Mouse α -macroglobulin

Structure, function and a molecular model

Norman W. HUDSON,* J. Michael KEHOE† and Peter H. KOO†

*Division of Hematology/Oncology, Department of Medicine, Indiana University School of Medicine, 541 Clinical Drive, Room 379, Indianapolis, IN 46223, U.S.A., and †Department of Microbiology and Immunology, Northeastern Ohio Universities College of Medicine, Rootstown, OH 44272, U.S.A.

Mouse α -macroglobulin (M-AMG) is believed to be a functional homologue of human α_2 -macroglobulin (h- α_2 M). The subunit composition, the tryptic cleavage pattern before and after methylamine incorporation and the two-dimensional tryptic-peptide mapping, however, indicate that these two proteins are structurally distinct. M-AMG is composed of two major types of polypeptides (M_r 163000 and 35000) together with a minor polypeptide (M_r 185000), whereas h- α_2 M has only one type of polypeptide (M_r 185000). After incorporation of methylamine, there is no change in the normal tryptic-cleavage pattern of M-AMG; however, tryptic cleavage of h- α_2 M is severely retarded [Hudson & Koo (1982) *Biochim. Biophys. Acta* **704**, 290–303]. The *N*-terminal sequence of the 163000- M_r polypeptide of M-AMG shows sequence homology with the *N*-terminal sequence of h- α_2 M. The amino acid compositions of M-AMG and its two major polypeptide chains are compared. Thermal fragmentation studies show that the 163000- M_r polypeptide is broken down into 125000- M_r and 29000- M_r fragments. Trypsin-binding studies show that M-AMG can bind two molecules of trypsin/molecule. Inactivations of the trypsin-binding property of M-AMG and h- α_2 M with methylamine show similar kinetics of inhibition at 4 °C. A structural model of M-AMG is proposed, based on accumulated data.

INTRODUCTION

α_2 -Macroglobulin (α_2 M) is a major proteinase inhibitor in humans and other animals. The concentration of this protein in serum varies from 2 to 4 mg/ml, depending on age, sex and physical state of the individual. α_2 M has also been found in semen, colostrum, lymph and other extravascular fluids. The absolute physiological function of α_2 M is still unknown, but as yet no human has been reported to lack this protein. It has been shown that lowered or elevated concentrations of α_2 M are associated with certain disease states in humans (Heimbürger, 1975; James, 1980). The primary structure of h- α_2 M has been reported (Sottrup-Jensen *et al.*, 1983; Jensen & Sottrup-Jensen, 1986).

M-AMG was first purified by density centrifugation (Greene *et al.*, 1971). It was shown to be antigenically distinct from h- α_2 M but exhibited a similar trypsin-protein esterase activity. Several reports suggest that protein fractions from serum containing M-AMG or h- α_2 M convey a radioprotective effect on mice (Hanna *et al.*, 1967; Nettesheim *et al.*, 1968; Graham, 1975; Olinescu *et al.*, 1976, 1980). One study has shown that both M-AMG and h- α_2 M inhibit mitogen-stimulated mouse lymphocyte and mouse tumour growth *in vitro* (Koo, 1982, 1983).

M-AMG has been purified to homogeneity and its subunit structure elucidated (Hudson & Koo, 1982). In contrast with h- α_2 M, which contains only one type of polypeptide chain (M_r 185000) (Swenson & Howard, 1979a), the mouse protein contains two major (M_r 163000 and 35000) and one minor (M_r 185000) polypeptide chains. Upon digestion with trypsin M-AMG is

cleaved into multiple fragments (M_r 86000, 63000, 61000 and 33000), whereas h- α_2 M is cleaved into 85000- M_r fragments (Harpel, 1973). Two-dimensional tryptic-peptide map analysis of M-AMG polypeptide chains showed that the 185000- M_r polypeptide was a composite, or near-composite, of the 163000- M_r and 35000- M_r polypeptides. This was the first indication that the 35000- M_r polypeptide was neither a contaminant nor a carried protein. We now describe additional structural and functional characteristics of M-AMG and propose a structural model that is based on our accumulated data.

EXPERIMENTAL

Animals

C3HeB/FeJ mice were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A.

