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Thiol Reduction of Human α_2-Macroglobulin

THE SUBUNIT STRUCTURE

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1. Human α_2-macroglobulin was prepared from a fraction obtained during the large-scale separation of normal human plasma proteins for clinical use. 2. Sedimentation-equilibrium measurements indicated a molecular weight of 725000. A value of 18.1S was obtained for $s^0_{20,w}$. 3. The dissociation that occurs in the pH range 4.5–2.5 and in the region of neutrality in urea-containing solutions is consistent with a dimeric structure of the molecule. 4. The effects of the thiol reagents mercaptoethanol, mercaptoethylamine and *N*-acetylcysteine were investigated over a range of experimental conditions. Distinct components having sedimentation coefficients of 15, 12 and 8.5S were identified. 5. Conditions were found under which limited reduction with thiol liberated a subunit with a molecular weight approximately one-quarter of that of the intact molecule. This subunit retains the serological specificity of the whole molecule.

In normal human serum α_2-macroglobulin (α_2M)† is quantitatively one of the more prominent of the minor protein constituents; the serum from adult males contains 203±11 and that from females 209±19mg/100ml, with an overall range of 130–334mg/100ml (Wilding *et al.*, 1967).

Although its biological function is not well understood, α_2-macroglobulin is known to inhibit the activity of plasmin and trypsin with respect to protein substrates, but not the esterolytic activity of these enzymes (James *et al.*, 1966; Steinbuch *et al.*, 1967*a*; Ganrot, 1967). It acts as a progressive antithrombin but has no heparin-cofactor activity (Steinbuch *et al.*, 1967*b*). There is evidence that it promotes the growth of mammalian cells in suspension culture (Tozer & Pirt, 1964) and that it stimulates the regeneration of the thymus and of bone-marrow cells in irradiated mice (Berenblum *et al.*, 1968).

Several reports have appeared concerning the partial dissociation of α_2-macroglobulin but no clear picture of its subunit structure has emerged. Schönenberger *et al.* (1958) reported that α_2M (sedimentation coefficient approx. 19S) dissociates to the extent of 42% into an 11S component in phosphate buffer, pH7.0, containing 5M-urea. Removal of the urea by dialysis effectively reversed the dissociation but substantial amounts of aggregates formed and some more slowly sedimenting substances were liberated. More recently Razafimahaleo *et al.* (1969) reported that in 0.1M-tris–HCl buffer, pH7.5, three components resolved in 5M-urea (9, 11 and 15S) and in 8M-urea (2.7, 3.9 and 5.75). In 8M-guanidine hydrochloride they observed two (1.5 and 2.1S) and in 2% sodium dodecyl sulphate four components (2.1, 3.3, 3.65, 4.2S). None of these sedimentation-coefficient values was corrected for the effect of solvent viscosity and density.

In sedimentation studies of unbuffered solutions of α_2M in water over the pH range 1.5–11.5 Gentou (1965) found various proportions of two components, sedimentation coefficients 12 and 17.5S. In the range pH3.5–7.5 the 17.5S component predominated but above pH10 only traces of this were present. Up to pH10 the effects observed were reversible and were interpreted in terms of the p*K* values of ionizing groups.

No systematic studies of the cleavage of α_2M by thiol compounds have been described and the brief reports in the literature are conflicting. Schultze *et al.* (1962) claim, from ultracentrifuge examinations, that α_2M remains unaffected by treatment at pH7.0 with 0.1M-mercaptoethanol for 48h, presumably at room temperature. Poulik (1960) treated α_2M, dissolved in 0.1M-sodium borate (pH8.5)–8M-urea, with 0.02M-mercaptoethanol for 2–3h at room temperature and then with 0.05M-iodoacetamide. From analyses of the native and treated protein by electrophoresis in starch gel [0.05M-formate (pH3.1)–8M-urea] he suggested that human α_2M probably contains some disulphide-linked subunits, although the evidence presented appears rather inconclusive.

Treating solutions of α_2M with 1% (v/v) cysteamine (Isliker, 1958) led to the formation of '6S' components and simultaneously to a decrease in

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† Abbreviations: α_2M, α_2-macroglobulin; IgM, immunoglobulin M (World Health Organization, 1964).

viscosity of over 50%. During exposure of the reduced solution to air the viscosity increased over a period of hours but this effect was prevented by adding iodoacetamide. These results indicate definitely that human α_2M may be broken into subunits by these reagents.

The effects of reduction for 3h with 0.25M-mercaptoethanol in the presence of urea or guanidine hydrochloride, terminated by the addition of iodoacetamide, were examined by Razafimahaleo *et al.* (1969). Two sedimenting components, 0.7 and 2.4S, were resolved in urea-containing buffer and six components in urea–starch gel by electrophoresis at pH3.3. Under such drastic conditions of reduction both inter- and intra-chain disulphide bonds would be broken and the products would be the ultimate constituent polypeptide chains rather than multichain structural subunits.

In this paper details are provided of a procedure used for the preparation of human α_2-macroglobulin from normal human citrated plasma. Some definitive physicochemical characteristics of this material are reported. The conditions leading to limited dissociation of the molecule are described together with the properties of a well-defined subunit.

Experimental

Materials

Reagents. Most reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., the following being AnalaR grade: diethyl ether, urea, acetic acid, formic acid, $NaH_2PO_4,2H_2O$. Ethyleneimine was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were Eastman Chemicals from Kodak Ltd., London W.C.2, U.K. Ampholine carrier ampholyte, pH3–10, was purchased from LKB Instruments Ltd., South Croydon, Surrey, U.K.

