Physical and Chemical Properties of Human Plasma α_2 -Macroglobulin

By PHILIP K. HALL* and RONALD C. ROBERTS†

Marshfield Medical Foundation Inc., 510 North St. Joseph Avenue, Marshfield, WI 54449, U.S.A.

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α_2 M (α_2 -macroglobulin) was purified from human plasma by two different procedures. As well as having no detectable impurities by the usual criteria for testing the homogeneity of protein preparations, these α_2 M preparations showed a single component, after reduction in urea, of 185000 daltons by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The molecular weight of the α_2 M was found to be 718000 by sedimentation-equilibrium experiments by using the gravimetrically determined $\bar{v}$ of 0.731 ml/g. The interaction of several proteinases with α_2 M was studied by using a novel discontinuous polyacrylamide-gel system, which showed clear separation of the enzyme-complexed α_2 M from the free α_2 M. These studies indicated that urokinase, as well as trypsin, chymotrypsin, plasmin and thrombin forms complexes with α_2 M. The cleavage of the 185000-dalton subunit to a 85000-dalton species on interaction of trypsin with α_2 M was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after reduction of the α_2 M–trypsin complex in urea. The amino acid composition, carbohydrate content, absorption coefficient at 280 nm, the specific refractive increment and the sedimentation coefficient for these α_2 M preparations were measured. The stability of the trypsin-binding activity of the α_2 M preparations was also studied under several storage situations.

α_2 -Macroglobulin is one of the major proteinase inhibitors of blood plasma. Unlike with other proteinase inhibitors, proteinases bound to α_2 M retain their peptidase or esterase activity toward low-molecular-weight substrates (Mehl *et al.*, 1964). This property has led Barrett & Starkey (1973) to hypothesize that on attack of a proteinase on α_2 M, a major molecular rearrangement occurs resulting in the entrapment of the proteinase inside the α_2 M molecule. Harpel (1973) has shown that a peptide bond in α_2 M is cleaved when proteinases interact with it, by the appearance of a new peptide chain demonstrable by SDS/polyacrylamide-gel electrophoresis. Other remarkable properties of α_2 M as a proteinase inhibitor are its large size and the broad range of proteinases that it binds and inhibits (Barrett & Starkey, 1973). These studies all suggest that rearrangement of quaternary structure is involved in proteinase binding. A careful definition of the physicochemical properties of the native molecule is a necessary starting point in detecting such molecular rearrangements.

The reported physical properties of human α_2 M vary significantly. The molecular-weight values

range from 650000 (Saunders *et al.*, 1971) to a high of 820000 (Schönenberger *et al.*, 1958). At least five values of the absorption coefficient have been reported, ranging from 6.9 (Schönenberger *et al.*, 1958) to 11.1 $A_{280}/10$ mg per ml (or A_{280}/g per dl) (Brown *et al.*, 1954). As one might expect, there are differences in the reported amino acid compositions.

Because these physical properties have an important influence on elucidation of the molecular structure, the binding mechanisms and ultimately the biological function of α_2 M, the following studies report the physicochemical properties of α_2 M preparations isolated to a high degree of purity and actively able to bind a variety of proteinases. The molecular weights under a variety of conditions, partial specific volume, absorption coefficient, amino acid and subunit composition of the α_2 M preparations are reported. The effects of freezing, age and temperature on the proteinase-binding activity of α_2 M were also determined.

Experimental

General

Unless otherwise stated, all chemicals were analytical-reagent grade. All dialyses, concentration steps and chromatographic separations were carried out in a cold-room at 4°C. All buffers contained 0.02% NaN_3 (Mallinckrodt, St. Louis, MO, U.S.A.).

Abbreviations used: α_2 M, α_2 -macroglobulin; SDS, sodium dodecyl sulphate.

* Permanent address: University of Wisconsin, Marshfield/Wood Campus, Marshfield, WI 54449, U.S.A.

† To whom reprint requests should be addressed.

A Beckman model J-21 refrigerated centrifuge and a model L3-50 preparative ultracentrifuge were used in the isolation procedure.

Isolation

The reported instability of the proteinase-binding activity of α_2 M to freezing (Jones *et al.*, 1972), freeze-drying or precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Schönenberger, 1955; Saunders *et al.*, 1971) at neutral pH values led us to adopt isolation methods avoiding these procedures. α_2 M from fresh human serum (haptoglobin type 1-1) was isolated by a procedure adapted from that of Frenoy *et al.* (1972) and previously reported (Roberts *et al.*, 1974).

α_2 M was prepared from human plasma by two methods. Method 1 was a modification of the procedure of Harpel (1973) in which α_2 M was protected from proteolytic enzymes by the addition of soya-bean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, U.S.A.) immediately after the fresh plasma was drawn: 1 vol. of a solution of the inhibitor [0.5 mg/ml in 0.13 M-sodium citrate (38 g/litre)] was added to 9 vol. of whole blood. After centrifuging at 2000g at 4°C to remove cells, the plasma was treated with BaCl_2 and BaSO_4 to remove the prothrombin complex. The addition of 4% poly(ethylene glycol) (Pierce Chemical Co., Rockford, IL, U.S.A.) to the supernatant in 0.02 M-sodium phosphate (pH 7.4)/0.1 M-NaCl precipitated the fibrinogen. The supernatant was made 12% with poly(ethylene glycol) to precipitate an α_2 M-rich fraction. The lipoproteins were removed by ultracentrifugation in a KBr solution (density 1.087 g/ml) at 82000g for 16 h at 4°C. The contents of the lower one-third of each centrifuge tube were dialysed against 30 vol. of 0.05 M-Tris/HCl buffer, pH 8.0, changed three times at 6–8 h intervals, and applied to a column (2 cm $\times$ 64 cm) of DEAE-cellulose (Whatman DE-52; Whatman, Clifton, NJ, U.S.A.). The sample was eluted by a 1-litre linear salt gradient at constant pH (starting buffer 0.05 M-Tris/HCl, pH 8.0, and final buffer containing 0.4 M-NaCl). α_2 M-containing fractions as detected by double immunodiffusion by using commercial anti- α_2 M serum were dialysed against 30 vol. of 0.1 M- NaH_2PO_4 , pH 6.5, changed three times at 6–8 h intervals, and applied to a column (3 cm $\times$ 82 cm) of Bio-Gel A5m (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Haptoglobins were removed by adsorption on a haemoglobin-agarose column by the procedure of Klein & Mihaesco (1973). Final purification was obtained by gel filtration on a column (1.8 cm $\times$ 75 cm) of Sephadex G-200 (Pharmacia, Piscataway, NJ, U.S.A.) in 0.1 M- NaH_2PO_4 , pH 6.5.

