive dissociation had not been quite complete, as some ('slow') α_2M molecules remained with the quarter-subunits. Nevertheless 3–4 thiol groups were detected for each unit of 182000 mol.wt. in two separate experiments.

It was concluded that treatment with dithiothreitol sufficient to cause complete dissociation of α_2M into quarter-subunits is associated with the reduction of about four disulphide bonds per half-molecule. We cannot say, however, whether all of these are inter-subunit bonds or whether some may be internal.

Reoxidation of quarter-subunits. In further experiments reduction of S-α_2M with 1mM-dithiothreitol at pH8 was followed, not by alkylation of thiol groups, but by addition of 2-hydroxyethyl disulphide (to 2mM), to allow re-formation of disulphide bonds. Pore-limit electrophoresis of samples taken after 16h at 4°C and alkylated with iodoacetate showed the disappearance of the quarter-subunits, and the reappearance of S-form tetramer with a faint band of the intermediate mobility characteristic of half-molecules. As was expected, no reassembly of tetramers occurred when alkylated quarter-subunits were treated with hydroxyethyl disulphide.

Alkylated and non-alkylated quarter-subunits and re-formed tetramers were tested for inhibitory activity against trypsin and thermolysin (with the hide powder substrate). The quarter-subunits showed no measurable inhibitory activity, whereas the reoxidized samples showed 50–90% of the initial inhibitory activity of the α_2M (four experiments).

The recovery of activity after reoxidation of the quarter-subunits indicated that the dissociation of α_2M by mild reduction liberated quarter-subunits in their native state. In an earlier study of reductive dissociation of α_2M, Jones *et al.* (1972) noted that the subunits retained the optical-rotary-dispersion characteristics of the parent molecule, and therefore probably retained their native structure.

Effect of mild reduction on F-α_2M. F-α_2M that had been formed by saturation with trypsin or treatment with 200mM-NH_4^+ or -methylammonium ions at pH8 was treated with 1mM-dithiothreitol at pH7.1 for 30min, as described for the S-form. Electrophoresis in the rate and pore-limit systems showed no dissociation (after trypsin) (Fig. 3, track *f*) or very little (after methylamine). Nevertheless SDS/polyacrylamide-gel electrophoresis without reduction showed that the disulphide bonds linking quarter-subunits had been cleaved by the dithiothreitol, just as they were with the S-form. The α_2M that had been treated with trypsin showed 'intact' quarter-subunits when samples were not reduced in SDS, but the expected smaller fragments after reduction.

We concluded that the conformation of the subunits in the 'fast' form of α_2M is such that there are important non-covalent interactions between them, and the disulphide bonds, though not protected from reduction, are no longer essential for the integrity of the tetrameric molecule. Disulphide bonds within subunits (i.e. intra-chain bonds) link the peptides produced by tryptic cleavage, and at least some of these are resistant to reduction by 1mM-dithiothreitol.

Effect of proteinases or ammonium salts on free quarter-subunits. A preparation of free subunits was obtained by treatment of S-α_2M with 1mM-dithiothreitol, at pH8.0, as described above. Part of the preparation was alkylated with 3mM-sodium iodoacetate for 30min. Samples were treated with trypsin (40μg/mg of protein) or thermolysin (40μg/mg of protein) or papain (6μg/mg of protein) as described for intact α_2M above, and others were made 200mM with respect to methylammonium chloride and left at 20°C. Samples were applied to pore-limit electrophoresis gels at various times.

When unblocked quarter-subunits were treated either with the proteinases or with methylammonium chloride for 30min, pore-limit electrophoresis of the products showed a strong band of F-form tetramers and a weaker band of intermediate mobility attributable to half-molecules. When papain was used to bring about the reassembly of unblocked quarters, staining for enzymic activity showed papain both in the F-α_2M zone and (more weakly) in the zone corresponding to half-molecules.

Similarly the alkylated subunits re-formed F-form tetramers when treated with trypsin or thermolysin, and also in the presence of methylammonium

chloride, although 3h incubation was required for substantial conversion by the amine.

SDS/polyacrylamide-gel electrophoresis with reduction of F-α_2M formed from quarter-subunits by treatment with trypsin showed fragments indistinguishable from those produced by proteolytic cleavage of intact α_2M. SDS/polyacrylamide-gel electrophoresis without reduction showed that the tetramers were not disulphide-bonded.