Chromatographic materials. DEAE-cellulose (Cellex D) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Sephadex G-200 and Sepharose 4B (4% agarose gel) from Pharmacia, Uppsala, Sweden; Biogel A 0.5m (10% agarose gel) and Biogel A 1.5m (8% agarose gel) from Calbiochem Ltd., London W.1, U.K.

Buffers. Phosphate buffer, pH8.0, *I* 1.0, contained 0.0219M-NaH_2PO_4 and 0.326M-Na_2HPO_4. Phosphate buffer, pH6.0, *I*1.0, contained 0.723M-NaH_2PO_4 and 0.0923M-Na_2HPO_4. Phosphate buffer, pH6.8, *I*0.1, contained 0.027M-NaH_2PO_4 and 0.024M-Na_2HPO_4. Dilutions to lower ionic strengths were made appropriately. Phosphate–acetate buffer, pH5.0, *I*0.01, contained 7.6mM-acetic acid and 4.9mM-Na_2HPO_4; formate buffer, pH3.6, *I*0.1, contained 0.20M-formic acid and 0.10M-NaOH; formate–NaCl buffer, pH3.6, *I*0.1, contained 0.10M-formic acid, 0.05M-NaOH and 0.05M-NaCl.

Methods

Preparation of N-acetylcysteine solutions. These were freshly prepared for each experiment. The appropriate amount of *N*-acetylcysteine was dissolved in water and 2.5ml of phosphate buffer, pH6.0 or 8.0, *I*1.0, added followed by an amount of NaOH equivalent to the *N*-acetylcysteine. The mixture was made up to 25ml with water. Solutions of other thiols were prepared similarly but the NaOH was omitted.

Preparation of iodoacetamide solutions. An amount of iodoacetamide was dissolved in phosphate buffer, pH6.0 or 8.0, *I*1.0, to give twice the molar concentration of the thiol reagent used in a particular protein reduction. The high-ionic-strength buffer was used to maintain solutions at the desired pH during the reaction between excess of thiol reagent and iodoacetamide.

Thiol treatment of protein. The solution (1.0vol.) containing protein (15mg/ml) in phosphate buffer, pH6.0 or 8.0, *I*0.1, was equilibrated for 5min at 37°C and 0.25vol. of thiol solution in the same buffer at 37°C was added. The reaction was terminated after a desired period by adding 0.25vol. of iodoacetamide solution of double the molarity and of the same pH as the thiol solution. The reaction vessel was maintained at 37°C for 1h in the dark after which the reaction mixture was dialysed against the appropriate buffer for 36h at 4°C.

Electrophoresis in polyacrylamide gel. Electrophoresis was carried out by following the general directions of Davis (1964), with tris–HCl running buffer, pH8.9, and tris–glycine, pH8.3, as electrode buffer.

Preliminary experiments showed that solutions containing 5% (w/v) acrylamide, 0.2% (w/v) *NN'*-methylenebisacrylamide and 0.05% (w/v) ammonium persulphate polymerized to give gels in which satisfactory migration of native α_2M occurred and these also allowed good resolution of derived subunits.

Electrophoresis experiments were done at room temperature by using a separation gel only. Into tubes (0.5cm×7.5cm) 1.3ml of gel solution was pipetted and after over-layering with water 25min was allowed for polymerization to occur in subdued light. The apparatus (Shandon Ltd., London N.W.10, U.K.) was assembled and the electrode vessels were filled with buffer. The solution to be examined, usually containing 1g of protein/100ml in phosphate buffer, pH6.0 or 8.0, *I*0.1, was diluted with an equal volume of 20% (w/v) sucrose, and 10μl of the resulting solution was pipetted on to the top of the running gel under the electrode buffer. A current of 1mA/gel

was passed for 5min to stack the protein on to the top of the gel and electrophoresis was then continued for a further 1.5h at a constant current of 2mA/gel.

After the removal of the gels from the tubes they were immersed in Amido Black solution (1%, w/v, in 7%, v/v, acetic acid) for 1h. Excess of dye was removed by washing the gels repeatedly in 7% (v/v) acetic acid.

Isoelectric focusing in polyacrylamide gel. Isoelectric focusing was carried out in gels polymerized from solutions containing 4% (w/v) acrylamide, 0.15% (w/v) *NN'*-methylenebisacrylamide, 0.06% (w/v) potassium persulphate and 2% (w/v) Ampholine, pH3–10. Into tubes (0.5cm×7.5cm) was pipetted 1.3ml of gel solution containing also 50μg of protein and after overlayering with water, 30min was allowed for polymerization in subdued light at room temperature. All the subsequent electrophoresis operations were conducted in a cold-room at 4°C. The gel tubes were equilibrated at 4°C for 20min and after the removal of the overlayering water were mounted in the standard polyacrylamide-gel-electrophoresis apparatus. The anode fluid was 5% (v/v) orthophosphoric acid and the cathode fluid 5% (v/v) ethylenediamine. A constant current of 1mA/gel was applied until the potential across the apparatus rose to 200V, this usually taking about 1h. Thereafter a constant voltage of 200V was maintained for a further 2h during which time a steady state was reached with the current falling to about 0.5mA/tube. The gels were removed from the tubes and washed in repeated changes of 5% (w/v) trichloroacetic acid to fix the protein and to elute the ampholyte from the gels. The gels were then stained with 1% (w/v) Amido Black in 7% (v/v) acetic acid for 1h and the excess of dye was then removed by repeated washing in 7% (v/v) acetic acid.

Ultracentrifuge methods. All ultracentrifuge measurements were made with a Beckman model E ultracentrifuge.