Method 2, a much shorter procedure, was used in the later stages of the project to isolate α_2 M from outdated blood-bank plasma. Plasma (200 ml) was applied to a Blue Dextran–Sephadex 4B column

(5 cm $\times$ 76 cm) prepared by the method of Travis & Pannell (1973) and the albumin-free sample was eluted with 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl. In most runs, careful pooling of α_2 M fractions near the void volume ($V_0 = 522$ ml) provided samples containing no proteins of electrophoretic mobility greater than that of α_2 M on polyacrylamide-gel electrophoresis (conditions are given below). If proteins migrating faster than α_2 M did appear, they were removed by gel filtration on a column (3.0 cm $\times$ 80 cm) of Sepharose 4B (Pharmacia) with 0.1 M- NaH_2PO_4 buffer, pH 6.5. Fractions containing haptoglobin-free α_2 M samples were identified by double diffusion against commercial rabbit antisera to human α_2 M and human haptoglobin (Hoechst Pharmaceutical Co., Kansas City, MO, U.S.A.). The proteins migrating slower than α_2 M on polyacrylamide-gel electrophoresis were removed by stepwise DEAE-cellulose chromatography on a column (3.0 cm $\times$ 30 cm) of DEAE-cellulose (Whatman DE-52). After dialysis against the initial column buffer, 0.05 M-Tris/HCl, pH 8.0, the sample was applied to the column. The column was first rinsed with 200 ml of 0.05 M-Tris/HCl, pH 8.0, containing 0.042 M-NaCl, and then with the same buffer containing 0.053 M-NaCl, which eluted the α_2 M.

Polyacrylamide-gel electrophoresis

Slab polyacrylamide electrophoresis was carried out in an E-C model 470 vertical gel apparatus (E-C Apparatus Corp., St. Petersburg, FL, U.S.A.). For this, 5% gels were prepared from a mixture of 95% acrylamide and 5% N,N' -methylenebisacrylamide (Cyanogum-41; Fisher Scientific Co., Pittsburgh, PA, U.S.A.) in Tris/EDTA/borate buffer, pH 9.2 (Raymond & Wang, 1960). The Ortec electrophoresis system (Ortec, Oak Ridge, TN, U.S.A.) was also used with discontinuous gradient gels of 4½, 6, and 8% acrylamide in Tris/EDTA/borate buffer, pH 9.2 (Zais & Roberts, 1977). The 10-times-concentrated stock Tris/EDTA/borate buffer for the two slab-gel-electrophoresis systems contained 107.5 g of Tris (Sigma), 9.02 g of EDTA (disodium salt) and 5.45 g of boric acid (Mallinckrodt) per litre of distilled deionized water. SDS/polyacrylamide-disc-gel electrophoresis was carried out in Bio-Phore SDS/polyacrylamide gels at pH 6.6, in 0.2 M-Tris/0.2 M-acetic acid/0.1% SDS buffer (Bio-Rad). Standard molecular-weight marker proteins were ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (45 000) and aldolase (158 000) obtained from Pharmacia, and bovine serum albumin treated with glutaraldehyde by the method of Payne (1973) to give a mixture of monomers and oligomers of increasing molecular weight. The molecular weights of the α_2 M subunits were determined by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn,

1969) after treatment of the purified sample with trypsin (0.1 mg/ml) and incubation at 37°C in 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.85% NaCl for 1 h. Reduction was carried out by adding to the mixture an equal volume of a solution containing 10 M-urea, 1% SDS and 14 mM-dithioerythritol and incubating at 37°C for 45 min (Harpel, 1973).

All gels were stained with Coomassie Brilliant Blue G-250 (Wilson Diagnostics, Glenwood, IL, U.S.A.) by the method of Diezel *et al.* (1972).

Binding activity

The trypsin-binding activity was assayed by the method of Ganrot (1966), by using freshly prepared solutions of 0.003 M- α -N-benzoyl-DL-arginine *p*-nitroanilide (Sigma), 0.1% soya-bean trypsin inhibitor (ICN Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.), and 0.1% bovine trypsin (EC 3.4.21.4; twice crystallized; lot no. 124C-0417; Sigma). The degree of purification was followed by estimating the specific activity of α_2 M as the ratio of the A_{410} obtained in the trypsin-binding assay to the A_{280} of the protein solution.

Immunoelectrophoresis

The micro-method of Scheidegger (1955) was used. Antisera were obtained from Behring Diagnostics, Woodbury, NY, U.S.A.