Purification of the M-AMG 163000- M_r and 35000- M_r polypeptides

Homogeneous M-AMG and h- α_2 M were prepared as described previously (Hudson & Koo, 1982). The purified 163000- M_r and 35000- M_r polypeptides were obtained by preparative electrophoresis in 5% polyacrylamide gels in the presence of SDS and urea as described below. Purified M-AMG (3–7 mg in 0.5–1.5 ml) was applied to the top of 20 ml (1.3 cm $\times$ 15.5 cm) preparative gels (Gelman apparatus) and electrophoresed under constant current (20 mA/tube). After electrophoresis the protein bands were located by staining with ethidium bromide (1 μ g/ml) by the method of Vincent & Scherrer (1979).

Abbreviations used: M-AMG, mouse α -macroglobulin; h- α_2 M, human α_2 -macroglobulin.

* To whom correspondence should be addressed.

The protein bands were removed from the gel and immediately frozen (-80°C). The proteins were extracted from the gel by using a 15 ml Wheaton homogenizer containing 50 vol. of protein extraction buffer (0.4 M-urea, 0.05% SDS and 3.2 mM-dithiothreitol in 0.16% sodium citrate/150 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0). The extracted protein was concentrated by vacuum dialysis and exhaustively dialysed into 0.1 M-propionic acid followed by dialysis into deionized water before being freeze-dried.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

Discontinuous polyacrylamide-gel electrophoresis was performed by the method of Davis (1964). Discontinuous SDS/urea/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) with the following modifications. Resolving and separating gels contained 0.5 M-urea. Samples were dissolved in an equal volume of urea buffer (8 M-urea and 1.0% SDS in 10 mM-sodium phosphate buffer, pH 7.0) and maintained at 37°C for 30 min before electrophoresis. Gels were stained and destained as described by Weber & Osborn (1969).

Amino acid composition analysis

Ampoules (1.0 ml; Wheaton Scientific, Millville, NJ, U.S.A.) containing 0.2–0.5 mg of M-AMG or h- α_2 M with 200 μl of 4 M-methanesulphonic acid containing 2% (v/v) 3-(2-aminoethyl)indole (Pierce Chemical Co., Rockford, IL, U.S.A.) were sealed and the contents hydrolysed *in vacuo* [4 Pa (0.03 Torr)] by the method of Simpson *et al.* (1976). The sealed tubes were maintained at 115°C for 24, 48 or 72 h. The material was partially neutralized with 180 μl of 4 M-NaOH and adjusted to pH 2.2 with sodium citrate sample-dilution buffer (Pierce Chemical Co.). Hydrolysis of 163000- M_r and 35000- M_r polypeptides with 5.7 M-HCl was as described above except that hydrolysis was for 18 h at 106°C . The N_2 -dried residues were resuspended in sample-dilution buffer, pH 2.2. The hydrolysate was analysed on a Durrum 500 analyser with a DC $\times$ 10-10 resin column (0.175 cm $\times$ 48 cm).

N-Terminal sequence analysis

The N-terminal sequence of M-AMG was determined by automated Edman degradation in a Beckman 890C sequencer equipped with a cold trap (Kaplan *et al.*, 1977). The first cycle of the DMAA programme no. 102974 was used as a wash cycle (no phenyl isothiocyanate). Fractions were collected in butyl chloride and evaporated to dryness under N_2 in a 50°C water bath. The residues were manually converted into amino acid phenylthiohydantoin derivatives by the addition of 1.0 M-HCl followed by incubation at 80°C for 10 min. The converted residues were extracted twice with ethyl acetate and dried under N_2 . The aqueous residues were also dried under N_2 . The amino acid phenylthiohydantoin derivatives were dissolved in 100 μl of h.p.l.c.-grade methanol and analysed by h.p.l.c. in a Zorbax C-8 column (0.46 cm $\times$ 25.0 cm). The amino acid phenylthiohydantoin derivatives (10 μl) were separated by using a 10 mM-sodium acetate (pH 5.0)/methanol buffer system. The

methanolic amino acid phenylthiohydantoin derivatives were converted back into free amino acids by the method of Mendez & Lai (1975), with modification. The methanolic amino acid phenylthiohydantoin derivatives (90 μl) were dried in hydrolysis ampoules under N_2 . Portions (200 μl) of 5.7 M-HCl containing 1% TiCl_3 were added to the dry phenylthiohydantoin residues. The ampoules were sealed under vacuum (< 4 Pa) and heated at 150°C for 4 h. Under these conditions threonine is converted into α -aminobutyric acid. Residues were analysed on a Durrum 500 amino acid analyser after dilution in sodium citrate sample-dilution buffer.