Sedimentation-velocity experiments, measurement of records and calculation of sedimentation coefficients were made as previously described (Creeth, 1964). For the determination of the proportions of sedimenting components in mixtures the schlieren profiles were projected optically with a magnification of 8 diameters on to graph paper. The curves were traced in, analysed geometrically and component areas measured planimetrically. In calculating the proportions of components corrections were made for radial dilution but not for the Johnston–Ogston effect. Analytical density-gradient experiments in caesium chloride followed the procedure of Creeth & Denborough (1970). Sedimentation-equilibrium experiments were carried out either by the high-speed method (Yphantis, 1964) or at an intermediate speed (J. M. Creeth & J. C. Holt, unpublished work) characterized by the condition $A = 1.2$, where $A = M(1-\bar{v}\rho)\omega^2/2RT$, the symbols having their usual significance. Molecular weights were obtained from the limiting slopes of logarithmic plots in the usual manner (see, e.g., Creeth & Pain, 1967). Data were processed automatically by a series of programs written for an Olivetti P.101 desk-top computer.

Optical rotatory dispersion. Measurements of the specific optical rotation, [α], were made at six wavelengths in the range 365–578nm, by using a spectropolarimeter constructed to the design of Malcolm & Elliott (1957). The [α] values were converted into mean residue rotations by using the assumed value of 115 for the mean residue weight. Dispersion data for water were obtained from International Critical Tables and the specific refraction increment for urea was taken as 8.608×10^{-3} litre/mol (Gosting & Akeley, 1952). The results were plotted according to the Moffitt & Yang (1956) equation with $\chi_0 = 212$nm; straight lines were found yielding a_0 and b_0 as the intercept and slope respectively.

Preparation of antisera. To 0.4ml of Freund's 'complete' adjuvant was added 0.25ml of a solution containing 2g of protein/100ml in phosphate buffer, pH7.0, I 0.2. The mixture was homogenized, divided into four equal volumes and injected intramuscularly into the fore- and hind-quarters of a rabbit. At 2 and 8 weeks after the initial injection this procedure was repeated. The animals were bled 2 weeks after the third injection, giving satisfactory antisera. Booster doses were given 3 months later and antisera collected 2 weeks after the injection, and so on if required.

Antisera to fraction G2.2.P contained antibodies to α_2M, IgM and to four or five other proteins. The initial bleeding from animals immunized with purified α_2M contained only antibody specific to α_2M but bleedings taken after booster doses occasionally contained traces of antibody to minor protein constituents of fraction G2.2.P.

General methods. All dialyses, vacuum dialyses and chromatographic separations were carried out in a cold-room at 4°C.

A Beckman model L ultracentrifuge was used for high-speed preparative separations.

Preparation of human α_2-macroglobulin (α_2M)

When normal human plasma is fractionated by the procedure of Kekwick & Mackay (1954), the α_2- and γ-macroglobulins (α_2M and IgM) separate predominantly into the globulin fraction G2. This fraction, prepared at the Blood Products Laboratory, Lister Institute, Elstree, from 30-litre pools of fresh normal human citrated plasma was the source for the preparation of α_2M.

Preliminary subfractionation in aqueous ethyl ether. The amount of centrifuged G2 precipitate derived

from 10 litres of plasma was suspended in 10 litres of cold (4°C) phosphate–acetate buffer, pH5.00, I0.01. The mixture was transferred into a closed 15-litre precipitation vessel (Kekwick *et al.*, 1955) and stirred for 3h, the vessel standing in a thermostat-controlled refrigerated bath at 0.0±0.2°C. Ethyl ether (500ml) was then added slowly through a jet with continuous stirring and the mixture equilibrated at 0°C overnight. The translucent supernatant was siphoned off and the precipitate slurry was centrifuged in transfusion bottles in a refrigerated-bucket centrifuge (Hearson Ltd., London) at 0±0.2°C, 1500rev./min ($r_{av.}$ 22cm) for 1h and the supernatants were added to the main bulk.

The precipitate (fraction G2.1) contains α- and β-lipoproteins, plasminogen and much of the fibrinogen present in fraction G2, the macroglobulins remaining in the supernatant with other proteins.

For each litre of supernatant 60ml of the ethyl ether was added as described above, and the mixture equilibrated overnight at 0°C. After removal of most of the clear supernatant, the small precipitate was resuspended in the remainder (1–2 litres) and collected by centrifuging at 0.0±0.2°C. The precipitate was dissolved in 100ml of phosphate buffer, pH8.0, I 0.2, and dialysed at 4°C against several changes of 1 litre volumes of this buffer to remove the ether. During the dialysis a fibrin-containing precipitate formed. This was centrifuged down at 4°C, and after removing the supernatant the fibrin precipitate was washed by suspension in a small volume of pH8.0 buffer and centrifuging again, the washings then being added to the original supernatant. The solution was then diluted with buffer to a protein content of 2g/100ml on the basis of refractive-index measurements. This fraction is designated G2.2.

From fraction G2 derived from 10 litres of plasma the mean recovery of total protein in fraction G2.2 was about 6.5g. Examination in the ultracentrifuge showed 60% (range 55–65%) of the total protein to be macroglobulin, the remainder, apart from traces of aggregate, having a sedimentation coefficient of about 7S.