Protein concentrations

These were measured, for use in calculation of the partial specific volume and absorption coefficient, by a modification of Hunter's (1966) dry-weight technique. Triplicate 110.0 μ l samples of dialysed pure α_2 M solutions in 0.1 M- NaH_2PO_4 , pH 6.5, were transferred at 20°C by using a previously calibrated pipette (Pipetman P 200) to 12 mm $\times$ 25 mm aluminium capsules previously weighed on an electrobalance (Cahn Instrument Co., Paramount, CA, U.S.A.). The solutions were freeze-dried under vacuum at 110°C for 24 h, cooled for 10 min in a desiccator containing P_2O_5 , and weighed to the nearest microgram. Measurements were repeated to constant weight. Diffusate samples were treated similarly. The absorption coefficient was calculated from A_{280} measurements of samples of these same solutions measured with a Zeiss PMQ II spectrophotometer.

Partial specific volume

The partial specific volume, $\bar{v}$, of the purified α_2 M samples was determined by three methods. (a) The differential density technique used a Cahn electrobalance equipped with a glass plummet and water-jacketed density cell maintained at $20.00 \pm 0.01^\circ\text{C}$ (Elgert & Cammann, 1967). The $\bar{v}$ of α_2 M was determined by using 1.5 ml of a solution containing

11.57 mg of protein/ml. This solution was dialysed against two 1-litre changes of 0.10 M- NaH_2PO_4 , pH 6.5, for 24 h. The solvent density was determined by means of an 11 ml Ostwald pycnometer. (b) Sedimentation-equilibrium experiments carried out at three temperatures in 0.10 M-sodium citrate buffer, pH 6.0, in either $^2\text{H}_2\text{O}$ (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) or H_2O were used to calculate $\bar{v}$ by the density-perturbation method of Edelstein & Schachman (1967). A value of 1.0155 was used for the deuterium-exchange coefficient. (c) The partial specific volume was also calculated from the amino acid and carbohydrate composition (Cohn & Edsall, 1943).

Analytical ultracentrifugation

All experiments were performed with a Spinco model E analytical ultracentrifuge equipped with an electronic speed control, an RTIC temperature-control unit, and a schlieren interference optical system. A double-sector cell equipped with a 12 mm aluminium centrepiece was used to measure sedimentation coefficients at 56000 rev./min and 20°C. The relative viscosity of the solvent buffers was measured with a 10 ml Ostwald viscometer at 20°C. Sedimentation-equilibrium experiments were performed by the boundary-depletion method (Yphantis, 1964) at various temperatures between 6 and 20°C. Rotor speeds (7000–9000 rev./min) and protein concentrations (0.2–0.5 mg/ml) were selected by criteria outlined by Teller *et al.* (1969) to achieve true meniscus depletion, yet maintain a sufficient concentration distribution across the cell to provide optimum association conditions. Interference patterns were recorded on Kodak II-G spectroscopic plates after 24–26 h runs and measured on a two-dimensional micro-comparator (model 6C; Nikon Division of Ehrenreich Photo-Optical Industries, Garden City, NY, U.S.A.). A Polaroid filter was placed over the light-source, as suggested by Ansevin *et al.* (1970), to sharpen the fringe pattern near the base of the cell. Computations of the weight- and number-average molecular weights direct from fringe-displacement data were done with the aid of a programmable calculator (Olivetti Programma 101). The refractive increment was determined by the synthetic-boundary method (Babul & Stellwagen, 1969).

Amino acid and carbohydrate analysis

Amino acid composition was determined with a Beckman model 120B amino acid analyser after hydrolysis for 22 h at 110°C with 3 M-mercaptoethanesulphonic acid (Pierce) (Penke *et al.*, 1974) and compared with results obtained after 22, 48 and 72 h hydrolysis in constant-boiling HCl. Total neutral sugars were determined by using the phenol/ H_2SO_4 technique (Dubois *et al.*, 1956), with galactose

(Sigma) as a standard. Hexosamine was determined by the technique of Good & Bessman (1964) after hydrolysis of the glycoprotein in 4M-HCl for 4h at 100°C, with glucosamine (Calbiochem, Los Angeles, CA, U.S.A.) as a standard. Sialic acid was determined by the thiobarbituric assay method of Warren (1959), after hydrolysis of the protein in 0.05M-H₂SO₄ at 80°C for 1h, with *N*-acetylneuraminic acid (Aldrich) as a standard.

Results

Isolation and criteria of purity

Several criteria of purity were utilized to demonstrate the homogeneity and activity of the final α_2 M preparations.

Activity. The specific trypsin-binding activity of the purified α_2 M samples was 1.2–1.4 (A_{420}/A_{280}). This represents 30–35 times the specific activity of the starting plasma (0.04).

Polyacrylamide-gel electrophoresis. As shown in Fig. 1(a), a single protein band appears in the 5% slab-gel-electrophoresis experiment after the final purification step. If the purified sample is applied to a discontinuous 4½%/6%/8% polyacrylamide gel, an α_2 M double band appears (Zais & Roberts, 1977) [Fig. 1b, (1)]. The amount of the faster electrophoretic band varied from batch to batch and may represent inactive α_2 M. However, this component does not represent α_2 M–proteinase complexes, since, as shown in Fig. 1(c), no detectable 85000-dalton band occurs when the preparations were examined by SDS/polyacrylamide-gel electrophoresis after reduction of the disulphide bonds. All preparations of α_2 M isolated from plasma used in this study migrated as a single component of 185000 daltons during disc gel electrophoresis in the presence of SDS and after treatment with the reducing buffer (10M-urea/1% SDS/14mM-dithioerythritol). This finding is in agreement with the results obtained by Harpel (1973) for α_2 M isolated from plasma and indicates that no detectable interaction with proteinases has occurred during the isolation. Fig. 1(b) also shows the ability of the α_2 M preparation to bind proteinases by the

conversion of the slower-moving band into the faster-moving form on addition of the proteinase, which is discussed further below.