Saturation of α -macroglobulins with trypsin

M-AMG (23.5 μg) or h- α_2 M (26.2 μg), which could incorporate 3.9 or 3.2 mol of methylamine/mol of macroglobulin respectively, was incubated in the presence of increasing amounts of trypsin [*N*-tosyl-L-phenylalanyl-chloromethane ('TPCK')-treated; Worthington Biochemical Corp., Freehold, NJ, U.S.A.] (0.082–3.0 μg in 1 mM-HCl) in a total reaction volume of 300 μl (containing 33 mM- CaCl_2 , 17 mM-NaCl and 83 mM-Tris/HCl buffer, pH 8.0) for 5 min at room temperature. At the end of each incubation period 100 μl of soya-bean trypsin inhibitor (0.1 mg/ml in water) was added and the mixture was allowed to incubate for an additional 5 min at room temperature. Next 500 μl of *N*-tosyl-L-arginine methyl ester (0.63 mg/ml in water) was added, and the change in absorbance at 247 nm was recorded. (The greater the esterolytic activity the greater the rate of change in absorbance.) The positive control contained trypsin (3.30 μg), 500 μl of tosylarginine methyl ester and the salts and buffering elements at the concentrations described above. The negative control contained trypsin (3.30 μg), soya-bean trypsin inhibitor (10.0 μg), 500 μl of tosylarginine methyl ester and the salts and buffering elements at the concentrations described above. The concentration of trypsin was determined in accordance with the Worthington Enzyme Manual (1977) by using a value of $A_{280}^{1\%} = 14.3$ at pH 8.0. Generally, we found only 85% of the activity reported on the container by using the assay described by Worthington. We assumed that the loss of activity was a result of irreversible denaturation of the enzyme preparation, as reported previously (Bender *et al.*, 1966). Trypsin solution (1.0 mg/ml in 1 mM-HCl) was divided into small portions and stored at -80°C until used.

Methylamine inactivation of the trypsin-binding activity of M-AMG

M-AMG (0.270 mg/ml) or h- α_2 M (0.32 mg/ml) was incubated for various periods of time on ice or at 4°C in the presence of 25 mM-methylamine in 50 mM- CaCl_2 /25 mM-NaCl/125 mM-Tris/HCl buffer, pH 8.0. After the allotted period, 200 μl of incubation solution was removed and further incubated in the presence of 100 μl of trypsin (25 μg /ml in 1.0 mM-HCl) for 5 min at room temperature. After 5 min 100 μl of soya-bean trypsin inhibitor (0.1 mg/ml of water) was added. The incubation mixture was assayed 5 min later in the presence of 500 μl of *N*-tosyl-L-arginine methyl ester (0.63 mg/ml of water) at 247 nm. Both positive and negative controls were run at each time point in the presence of methylamine as described above in order to monitor the specific activity of trypsin. The mouse and human proteins were able to

react with 3.9 and 3.2 mol of methylamine/mol of macroglobulin respectively.

Covalent labelling of α -macroglobulins with [^{14}C]methylamine

M-AMG (1.35 mg, 1.75 nmol) and h- α_2 M (1.60 mg, 2.08 nmol) in Tris I buffer (50 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0) were incubated in the presence of 20 mM-[^{14}C]methylamine (Amersham Corp., Arlington Heights, IL, U.S.A.) at 4 °C in a total volume of 600 μl . After 24 h of incubation the materials were desalted on a Sephadex G-25 (superfine grade) column (1.5 cm $\times$ 30.0 cm). Under these conditions M-AMG and h- α_2 M reacted with 3.9 and 3.2 mol respectively of methylamine/mol of macroglobulin.