Fractionation in the preparative ultracentrifuge. The solution of fraction G2.2 (2g/100ml in phosphate buffer, pH8.0, I0.2), was centrifuged (Beckman model L, rotor 40) at 40000rev./min for 6h at 10°C. The solution separated into a small green–blue pellet covered by a thin layer of similarly coloured concentrated protein solution (heavy layer); above this a colourless region extended to about 1cm from the top of the tube, which was occupied by a lipaemic layer. The contents of the tubes were removed as far as the heavy layer which was pooled, diluted with buffer to 2g of protein/100ml and recycled, providing more pellets. The combined pellets were dissolved in sufficient phosphate buffer, pH8.0, I0.2, containing 0.02% (w/v) sodium azide, to a final concentration of 2g of protein/100ml, and, if not used immediately for further purification stages, the solution was stored frozen at −25°C.

About 30% of the total protein from fraction G2.2 was recovered in this fraction (G2.2P). Sedimentation analysis gave a mean value of 89% (range 87–91%) for the proportion of macroglobulins, the remainder being '7S' protein. Therefore approx. 45% of the macroglobulin content of fraction G2.2 was recovered in fraction G2.2P.

Recycling fraction G2.2P in the preparative ultracentrifuge gave a product containing 96% macroglobulin, an enhancement in purity not sufficiently advantageous to be useful. Moving-boundary electrophoresis of such preparations in phosphate buffer, pH8.0, I0.2, gave a cathode-limb analysis of 82% α_2-globulin and 18% γ-globulin. The anode-limb values diverged considerably from these (61 and 39% respectively) indicating marked protein–protein interaction.

Chromatographic separations. (*a*) Sephadex G-200. The macroglobulins of fraction G2.2P were separated from the '7S' proteins by passage through a column of Sephadex G-200 equilibrated against phosphate buffer, pH8.0, I0.2. Initially a column in a glass envelope (100cm×4.5cm) was used, with downward flow stabilized with a Mariotte bottle to give a flow rate of about 20ml/h, fractions being collected every 20min. To this column, which had a void volume of 300ml, 20ml of fraction G2.2P (2g/100ml) was applied. The protein content of the individual fractions was determined by measuring their absorption at 280nm with a Unicam SP.500 spectrophotometer. For later separations an LKB Recychrom (LKB Produkter AB, Bromma, Sweden) equipment, with a plastic envelope (100cm×3.5cm), was used, providing a column with a void volume of 230ml to which 15ml of solution was applied. In this system a pumped upward flow rate of 14ml/h was maintained, the effluent being monitored with a Uvicord 1 analyser at 254nm and fractions were collected every 30min. The elution profile showed a main peak followed by a broader shallow secondary peak, in conformity with the pattern obtained by sedimentation-velocity analysis.

From sedimentation-velocity runs on individual fractions, any aggregated material present was shown to segregate into the leading fractions of the main peak. The onset of contamination of fractions from the trailing edge of the main peak with traces of '7S' protein associated with the secondary peak was detected by immunodiffusion against rabbit antiserum to fraction G2.2P. As a consequence of these tests samples were pooled over the main peak from E_{280}^{1cm} 2.0 on the leading edge to 1.0 on the trailing edge. Such pools contained 3.7–4.3mg of protein/ml, accounting for 75–80% of the protein applied to the column. In addition to α_2M and IgM they contained

traces of another unidentified protein detected by immunodiffusion.

(*b*) DEAE-cellulose. Two slightly different procedures were used to obtain pure α_2M from the Sephadex G-200 macroglobulin pool. For earlier preparations the pool was concentrated by vacuum dialysis to 2g of protein/100ml and dialysed against phosphate buffer, pH6.0, *I* 0.05; the small precipitate that formed, accounting for 12–15% of the initial protein, was removed by centrifuging at 4°C. The supernatant was dialysed against phosphate buffer, pH6.0, *I* 0.1, and applied to a column of DEAE-cellulose equilibrated against the same buffer. Under these conditions α_2M passes straight through the column whereas IgM and other trace contaminants are retained. The precipitate formed at pH6.0, *I* 0.05, contains contaminants that otherwise pass through the column at pH6.0, *I* 0.1, with the α_2M. At pH6.0, *I* 0.05, α_2M itself is adsorbed by DEAE-cellulose.

In the second and preferred procedure, the Sephadex G-200 macroglobulin pool was dialysed without a preliminary concentration stage, initially against phosphate buffer, pH6.8, *I* 0.1, and then against phosphate buffer, pH6.8, *I* 0.01. The precipitate that formed was removed by centrifuging at 4°C and contained almost all the IgM (C. M. Butterworth & R. A. Kekwick, unpublished work). The supernatant was dialysed against sodium phosphate, pH6.0, *I* 0.1, concentrated to 2g of protein/100ml by vacuum dialysis and applied to a column of DEAE-cellulose as in the first procedure. About 60% (range 55–64%) of the protein applied to the DEAE-cellulose column was recovered in the eluate.

The samples containing the purified α_2M eluted from the column were pooled, concentrated to 2g of protein/100ml by vacuum dialysis and stored at 4°C. If such solutions of α_2M are frozen a proportion of the protein tends to aggregate to dimers after prolonged storage.

The α_2M prepared in this manner sediments in the region of neutrality as a single peak in the ultracentrifuge (Fig. 1*a*), and displays a single band on electrophoresis in 5% (w/v) polyacrylamide gel at pH8.9. When subjected to isoelectric focusing in a pH3–10 gradient in 4% polyacrylamide gel a very intense sharp band forms at pH5.4 and one to three extremely faint bands between pH5.4 and 5.6. It gives a single precipitin line by immunoelectrophoresis and immunodiffusion when tested against wide-spectrum antisera to human plasma proteins.