Immunoelectrophoresis. Immunoelectrophoresis against anti-(whole human plasma) gave a single precipitin line analogous to the band developed with anti- α_2 M serum, as shown in Fig. 2. Double diffusion in agar against anti-(immunoglobulin M) sera and anti-(human haptoglobin) sera showed no precipitin lines.

Sedimentation-velocity experiments. These were carried out on several preparations by using a concentration range of 5–10mg/ml in 0.10M-sodium citrate buffer, pH6.0, and always showed a single symmetrical peak (Fig. 3). Extrapolation of the results gave $s_{20,w}^0 = 18.7S$.

Effect of storage and temperature on trypsin-binding activity

The trypsin-binding activity of α_2 M preparations stored at 4°C was checked regularly. After 20 months

Fig. 2. Immunoelectrophoresis of human α_2 M and normal human serum

Electrophoresis was carried out for 2h at 8mA in 0.05M-sodium veronal buffer, pH8.6, followed by immunodiffusion for 16h at 4°C. Wells 1 and 3, normal human serum; well 2, α_2 M; slot A, goat anti-(whole human serum); slot B, goat anti-(human α_2 M).

Fig. 1. Various methods for evaluating the purity of α_2 M

(a) Polyacrylamide-gel electrophoresis of a purified α_2 M preparation in a 5% gel, in Tris/EDTA/borate buffer, pH9.2; (1) normal human serum, 10µl; (2) α_2 M, 63 µg of protein. (b) Polyacrylamide-gel electrophoresis of 82.5 µg of purified α_2 M in a three-layer discontinuous gradient gel (4½%, 6% and 8% acrylamide), in Tris/EDTA/borate buffer, pH9.2. (1) and (7), α_2 M alone; (2) α_2 M with 0.075 caseinolytic unit of impure plasmin (Sigma lot 276-0152) added; (3) α_2 M with 0.24 benzoyltyrosine ethyl ester international unit of α -chymotrypsin (Worthington CDI-OBK); (4) α_2 M with 600 CTA units of urokinase (Winthrop R019H0); (5) α_2 M with 6 NIH units of thrombin (Parke Davis Co. lot 903975A); (6) α_2 M with 68.6 benzoylarginine ethyl ester international units of bovine trypsin (Sigma 124C-0417). (c) SDS/polyacrylamide-disc-gel electrophoresis (4% gel) of (1) purified α_2 M and (2) α_2 M treated with reducing buffer (10M-urea/1% SDS/14mM-dithioerythritol) and incubated at 37°C for 45min. Running buffer was 0.2M-Tris/acetic acid, pH7.0, containing 0.1% SDS.

storage at this temperature, samples still retained 90% of their initial specific activity. No loss of activity was detected in samples that had been frozen (-20°C) overnight and thawed to room temperature (22°C) once. One sample which had been quickly frozen in liquid N_2 (-195°C), stored for 1 week at that temperature and quickly thawed to 37°C retained 85% of its initial activity. A 90% loss of activity, however, was observed in a sample heated to 56°C (5 min) and returned to room temperature.

Physicochemical properties of the native molecule

Absorption coefficient at 280 nm. This ($A_{1\text{cm}}^{1\%}$) is 8.93 ± 0.09 , calculated from the results of three dry-weight determinations on $\alpha_2\text{M}$ in 0.10M- NaH_2PO_4 buffer, pH 6.5.

Refractive increment. The refractive increment measured from the interference-fringe displacement of a 2.05 mg/ml solution of $\alpha_2\text{M}$ at 20°C in 0.10M-

NaH_2PO_4 , pH 6.5, is 4.38 fringes/mg per ml. This value used in the sedimentation-equilibrium experiments compares with the average refractive increment of 4.10 fringes/mg per ml reported by Babul & Stellwagen (1969) for seven non-haem proteins. This value gives 2.01×10^{-4} ml/mg at 546 nm for the specific refractive increment of $\alpha_2\text{M}$ (Schachman, 1959).

Partial specific volume. The results of the partial-specific-volume measurements for the three methods used are summarized in Table 1. The value ($\bar{v}_{20^{\circ}\text{C}} = 0.731$ ml/g) obtained from differential-density experiments agrees closely with the often quoted value ($\bar{v}_{20^{\circ}\text{C}} = 0.735$ ml/g) measured by pycnometry (Schönenberger *et al.*, 1958). The differential sedimentation-equilibrium technique of Edelstein & Schachman (1967) produced a value of $\bar{v} = 0.744 \pm 0.002$ ml/g as the average of three determinations. Although the method offers the advantage of determining $\bar{v}$ without knowledge of the initial protein concentration, k , the deuterium-exchange coefficient, was estimated as the average value for several proteins ($k = 1.0155$) (Hvidt & Nielsen, 1966). An approximate value of

Fig. 3. Sedimentation-velocity centrifugation of human $\alpha_2\text{M}$. Centrifugation was in 0.10M-sodium citrate buffer, pH 6.0, for 32 min at 56000 rev./min and 20°C , with a 4.5 mg/ml solution. Sedimentation is from left to right.

Table 1. Apparent partial specific volume of human α_2 -macroglobulin

Summary of the experimental values obtained for the partial specific volume ($\bar{v}$) of $\alpha_2\text{M}$ by various techniques and at various temperatures.