It seemed probable from these results that isolated quarter-subunits of S-α_2M are affected by exposure to $(NH_4)_2SO_4$ or methylammonium chloride, or by limited proteolysis, just as they are in intact S-form tetramers. They are converted into a state in which they have the capacity to form strong non-covalent interactions with other subunits, and the assembly of dimers and tetramers is favoured. When a proteinase is used, some enzyme molecules may be trapped as F-form tetramers re-form, and binding by half-molecules also may occur.

Conclusions

The aim of the present study was to obtain a clearer idea of the molecular structure of α_2M, and of the mechanism of interaction with proteinases. If it is true that the molecule acts literally as a trap one would like to know the mechanics of this.

We agree with previous reports (e.g. Hall & Roberts, 1978) that the subunits of α_2M seem to be identical. They give a single band in pore-limit electrophoresis after reductive dissociation, and are antigenically homogeneous (Jones *et al.*, 1972; McConnell & Loeb, 1974; Starkey & Barrett, 1977). It remains to be established whether the two-thirds/one-third cleavage of the polypeptide chain that is seen in electrophoresis in the presence of SDS is a natural phenomenon or an artifact of the method. It is also unclear why this fragmentation is not seen with F-α_2M produced by ammonium compounds.

Properties of subunits liberated by dissociation of S-α_2M in urea or at pH3, or in SDS without reduction, plainly point to the existence of identical half-molecules, again in agreement with Harpel (1973), McConnell & Loeb (1974) and Hall & Roberts (1978).

In S-α_2M it seems that the disulphide bonds stabilizing the half-molecules are a major element in the structure of the tetramer. These are rather easily reduced, with the result that the quarter-subunits separate. Active S-α_2M can be regenerated in high yield by reoxidation, however. The areas of contact between half-molecules in S-α_2M may not be extensive, since glutaraldehyde cross-links did not tend to be formed.

S-α_2M was converted into F-α_2M by two different kinds of treatment, namely reaction with a proteinase or exposure to an ammonium salt. The F-α_2M molecules formed are not identical, because reaction with a proteinase involves peptide-bond cleavage in some or all of the subunits. Nevertheless the properties of F-α_2M molecules formed in the different ways were very similar: their electrophoretic mobilities were indistinguishable, they lacked proteinase-inhibiting activity and there was a characteristic tightening of subunit interactions. Thus reduction of inter-chain disulphide bonds did not cause dissociation of the F-tetramers, and separation of half-molecules occurred less readily in the presence of chaotropic agents and at low pH. The tightening of subunit interactions was tather more clear-cut in the proteinase-generated α_2M, but both types readily formed cross-linked tetramers with glutaraldehyde, and we consider that it is a reasonable working hypothesis that F-α_2M produced by the action of ammonium salts has a very similar structure to that of α_2M–proteinase complexes. This implies that the conformational change in the subunits that causes them to interact as they do in the F-tetramer can occur without any proteolytic cleavage.

It seems unlikely that the proteinase molecules themselves are directly responsible for holding the subunits of F-α_2M together, as was suggested by Krebs *et al.* (1978). Rather, it seems that each individual quarter-subunit as existing in S-α_2M can respond to proteolysis or an environmental change (e.g. a high concentration of ammonium salts) by a conformation transition that creates strong non-covalent binding sites for other F-subunits. The new interactions can occur whether or not the S-subunits are already linked in pairs by disulphide bonds in the S-tetramer, but once they have occurred the disulphide bonds are no longer required to hold an F-tetramer together.

We do not yet know what is the minimum number of subunits that must be proteolytically cleaved to trigger the binding of a proteinase molecule and the complete transition of S-α_2M into F-α_2M. It is, however, most improbable that all four subunits must be cleaved, so presumably some subunits normally adopt the F-conformation without peptide-bond cleavage. Perhaps the transition from S-form into F-form of a subunit in S-α_2M causes a change in the environment of the adjacent uncleaved subunits such that they tend also to undergo the same transition. The ammonium salts may mimic this environmental influence.

Since the α_2M molecule is a 'dimer of dimers', each form of dimer behaving as a 'closed structure' in the terms of Cornish-Bowden & Koshland (1971), it seems certain that all of the subunit interactions are 'isologous' (Monod *et al.*, 1965), as they are indicated in Fig. 1. Closed tetramers can exist either as planar ('square') arrangements or as pseudo-tetrahedra. The data available for α_2M do not give any definite