Results and Discussion

Sedimentation-velocity behaviour

Effect of pH. The behaviour of the protein in sedimentation-velocity experiments was examined over the pH range 2.5–10.0 at ionic strengths from 0.1 to 0.3. Over the pH range 5.0–10.0 the protein sediments as a single peak (Fig. 1*a*), $s_{20,w}$ 17–18S at 1–3mg/ml. Below pH4.5, at temperatures near 4°C, a slower component is resolved, $s_{20,w}$ 10.6–11.4, the proportion of which increases as the pH is lowered; some '18S' component still remains at pH2.5.

In formate buffer, pH3.6, *I* 0.1, at a protein concentration of 10mg/ml, the slower component accounts for 33% of the total protein and about 13% is in the form of aggregates sedimenting ahead of the remaining '18S' component (Fig. 1*b*). Sodium chloride has an inhibitory effect on the dissociation since in formate–NaCl buffer, pH3.6, *I* 0.1 (NaCl 0.05M), 24% of the '11S' component is formed and also there is rather more aggregate (17%). The extent of dissociation is increased to some extent by lowering the total protein concentration.

The dissociation was found not to be reversible when the pH of solutions was raised relatively quickly by dialysing against buffers either within the dissociating region pH2.5–4.5 or from within the dissociating region to pH6.0. In these circumstances the amounts of both '11S' and '18S' components decreased and large amounts of polydisperse aggregates formed. Similar aggregation occurred when solutions in the dissociating range were kept for 24–48h at 25°C whereas at 4°C they were stable.

Since dissociation into the '11S' components begins at pH4.5 and is almost complete at pH2.5, the range of 2 pH units suggests that an ionizing group of p*K* about 3.5 may be involved in this effect. Such an explanation was proposed by Gentou (1965) to account for his observations with unbuffered aqueous solutions of α_2M at pH values below the isoelectric point (pH5.4). Gentou (1965) also described a similar dissociation increasing with pH above 5.4; from pH5.0 to 10 we found no dissociation into an '11S' component in buffered systems.

No dissociation was detected in phosphate buffer, pH6.0, *I* 0.1, containing in addition 1mM-EDTA (disodium salt), which suggests that polyvalent metal ions are not structurally involved in the subunit bonding.

Effect of urea. The results of other workers on the dissociating effects of urea on α_2M have been mentioned above. We examined the effects of 1–6M-urea on α_2M dissolved in phosphate buffer, pH8.0, *I* 0.1 and 0.2, at protein concentrations of 8–10mg/ml.

In phosphate buffer, pH8.0, *I* 0.2, no dissociation was detected in 1M-urea, but in 2M-urea 26% of the molecules dissociated (Fig. 1*c*) into a component of $s_{20,w}$ 10.2S (concn. approx. 2mg/ml), the value for the undissociated residue being 15.2S (concn. approx. 6mg/ml) uncorrected for the effect on its sedimentation velocity due to the dissociation

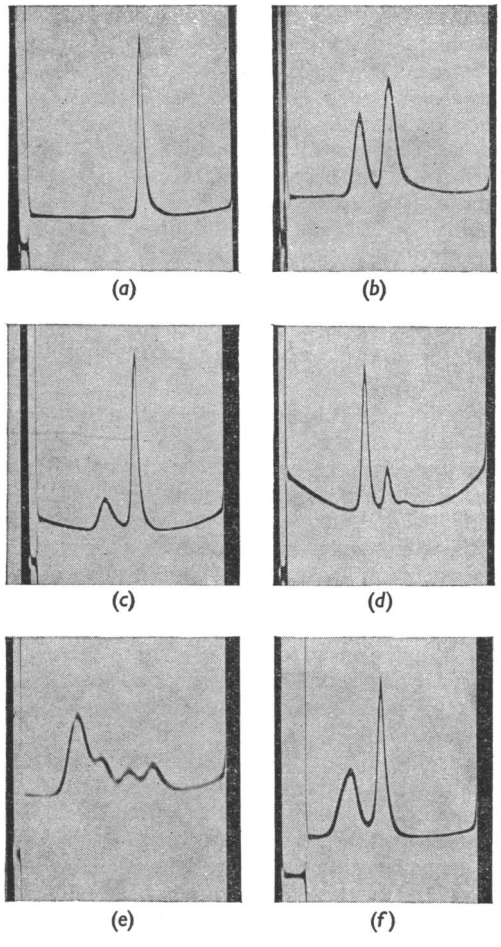

Fig. 1. *Sedimentation-velocity profiles of α_2M after treatment in various ways*

(*a*) Native α_2M in phosphate buffer, pH6.0, *I*0.1; 6mg/ml, 33min. (*b*) Native α_2M in formate buffer, pH3.6, *I*0.1; 10mg/ml, 33min. (*c*) Native α_2M in phosphate buffer, pH8.0, *I*0.2 + 2M-urea; 10mg/ml, 38min. (*d*) Native α_2M in phosphate buffer, pH8.0, *I*0.2 + 5M-urea; 8mg/ml, 70min. (*e*) Reduced carboxymethylated α_2M (0.05M-2-mercaptoethanol, pH6.0, 37°C, 1h) in phosphate buffer, pH6. 0, *I*0.1; 10mg/ml, 33min. (*f*) Reduced carboxymethylated α_2M (0.01M-*N*-acetylcysteine, pH8.0; 37°C, 3h) in phosphate buffer, pH8.0, *I*0.1; 10mg/ml, 21min. Direction of sedimentation was from left to right. Speed: 52640rev./min, except (*d*) 59780rev./min. Time (in min) is the period after reaching full speed.

product. In 3M-urea dissociation into the subunit was almost complete ($s_{20,w}$ 8.5S, concn. 8mg/ml) and little further increase in dissociation was apparent in 4M-urea. In 5M-urea the sedimentation profile indicated a partial reaggregation of the initial dissociation product, two faster-sedimenting components being formed (Fig. 1*d*); the extent of this reaggregation was greater in 6M-urea. When 5mM-iodoacetamide was included in the buffered 5M-urea solution reaggregation did not occur, the faster-sedimenting components being absent.