Experimental method	Apparent $\bar{v}$ (ml/g)	
Differential density	0.731	20°C
Sedimentation equilibrium	0.746	20°C
in 0.10M-sodium citrate buffer,	0.745	18.3°C
pH 6.0, in $^2\text{H}_2\text{O}$ and H_2O	0.742	15.3°C
Calculated from amino acid and carbohydrate composition	0.72	20°C

Table 2. Molecular-weight determinations of human α_2 -macroglobulin

Summary of the weight-average molecular weights of $\alpha_2\text{M}$ determined by the meniscus-depletion sedimentation-equilibrium method. The dependence of the molecular weights on $\bar{v}$ is illustrated by comparing the literature value, 0.735 (Schönenberger *et al.*, 1958) with our best experimental value, 0.731. For temperatures (t) significantly less than 20°C , $\bar{v}$ was corrected by the formula $\bar{v}_t = \bar{v}_{20^{\circ}\text{C}} - 0.00036(20^{\circ}\text{C} - t)$ (Hunter, 1966). The corrected values are indicated in the appropriate columns.

Source	Initial concentration (mg/ml)	Temperature ($^{\circ}\text{C}$)	M_w if $\bar{v} = 0.735$	M_w if $\bar{v} = 0.731$
Plasma	0.32	20.0	724.9×10^3 $\bar{v} = 0.735$	713.4×10^3 $\bar{v} = 0.731$
Serum	0.50	18.8	725.7×10^3 $\bar{v} = 0.735$	714.2×10^3 $\bar{v} = 0.731$
Plasma	0.32	15.3	734.1×10^3 $\bar{v} = 0.733$	722.0×10^3 $\bar{v} = 0.729$
Plasma	0.54	6.0	730.6×10^3 $\bar{v} = 0.730$	721.1×10^3 $\bar{v} = 0.726$
		Mean	728.8×10^3 $(\pm 4.3 \times 10^3)$	717.7×10^3 $(\pm 4.5 \times 10^3)$

0.72 ml/g for the apparent partial specific volume was calculated from the amino acid and carbohydrate composition of α_2 M. Because of the approximate nature of the calculated value, and the uncertainty of the exact value of the deuterium-exchange coefficient in the differential-sedimentation-equilibrium value, the value of $\bar{v}=0.731$ ml/g obtained by differential-density experiments is used in the molecular-weight calculations reported here.

Molecular-weight studies. Sedimentation-equilibrium experiments were performed on purified α_2 M samples isolated from both serum and plasma. Samples isolated from plasma were all studied in 0.1M-sodium citrate buffer, pH 6.0, whereas the sample isolated from serum was studied in 0.1M- NaH_2PO_4 buffer, pH 6.5. The results, summarized in Table 2, were calculated by using both the experimental and literature values for $\bar{v}$. The plots of $\log c$ versus r^2 for the experiments carried out at 20, 18.8 and 15.3°C showed no deviation from linearity over a c_0 (initial concentration) range of 0.32–0.54 mg/ml. The plot of $\log c$ versus r^2 for the experiment at 6.0°C tended to curve down slightly (Fig. 4). In Fig. 5 the weight-average, $M_w(r)$, and number-average, $M_n(r)$, molecular weights are given as functions of the protein concentration for the experiment at 6.0°C, where $M_n(r)$ was obtained from the relationship:

$$M_n(r) = \frac{c(r)}{\frac{c_m}{M_n(m)} + \frac{(1 - \bar{v}\rho)\omega^2}{2RT} \int_m^r c \, dr^2}$$

in which c_m and $M_n(m)$ are the protein concentration

Fig. 4. Sedimentation equilibrium of α_2 M. A 0.53 mg/ml solution was centrifuged at 6.0°C and 6815 rev./min in 0.10M-sodium citrate, pH 6.0. A plot of $\log c$ (cm of fringe displacement as measured on the photographic plate) versus the square of the radial distance is shown.

Fig. 5. Dependence of the molecular-weight averages on α_2 M concentration (mg/ml) calculated from the data in Fig. 4

O, Weight-average molecular weights. ●, Number-average molecular weights.

and number-average molecular weight at the meniscus (Aune & Timasheff, 1971), $c(r)$ is the concentration at radial distance r , ρ is the solvent density, ω is the angular velocity, R is the universal gas constant and T is the absolute temperature. The quotient $c_m/M_n(m)$ rather than $M_n(m)$ itself was estimated from the first experimental data points (Aune & Timasheff, 1971) and the integral was evaluated by trapezoidal integrations (Chervenka, 1969).

Amino acid and carbohydrate composition. The amino acid and carbohydrate composition of our preparations of human α_2 M is compared with the compositions reported by others in Table 3.

Interaction of α_2 M with various proteinases. The discontinuous polyacrylamide-slab-gel-electrophoresis system (Zais & Roberts, 1977) splits α_2 M into two bands. The addition of trypsin was found to convert α_2 M completely into the faster-migrating band. In Fig. 1(b), the ability of several proteinases to effect this conversion is shown. The quantity of enzyme used and their sources are indicated in the Figure legend. The enzymes were incubated with the α_2 M for 1.5 h at 37°C at pH 6.5 in 0.01M-sodium phosphate buffer. All the proteinases used, except urokinase, caused complete conversion into the band with faster mobility. The urokinase caused an increase in the amount of the faster band. Thrombin produced results analogous to those with the other proteinases, indicating that it reacted in a similar manner. The additional electrophoretic bands in gels (2) (plasmin), (4) (urokinase), and (5) (thrombin) are due to impurities in these enzyme preparations.