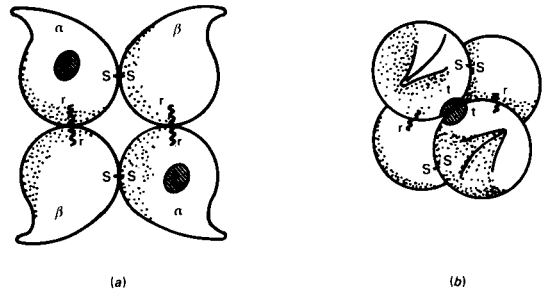


Fig. 7. *Planar and tetrahedral arrangements possible for the tetramer*
If the 'α'-faces of the subunits in the planar form of the tetramer (*a*) are imagined to carry potential sites of interaction (marked 't'), the transition to the pseudo-tetrahedral form (*b*) would involve two subunits coming up out of the plane of the paper to make the new interactions, and the other two going down to do likewise. The transition would seem to produce molecules that should pass more readily through sieving gels, would have more contacts between subunits and would enclose space. Again, the shapes indicated for the subunits and the whole molecule are not intended to resemble their actual shapes, which are far more complex. Moreover, there is an important oversimplification, because it is most improbable that the subunits are the same shape in the S- and F-forms of the tetramer.

information in favour of either arrangement, but it is tempting to speculate that the S-form may be planar, as it is represented in Fig. 1, whereas the F-form may be tetrahedral. In the planar form each subunit would interact with only two of the others, which would be consistent with the ease of dissociation of S-α_2M. It could then be suggested that the new conformation of each F-subunit allows binding to three others, to form a pseudo-tetrahedron. In Fig. 7 this would be represented by the two subunits of which we see the α-faces coming up out of the plane of the paper to form new interactions at the sites 't', while the other two go down, to bind similarly. The idea that the transition of S-form into F-form is a change from a planar to a tetrahedral arrangement of subunits is attractive for several reasons. Firstly, the tetrahedral molecule could well be more compact than the planar form. Electron microscopy has already shown that the F-α_2M molecule is more compact than that of S-α_2M (Barrett *et al.*, 1974), and this presumably explains why F-α_2M migrates more rapidly through gel media than does S-α_2M, although the molecular weights are the same. Secondly, the larger number of interaction sites between subunits could help to explain the greater resistance of the F-tetramer to dissociation. Finally, and most speculatively, it may be significant that the tetrahedron, being a three-dimensional array, can enclose space (an essential requirement for a physical trap), whereas the planar arrangement of subunits might not do so.

A crucial requirement of the 'trap' hypothesis for the action of α_2M was the conformational change representing closure of the trap (Barrett & Starkey, 1973). Such a change has already been indicated by electron microscopy and some less direct lines of evidence, but the existence of the S- and F-forms of α_2M, and their interconversion by proteinases, represents valuable confirmation. Since the hypothesis was put forward the interaction of α_2M with many more proteinases has been described, so that the idea that the reaction is rather general for endopeptidases is supported, although it is also clear that some very specific proteinases do not react at an appreciable rate. The original proposal that only 1 mol of proteinase is bound/mol of α_2M now seems to have been incorrect; instead, it seems that each molecule of α_2M can act as a double trap. Native quarter-subunits do not bind proteinases, and we were not able to isolate active half-molecules, but we believe that native half-molecules might well have activity. Nevertheless we have seen no electrophoretic evidence for the formation of molecules that are 'half-fast', in titration experiments, so the two traps may not act independently.

A consequence of the great structural stability of F-α_2M, seen in the individual subunits by the resistance to separation of components II and III, and in the whole molecule by the tight association of subunits, may well be that the molecule is rather resistant to proteolysis 'from within', and is thus an effective trap.

There are, however, some characteristics of the α_2M–proteinase reaction that were not predictable from the 'trap' hypothesis. These include the finding that some molecules of trypsin bound by α_2M are available to inhibition by soya-bean trypsin inhibitor (P. M. Starkey, C. A. Sayers & A. J. Barrett, unpublished work) and the apparently covalent binding of plasmin to α_2M (Harpel, 1978). The hypothesis can

be elaborated to explain such findings, however, and may remain the simplest hypothesis consistent with all of the data.

The rapid clearance of α_2M–proteinase complexes from the circulation (Ohlsson, 1971) is probably due to the recognition of the characteristic structure of F-α_2M by the reticuloendothelial cells. If so, F-α_2M produced by the action of NH_4^+ salts should also be cleared rapidly.

Note Added in Proof (Received 3 May 1979)

Recent experiments in which S- and F-α_2M have been subjected to isoelectric focusing in thin-layer polyacrylamide gel have given results qualitatively similar to those of Ohlsson & Skude (1976), in contrast with the lack of separation in sucrose gradients. It seems that one system or the other does not give a correct impression of the respective isoelectric points of the two forms.

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