A rational interpretation of these results, based on the values of the sedimentation coefficients of the components observed in urea and at low pH, is that partial unfolding and dissociation into half-molecules are brought about by urea, the dissociation being almost complete in 3M-urea. The value of 8.5S for the slower component, at a comparatively high concentration in 3M-urea, is considerably lower than that observed at low pH and at a lower concentration (approx. 11S), but the decrease in S value of the undissociated component in 2M-urea indicates a significant degree of unfolding, which is therefore presumed to occur also in the dissociation product. At higher concentrations of urea, further unfolding of the dissociation product occurs, rendering previously inaccessible thiol groups susceptible to oxidation and leading to the formation of disulphide-bond polymers. Such an effect would be inhibited by iodoacetamide.

Cleavage with thiol reagents

*Reductive effects of several thiols at pH*6.0 *and* 8.0. Preliminary experiments in which α_2M was treated at 37°C in pH6.0 buffer with 0.05M-mercaptoethanol, the products being stabilized by carboxymethylation with iodoacetamide, gave mixtures consisting of four components (Fig. 1*e*) with sedimentation coefficients ($s_{20,w}$) about 18, 15, 12 and 8.5S. The distribution of protein among these components (Table 1) shows that even after treatment for 9h the 18S component is still present. Progressive loss of protein occurred; after incubating for 3 and 9h, 12 and 27% respectively of the initial protein sedimented as grossly aggregated material and consequently is excluded from the analysis. As it appeared probable that this aggregation arose from further changes in the '8.5S' component during the longer periods of treatment, milder conditions of reduction were sought.

Since the susceptibility of disulphide bonds in proteins to cleavage by thiols might be influenced by local charge effects, a survey was made of the effects of treating α_2M with 2-mercaptoethanol, mercaptoethylamine and *N*-acetylcysteine at pH6.0 and 8.0. The results of ultracentrifugal analyses indicated (Table 2) that, with each reagent, at 0.01M a less complex mixture is formed at pH8.0 than at 6.0. In each instance, serial analysis showed no significant alterations in the distribution after 3h. With

Table 1. *Effect of treating α_2M with* 0.05M-*mercaptoethanol, pH*6.0, 37°*C*

The results indicate the percentage of total protein attributable to the designated component. The losses by aggregation were calculated from the sedimentation profiles by assuming that no such loss occurred after 1h treatment.

Period of treatment	Percentage of component present				Loss by aggregation
(h)	18S	15S	12S	8.5S	(%)
1.0	18	15	18	49	—
3.0	21	18	17	43	12
9.0	29	15	20	36	27

Table 2. *Effect of treating α_2M with various thiols at pH*6.0 *and* 8.0

The concentration of thiol was 0.01M and heating was at 37°C for 3.0h. The values indicate the percentage of the total protein attributable to the designated component. Abbreviation: tr., trace.

Thiols	pH	Component (approx. sedimentation coefficients) 18S	15S	12S	8.5S
Mercaptoethanol	6.0	38.5	33.4		28.1
	8.0	48.0	tr.	tr.	52.0
Mercaptoethylamine	6.0	30.3		69.7	
	8.0	67.1	—	—	32.9
N-Acetylcysteine	6.0	56.6	—	29.4	14.0
	8.0	58.8	—	—	41.2

mercaptoethanol and mercaptoethylamine, much more of the '18S' component remained after reduction at pH8.0 than at 6.0, suggesting a direct effect of pH on the extent of reduction. With *N*-acetylcysteine the amounts of residual '18S' component were nearly the same at these pH values; the stabilized 8.5S component aggregated extensively when redialysed to pH6.0.

Analysis of these mixtures by electrophoresis in polyacrylamide gel and then staining showed bands corresponding to each of the four sedimenting components; trace amounts of the '15S' and '12S' components were detected by this method even when they were not distinguishable in the ultracentrifuge. By splitting the gels longitudinally, staining one-half and immersing the other in antiserum specific to α_2M, the four components were shown by the formation of immunoprecipitation bands to retain the serological specificity of the native molecule.

Some exploratory experiments were made on the reductive effect of dithiothreitol at pH8.0 only. After treatment with 5mM-dithiothreitol much of the material sedimented in a grossly aggregated form and even with 1mM four components were present in the reduction mixture.

*Reduction with N-acetylcysteine at pH*8.0. Since the '8.5S' component appeared the most fundamental, conditions were sought that would provide a high proportion of this component with as little as possible of the '15S' and '12S' components. On the basis of the results summarized in Table 2, together with the observation that the product of reaction with mercaptoethanol gave a rather more diffuse '8.5S' peak, *N*-acetylcysteine at pH8.0 was selected. The effects of varying the thiol concentration and the temperature, and the effect of 1M-urea, were then determined (Table 3). It is apparent from these results that 1M-urea enhances the susceptibility of the protein disulphide bonds to reduction.

Separation of the '8.5S' component from the reduction mixtures. The preparation of purified '8.5S' component from the reduction mixtures at a concentration adequate for further physicochemical characterization proved to be unexpectedly difficult because of the inordinate tendency of this component once isolated to form aggregates.