Dissociation experiments. As illustrated by the SDS/polyacrylamide gel in Fig. 1(c), a 185000-mol.wt. subunit results when α_2 M is reduced in the SDS/urea/dithioerythritol system. Incubation of α_2 M samples at 37°C with increasing ratios of trypsin to α_2 M (approx. 0.5, 1.0, 2.0 and 3.0 mol of trypsin/mol of α_2 M) followed by reduction in urea causes the 185000-mol.wt. subunit to be partially converted

Table 3. *Amino acid and carbohydrate composition of human α_2 -macroglobulin*

Comparison of the amino acid composition of our α_2 M preparation with other compositions reported in the literature. Content in residues per molecule and column A are the values obtained for this paper, column B is from Dunn & Spiro (1967), column C is from Bourrillon & Razafimahaleo (1972) and column D is from Heimburger *et al.* (1964). The numbers in parentheses next to column A indicate the number of tests. Tryptophan was determined by two methods, basic hydrolysis in 6M-NaOH followed by analysis on a starch column in the amino acid analyser (Oelschlegel *et al.*, 1970), and also after hydrolysis with mercaptoethanesulphonic acid for 22h (Penke *et al.*, 1974). Half-cystine was determined as cysteic acid after performic acid hydrolysis.

Component	Content (residues/ molecule of mol.wt. 718000)	Residue wt. (g/100g of protein)			
		A	B	C	D
Aspartic acid	478	7.65 (3)	7.24	7.92	7.16
Threonine	370	5.21 (3)	5.47	5.11	7.35
Serine	452	5.49 (3)	5.35	5.77	5.50
Proline	356	4.81 (3)	4.21	2.75	2.75
Glutamic acid	679	12.21 (3)	12.60	13.5	12.30
Glycine	385	3.06 (3)	2.77	3.05	2.75
Alanine	368	3.64 (3)	3.49	3.94	3.44
Valine	506	6.98 (4)	6.98	6.52	6.80
Methionine	94	1.71 (3)	1.71	1.49	1.53
Isoleucine	199	3.13 (4)	3.26	3.04	3.09
Leucine	498	7.85 (6)	8.09	7.87	7.76
Tyrosine	212	4.81 (4)	4.76	5.3	4.84
Phenylalanine	229	4.69 (4)	4.96	5.12	4.56
Lysine	356	6.35 (5)	6.47	4.82	5.34
Histidine	148	2.83 (5)	3.03	2.55	2.70
Tryptophan	34	0.87 (2)	1.71	—	1.30
Arginine	181	3.94 (5)	4.27	3.58	3.82
½-Cystine	113	1.62 (2)	1.58	2.59	1.14
Total peptide		86.86	87.95	—	84.82
Total hexose		3.97	3.08	4.5	3.6
Hexosamine		2.47	3.62	3.95	2.9
Sialic acid		0.32	1.70	1.83	1.8
Total		93.62	96.59	—	93.22

into an 85000-mol.wt. fragment (Fig. 6a). Trypsin/ α_2 M molar ratios of 2 and 3 produced additional fragments with molecular weights lower than 85000. The molar ratios are not corrected for the absolute activity of the trypsin or α_2 M samples.

Fig. 6(b) shows that none of the 85000-dalton fragment is formed when α_2 M is incubated in a 0.1M-hydroxylamine hydrochloride solution followed by reduction in the SDS/urea/dithioerythritol system. This concentration of hydroxylamine will completely destroy the trypsin-binding activity of α_2 M and will completely convert α_2 M into the faster-migrating form in the discontinuous slab polyacrylamide-gel system. The same reducing buffer was used as in Fig. 6(a). The results indicate that only a very small portion of the 185000-dalton subunit was cleaved by hydroxylamine and that no fragments smaller than 107000 daltons appeared.

Discussion

The final preparations of α_2 M were shown to be free of detectable impurities by polyacrylamide-gel

electrophoresis and immunoelectrophoresis and by sedimentation-velocity and sedimentation-equilibrium ultracentrifugation. The purified α_2 M retained a high specific trypsin-binding activity. An additional sensitive indication of purity and freedom from interaction with proteinases of the α_2 M purified from plasma is the demonstration of a single 185000-dalton band by SDS/polyacrylamide-gel electrophoresis after reduction in urea. α_2 M isolated from serum shows multiple bands by this test (Roberts *et al.*, 1974), even though it appeared homogeneous by polyacrylamide-gel electrophoresis, immunoelectrophoresis and ultracentrifugation. The preferred method of isolation in our laboratory is method 2, by using a Sepharose 4B-Blue Dextran column to remove albumin and other lower-molecular-weight proteins simultaneously in the first step.

A summary of the physical properties of α_2 M determined in this investigation is shown in Table 4, together with the values reported by other authors. The reported absorption coefficient, 8.93, compares favourably with the $A_{1\text{cm}}^{1\%}$, 277nm values of 9.07 and 9.17 reported by Dunn & Spiro (1967). They had

Fig. 6. SDS/polyacrylamide-gel electrophoresis experiments with α_2 M

The numbers on the left hand side are molecular-weight values for the indicated protein bands. (a) The effect of reduction of disulphide bonds and preincubation with various amounts of trypsin on α_2 M. Samples were incubated with the specified amount of trypsin for 1 h at 37°C. After incubation, the samples were diluted with an equal volume of 10M-urea/1% SDS with or without 14mM-dithioerythritol, depending on whether or not the disulphide bonds were to be reduced. (A) α_2 M with no trypsin or reduction; (B) α_2 M with no trypsin, but reduction of the disulphide bonds; (C) α_2 M with reduction and 0.5mol of trypsin/mol of α_2 M; (D) α_2 M with reduction and 1.0mol of trypsin/mol of α_2 M; (E) α_2 M with reduction and more than 2.0mol of trypsin/mol of α_2 M; (F) α_2 M with no reduction and more than 2.0mol of trypsin/mol of α_2 M. (The molar ratios of trypsin to α_2 M are uncorrected for inactive trypsin or α_2 M.) (b) SDS/polyacrylamide-gel electrophoresis of α_2 M after incubation in various concentrations of hydroxylamine and reduction with 10M-urea/1% SDS/14mM-dithioerythritol. Conditions were the same as in (a) except that no trypsin is present in (C)–(E). (A) bovine serum albumin standards; (B) 1:1 molar ratio of trypsin/ α_2 M; (C) aq. 0.05M- NH_2OH ; (D) aq. 0.10M- NH_2OH ; (E) aq. 0.20M- NH_2OH .