By using the mixture obtained by reduction at pH8.0 with 0.01M-*N*-acetylcysteine and stabilized by carboxymethylation with iodoacetamide, various types of molecular sieving gels were tested by loading columns (60cm×3.2cm) with 5ml samples of solutions containing 10mg of total protein/ml in phosphate buffer, pH8.0, *I*0.2. No resolution of the components was obtained on Sephadex G-200 or

Table 3. *Effect of concentration of N-acetylcysteine and urea on the cleavage of α_2M at pH8.0*

The values indicate the percentage of the total protein attributable to the designated component. Reaction times were: at 37°C, 3h; at 0°C, 8h.

N-Acetylcysteine (M)	Temp. (°C)	Urea (M)	Component (approx. sedimentation coefficient) 18S	15S	12S	8.5S
0.005	37	—	84.8	—	—	15.2
0.010	37	—	56.9	—	—	43.1
0.015	37	—	42.6	2.9	54.5	
0.020	37	—	40.6	4.4	55.0	
0.030*	37	—	37.0	6.8	56.2	
0.050*	37	—	23.0	13.1	16.9	47.0
0.010	0	—	65.6	—	—	34.4
0.010	37	1.0	32.7	5.4	3.8	58.0
0.020	37	1.0	20.3	12.3	20.3	47.0

* A small proportion of aggregated material sedimented ahead of the 18S component and is excluded from the analysis.

Sepharose 4B (4% agarose), but separation occurred with Biogel A 1.5m (8% agarose) and Biogel A 0.5m (10% agarose).

With these materials, a sharp leading peak was followed by a broader second peak; when monitored by gel electrophoresis, it was found that the first peak was pure 18S component and that the slower was predominantly 8.5S. However, concentration of the slower fractions caused extensive aggregation, leading mainly to 12 and 18S components. The addition of 2M-urea decreased the extent of aggregation, but even in 4M-urea, four sedimenting components were still present. Adding iodoacetamide (5mM) to a pool of 8.5S fractions before concentration did not prevent aggregation. Similar results were obtained when iodoacetate or ethyleneimine was substituted for iodoacetamide when terminating the initial reduction, in spite of the modification in charge that these reagents produced.

When urea was present throughout the reduction, chromatographic and concentration stages, however, satisfactory preparations of 8.5S component were obtained. Thus, after reducing α_2M in phosphate buffer, pH8.0, *I*0.1, containing 1M-urea, with 0.01M-*N*-acetylcysteine and stabilizing with iodoacetamide, chromatography in the same buffer on Biogel A 0.5m gave two well-defined peaks. Fractions from the second peak, monitored as before, were concentrated to 10mg of protein/ml; sedimentation velocity analysis then indicated 92% of the 8.5S component, the remainder being 12 and 15S; no 18S component was present.

In later similar experiments with the same column, the resolution became progressively impaired. This was attributed to the deterioration of the molecular sieving properties of the Biogel A, presumably because prolonged contact with urea led to a breakdown in the hydrogen-bonded structure of the agarose-gel. Because of these unreliable characteristics this method of separation was abandoned. However, the initial results established satisfactorily that from reduction mixtures made in phosphate–urea buffer, stable preparations of 8.5S component could be separated.

The preparations of 8.5S component used for the physicochemical characterizations described below were obtained by high-speed preparative centrifugation from the reduction mixture obtained in phosphate (pH8.0, *I*0.1)–1M-urea. Samples (5ml) containing 10mg of protein/ml were centrifuged at 39500rev./min (Beckman model L, SW 39 rotor) for 9h at 3°C. With these conditions it was anticipated that the boundary of the '18S' component would be about 2cm, and that of the '8.5S' component 4cm from the bottom of the tube, so providing about 2ml of a solution containing 3–4mg of '8.5S' component/ml. The contents of the tubes were removed by upward displacement with 50% sucrose, monitored at 280nm with an automatic recorder (Isco) and collected in 0.3ml fractions. These were scanned by electrophoresis in polyacrylamide gel and appropriate fractions pooled for sedimentation-velocity, sedimentation-equilibrium and other measurements. The final 8.5S pool contained approx. 5% of heavier components.

Physicochemical characteristics of α_2M and the '8.5S' subunit

It has been mentioned above that, in the pH range 5.0–10.0 at ionic strength 0.1–0.3, α_2M sediments as a single component. In sodium phosphate buffer,

pH8.0, I0.1, the dependence of the sedimentation coefficient ($s_{20,w}$) on concentration (c in mg/ml) is given by the relation:

$$s_{20,w} = 18.06(1 - 0.0128c)$$

and in the same buffer containing 1M-urea by:

$$s_{20,w} = 17.62(1 - 0.0134c)$$

The difference of 0.44S between the values for the sedimentation coefficient at zero concentration is significant and could be interpreted as indicating a slight expansion of the molecule in the urea-containing medium.

The value of 18.1S for $s^0_{20,w}$ is lower than others published for human α_2M; Schönenberger *et al.* (1958) give 19.6S and Dunn & Spiro (1967) calculated a value of 20.6S for the main peak of a preparation containing 6% of a faster-sedimenting component having $s^0_{20,w} = 30.8$S. For pig α_2M Jacquot-Armand & Guinand (1967) give 19.1S. The present value is close to that of Nagasawa *et al.* (1970), who found 17.8S for highly purified bovine α_2M.