Table 4. *Physical properties of human α_2 macroglobulin*

Comparison of the literature values for various physical properties of α_2 M with the corresponding values obtained in this study.

Molecular weight of intact molecule	Sedimentation equilibrium	718 000	This paper
	Sedimentation equilibrium	725 000	Jones <i>et al.</i> (1972)
	Sedimentation equilibrium	620 000–680 000	Saunders <i>et al.</i> (1971)
	Sedimentation/diffusion	820 000	Schönenberger <i>et al.</i> (1958)
Subunit molecular weight	Sedimentation equilibrium	780 000	Frenoy <i>et al.</i> (1977)
	SDS/polyacrylamide-gel electrophoresis	185 000	This paper
	SDS/polyacrylamide-gel electrophoresis	185 000	Harpel (1973)
	Sedimentation equilibrium	176 000–226 000	Jones <i>et al.</i> (1972)
Sedimentation coefficient, $s_{20,w}^0$		18.7S	This paper
		18.1S	Jones <i>et al.</i> (1972)
		20.6S	Dunn & Spiro (1967)
		19.6S	Schönenberger <i>et al.</i> (1958)
Partial specific volume, $\bar{v}$ (ml/g)		0.731	This paper
		0.733	Dunn & Spiro (1967)
		0.735	Schönenberger <i>et al.</i> (1958)
		0.735	Schönenberger <i>et al.</i> (1958)
Absorption coefficient ($A_{1cm}^{1\%}$, 280)		8.93	This paper
		9.07	Dunn & Spiro (1967)
		6.9	Schönenberger <i>et al.</i> (1958)
		8.1	Schultze & Heremans (1966)
Refractive increment		11.1	Brown <i>et al.</i> (1954)
		2.01×10^{-4} ml/mg	This paper

determined the concentration of α_2 M by the method of Lowry *et al.* (1951). The earlier reported $A_{1cm,280}^{1\%}$ values of 6.9 (Schönenberger *et al.*, 1958) and 11.1 (Brown *et al.*, 1954) were determined with less-pure preparations of human α_2 M. The experimental molecular weight of 718 000 is based on $\bar{v} = 0.731$ ml/g. This value is identical with the molecular weight of 725 000 from sedimentation-equilibrium studies calculated by Jones *et al.* (1972) using $\bar{v} = 0.735$ ml/g. The higher value of 820 000 (Schönenberger, 1955) was calculated from sedimentation and diffusion data. The value of 780 000 reported by Frenoy *et al.* (1977) cannot be due to their lower $\bar{v}$ value as they suggest, since the molecular weight varies directly with $\bar{v}$, not inversely as they indicate. Their $\bar{v}$ value, 0.722, obtained from amino acid and carbohydrate content, is identical with the value that we obtained by the same method, suggesting close similarity in the chemical compositions of the two preparations. With proteins with molecular weights this high, the partial specific volume becomes the limiting factor in the accuracy of the ultracentrifuge technique. Application of the differential-density technique to obtain $\bar{v}$ requires accurate measurement of the deuterium-exchange coefficient (k) for the protein in question. We consider that the best value currently available for $\bar{v}$ is the one determined directly from density measurements, 0.731. The lower values found by Saunders *et al.* (1971) (620 000–680 000) may have been due to the presence of polyacrylamide in the

samples subjected to sedimentation-equilibrium centrifugation.

Although it has been reported (Jones *et al.*, 1972) that α_2 M tends to dissociate to half-molecules when solutions of this protein are frozen and thawed, we found no evidence of dissociation at 6°C, the lowest practical temperature at which we could run our ultracentrifuge. The $M_n(r)$ and $M_w(r)$ values did not change significantly with decreasing protein concentrations. The $M_n(r)$ values were constant with varying concentrations. The $M_w(r)$ values appeared to increase slightly at the lowest concentration, where the experimental values are the most uncertain. These data indicate that no concentration-dependent dissociation or association occurs under these conditions.

The amino acid composition of our α_2 M preparation agrees favourably with that found by others. The slightly higher value of proline reported here is the result of hydrolysis experiments carried out with both mercaptoethanesulphonic acid and constant-boiling HCl. Although our value for tryptophan is significantly lower than the other two reported, we determined this amino acid by direct measurement by both alkaline hydrolysis and mercaptoethanesulphonic acid hydrolysis. The values for tryptophan reported by the other authors (Table 4) were determined indirectly by spectrophotometry.

The results of the dissociation experiments with the urea/SDS/dithioerythritol reducing buffer confirmed the results of Harpel (1973) that α_2 M is made

up of four polypeptide chains each of 185 000 daltons. Jones *et al.* (1972), using the sedimentation-equilibrium technique, obtained a range of 176 000–226 000 for the subunit molecular weight after reduction with 0.01 M-*N*-acetylcystine and 1 M-urea. This preparation, however, contained 5% of heavier components. These chains are apparently cleaved near the middle by treatment with trypsin (and other proteinases) to yield the 85 000-mol.wt. fragments. Since this same cleavage did not occur on treatment with hydroxylamine hydrochloride, a susceptible ester bond does not explain how all the different proteinases appear to cleave at the same point, but presumably peptide bond(s) are being attacked. The increase in electrophoretic mobility seen on the discontinuous gradient-gel electrophoresis of the α_2 M-trypsin complex compared with that of the α_2 M reported here lends support to the conformational tightening of the molecule observed by Barrett & Starkey (1974) in electron micrographs on formation of α_2 M-trypsin complexes. If this mobility change was due to increased negative charge, then a similar electrophoretic-mobility shift would be observed in the single-concentration polyacrylamide-gel run under the same conditions. Saunders *et al.* (1971) reported up to five different electrophoretic components for α_2 M at pH 7.5. These components were found to be various enzyme-trypsin complexes as well as two forms of the native molecule. We have not been able to repeat these observations using their electrophoretic conditions and our α_2 M preparations.