The optical-rotatory-dispersion (ORD) parameters a_0 and b_0 for the native protein, for the protein in 1M-urea, and for the '8.5S' subunit in 1M-urea are given in Table 4. The values for the native protein closely resemble those for the immunoglobulins IgG and IgM (see, e.g., Jirgensons, 1969); a_0 is slightly lower than is the case with many single-chain globular proteins, and b_0 is close to zero, indicating the relative absence of helical structure. No previous values for human α_2-macroglobulin have appeared, but Jacquot-Armand & Guinand (1967) quote various ORD characteristics for the corresponding pig protein; their values are most easily compared in terms of the specific rotation $[\alpha]_{546}$ and the characteristic wavelength, λ_c, in the Drude equation. For the porcine material, $[\alpha]_{546} = -52°$, $\lambda_c = 230$nm. Our figures give $[\alpha]_{546} = -46.1°$, $\lambda_c = 222$nm; obviously, the two proteins are closely similar. The slight change in a_0 induced by 1M-urea is not in itself significant, although it is in the direction to be expected for the slight expansion suggested by the sedimentation-velocity results: thus these results confirm that the α_2-macroglobulin molecule is little affected by 1M-urea at pH8.0. Moreover, the values for the '8.5S' subunit differ little from those of the intact molecule, indicating that the dissociation is accompanied by minimal conformational change.

At pH3.4, within the region in which α_2M dissociates to give a second sedimenting component, measurements at several protein concentrations provided extrapolated values for the sedimentation coefficient ($s_{20,w}$) at zero concentration of 16.8S for the residual undissociated molecule and 11.1S for the slower component: this has a normal dependence of sedimentation coefficient on concentration. The ratio of the s^0 values is 1.52 and for a monomer–dimer system, assuming no change in frictional properties, this ratio should be 1.59. This dissociation product may with some confidence be regarded as half the original α_2M molecule.

Molecular weight values obtained by the Yphantis (1964) high-speed method, taking $\bar{v} = 0.735$ (Schultze & Heremans, 1966), are given in Table 5. The value of 725000 for the native protein, when combined with the value quoted for $s^0_{20,w}$, gives a value for the frictional coefficient of 1.57. The molecular weight found for the intact molecule is considerably lower than that of 820000 derived by Schönenberger *et al.* (1958) from measurements of the sedimentation and diffusion coefficients of their preparations.

The validity of the procedures used for these determinations was established by control measurements on bovine serum albumin; molecular-weight values of 68300 in the pH8.0, I0.1, buffer, and 68500 in this buffer containing 1M-urea, were obtained for this protein.

The molecular weight of the intact molecule in phosphate–urea not only confirms that obtained in the absence of urea but affords a direct comparison, in the same medium, with the molecular weight of the '8.5S' component. Sedimentation-velocity experiments on the preparations used for determining the molecular weight of the '8.5S' component showed trace amounts of '12S' material. This probably accounts for a slight degree of heterogeneity detected in the sedimentation-equilibrium measurements and for the range of values of molecular weight obtained. The ratio of the molecular weights suggests that the

Table 4. *Optical-rotatory-dispersion constants*

For experimental details see the text.

Protein	Buffer	a_0	b_0
Intact α_2M	Sodium phosphate, pH8.0, I 0.1	−231°	−24°
Intact α_2M	Sodium phosphate (pH8.0, I 0.1)−1M-urea	−237°	−24°
'8.5S' component	Sodium phosphate (pH8.0, I 0.1)−1M-urea	−238°	−41°

Table 5. *Molecular weights of α_2-macroglobulin and '8.5S' fragment*

Molecular weights were measured by the Yphantis (1964) high-speed method.

Buffer	Molecular weight	
	α_2M	'8.5S' fragment
Sodium phosphate (pH8.0, *I* 0.1)	724000	—
Sodium phosphate (pH8.0, *I* 0.1)–1 M-urea	725000*	196000† (Range 176000–226000)

* Mean of two values.
† Mean of six values obtained with two independent preparations.

'8.5S' component corresponds to a quarter of the original molecule.

From density-gradient-equilibrium experiments a value for the buoyant density (ρ^0) was obtained in CsCl buffered with phosphate buffer, pH8.0, *I* 0.1, for intact α_2M ($\rho^0 = 1.310$g/ml), and for the '8.5S' component in CsCl buffered with phosphate buffer, pH8.0, *I*0.1, + 1M-urea ($\rho^0 = 1.312$g/ml). These almost identical values indicate that the distribution of the carbohydrate moiety is the same in the '8.5S' subunits as in the intact α_2M; that is, in cleaving the molecule with thiol the carbohydrate moiety is not differentially broken away.

Conclusion

From the experimental results presented the general conception of the nature of human α_2-macroglobulin emerging is that of a relatively compact molecule of low helical content, having a molecular weight of 725000. The dissociation occurring below pH4.5 is consistent with a dimeric structure and that induced by 1–3M-urea in the region of neutrality appears to be similar in nature. The evidence suggests that the putative half-molecules are held together in the dimeric structure by electrostatic and hydrogen bonds.

Limited reduction with thiols leads to the formation of subunits whose estimated molecular weight of 196000 is close to one-quarter of that of the intact molecule. These probably represent a cleavage, into two, of the half-molecules produced by dissociation at low pH values or at neutrality by urea. They retain the serological properties of the whole molecule.

A more complete understanding of the peptide chain structure of the macromolecule may best be attained by studies extended to the controlled cleavage of the mol.wt. 196000 subunit, but the extreme tendency of this product to aggregate in aqueous solution makes it difficult to handle experimentally.

It is amply clear from the results presented that the fundamental polypeptide structure of α_2-macroglobulin is quite different from that of IgM.

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