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References

- Ansevin, A. T., Roark, D. E. & Yphantis, D. A. (1970) *Anal. Biochem.* **34**, 237–261
- Aune, K. C. & Timasheff, S. N. (1971) *Biochemistry* **10**, 1609–1617
- Babul, J. & Stellwagen, E. (1969) *Anal. Biochem.* **28**, 216–221
- Barrett, A. J. & Starkey, P. M. (1973) *Biochem. J.* **133**, 709–724
- Barrett, A. J. & Starkey, P. M. (1974) in *Bayer Symp. 5: Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J. & Truscheit, E., eds.), pp. 72–77, Springer-Verlag, Berlin
- Bourrillon, R. & Razafimahaleo, E. (1972) in *Glycoproteins* (Gottschalk, A., ed.), part A, vol. 5, pp. 699–716, Elsevier Publishing Co., Amsterdam
- Brown, R. K., Baker, W. H., Peterkofsky, A. & Kaufman, D. L. (1954) *J. Am. Chem. Soc.* **76**, 4244–4245
- Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, pp. 39–69, Beckman Instruments, Palo Alto
- Cohn, E. J. & Edsall, J. T. (1943) in *Proteins, Amino Acids, and Peptides* (Cohn, E. J. & Edsall, J. T., eds.), p. 372, Reinhold Publishing Co., New York
- Diezel, W., Kopperschlager, G. & Hofman, E. (1972) *Anal. Biochem.* **48**, 617–620
- Dubois, M., Gilles, K. A., Hamilton, J. L., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Dunn, J. T. & Spiro, R. G. (1967) *J. Biol. Chem.* **242**, 5549–5555
- Edelstein, S. J. & Schachman, H. K. (1967) *J. Biol. Chem.* **242**, 306–311
- Elgert, K. F. & Cämmann, K. (1967) *Z. Anal. Chem.* **226**, 193–198
- Frenoy, J.-P., Razafimahaleo, E. & Bourrillon, R. (1972) *Biochim. Biophys. Acta* **257**, 111–121
- Frenoy, J.-P., Bourrillon, R., Lippoldt, R. & Edelhoch, H. (1977) *J. Biol. Chem.* **252**, 1129–1133
- Ganrot, P. O. (1966) *Clin. Chim. Acta* **14**, 493–501
- Good, T. A. & Bessman, S. P. (1964) *Anal. Biochem.* **9**, 253–262
- Harpel, P. C. (1973) *J. Exp. Med.* **138**, 508–521
- Heimburger, N., Heide, K., Haupt, H. & Schultze, H. E. (1964) *Clin. Chim. Acta* **10**, 293–307
- Hunter, M. J. (1966) *J. Phys. Chem.* **70**, 3285–3292
- Hvidt, A. & Nielsen, S. O. (1966) *Adv. Protein Chem.* **21**, 287–386
- Jones, J. M., Creeth, J. M. & Kekwick, R. A. (1972) *Biochem. J.* **127**, 187–197
- Klein, M. & Mihaesco, C. (1973) *Biochem. Biophys. Res. Commun.* **52**, 774–778
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mehl, J. W., O'Connell, W. & DeGroot, J. (1964) *Science* **145**, 821–822
- Oelschlegel, F. J., Schroeder, J. R. & Stahmann, M. A. (1970) *Anal. Biochem.* **34**, 331–337
- Payne, J. W. (1973) *Biochem. J.* **135**, 867–873
- Penke, B., Ferenczi, R. & Kovacs, K. (1974) *Anal. Biochem.* **60**, 45–50
- Raymond, S. & Wang, Y. J. (1960) *Anal. Biochem.* **1**, 391–396
- Roberts, R. C., Riesen, W. A. & Hall, P. K. (1974) in *Bayer Symp. 5: Proteinase Inhibitors* (Fritz, H., Tschesche, H., Greene, L. J. & Truscheit, E., eds.), pp. 63–71, Springer-Verlag, Berlin
- Saunders, R., Dyce, B., Vannier, W. & Haverback, B. (1971) *J. Clin. Invest.* **50**, 2376–2383
- Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry*, p. 43, Academic Press, New York
- Scheidegger, J. J. (1955) *Int. Arch. Allergy* **7**, 103–110
- Schönenberger, M. (1955) *Z. Naturforsch. Teil B* **10**, 474–478
- Schönenberger, M., Schmidtberger, R. & Schultze, H. E. (1958) *Z. Naturforsch. Teil B* **13**, 761–772
- Schultze, H. E. & Heremans, J. F. (1966) *Molecular Biology of Human Proteins*, p. 204, Elsevier Publishing Co., Amsterdam

- Teller, D. C., Horbett, T. A., Richards, E. G. & Schachman, H. K. (1969) *Ann. N.Y. Acad. Sci.* **164**, 66-101
- Travis, J. & Pannell, R. (1973) *Clin. Chim. Acta* **49**, 49-52
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971-1975
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297-317
- Zais, D. P. & Roberts, R. C. (1977) *Clin. Chem.* **23**, 590-592