Incorporation of [^{14}C]methylamine into α -macroglobulins

[^{14}C]Methylamine-treated M-AMG or h- α_2 M was incubated at room temperature for up to 76 h in the presence of 200 mM unlabelled methylamine in a total volume of 700 μl of 50 mM-Tris/HCl buffer, pH 8.0. After the allotted period, 100 μl of incubation solution was mixed with 1.0 ml of DEAE-Sephadex A-25 (pre-swollen in 50 mM-Tris/HCl buffer, pH 8.0), followed by the addition of 5.0 ml of 50 mM-Tris/HCl buffer, pH 8.0. The mixture was left to stand for 5 min before it was filtered through a glass-microfibre disc (2.4 cm Whatman GF/A). The Sephadex-bound protein was washed with 200 ml of 50 mM-Tris/HCl buffer, pH 8.0, and then air-dried by an aspirator. The dried material was placed into a 20.0 ml scintillation vial, and 1.0 ml of 0.5 M-NaCl was added to release bound protein (Swenson & Howard, 1979b). Aquasol-2 (10 ml) was added to the gel slurry in order to quantify the amount of [^{14}C]methylamine bound by scintillation counting.

Two-dimensional t.l.c. analysis of [^{14}C]methylamine- and ^{125}I -labelled tryptic peptides

Radioiodination of peptides and two-dimensional t.l.c. were completed in accordance with Hudson & Koo (1982). [^{14}C]Methylamine-labelled M-AMG and h- α_2 M were carboxymethylated and then freeze-dried. The protein was digested with 0.5 ml of trypsin (*N*-tosyl-L-phenylalanylchloromethane-treated; 50 $\mu\text{g}/\text{ml}$ of 50 mM- NH_4HCO_3 , pH 8.0) for 8 h and then freeze-dried. The digested protein was resuspended in electrophoresis buffer and subjected to two-dimensional t.l.c. Samples (2–3 μl) containing 1200 c.p.m./ μl of electrophoresis buffer were applied to the pre-equilibrated t.l.c. plate.

Heat-induced fragmentation studies

M-AMG (0.60 mg/ml) and h- α_2 M (0.55 mg/ml) were incubated in capped containers for various periods of time (0–180 min) and at different temperatures (4, 37 and 98 °C) in 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0, containing 2.0% (w/v) SDS and 14 mM-dithiothreitol. At the end of the incubation period a portion was removed and added to an equal volume of 8 M-urea/1% SDS/5% (v/v) 2-mercaptoethanol. Protein (5 μg) from individual samples was analysed by 10%-polyacrylamide slab gels in the presence of SDS/urea.

RESULTS

Interactions of trypsin with M-AMG

It has been shown previously that both M-AMG and

Fig. 1. Binding-site titration of M-AMG and h- α_2 M

M-AMG or h- α_2 M (23.5 μg in 100 μl of Tris I buffer, pH 8.0) was added to 100 μl of CaCl_2 /Tris buffer, pH 8.0. To this was added 100 μl of increasing concentrations of trypsin (0.082–3.0 μg in 1 mM-HCl) with incubations at room temperature for 5 min. This was followed by the addition of 100 μl of soya-bean trypsin inhibitor (0.1 mg/ml of water) and incubated at room temperature for an additional 5 min. The amount of trypsin protected from soya-bean trypsin inhibitor by α -macroglobulin was assayed upon the addition of 500 μl of *N*-tosyl-L-arginine methyl ester by spectrophotometrically detecting the rate of increase in absorbance at 247 nm. The result of each assay determined a data point on the plot. The percentage of maximal activity is defined as the ratio of the esterase activity for a given trypsin/ α -macroglobulin ratio divided by the highest esterase activity found for trypsin- α -macroglobulin in this study. Symbols: ●, M-AMG; ■, h- α_2 M. The inset indicates several independent binding-site titration studies of M-AMG. Each symbol represents an independent study. The line has been drawn to indicate the maximal binding of 2 mol of trypsin/mol of M-AMG.

h- α_2 M can be purified to homogeneity and that they differ in their subunit structures (Hudson & Koo, 1982). More recently Saito & Sinohara (1985) have suggested a 1:1 molar binding ratio for trypsin-M-AMG. These authors did not fully explain the binding ratio discrepancy between trypsin-M-AMG (1:1) and trypsin-h- α_2 M (2:1).

To address this discrepancy we have investigated the molar binding ratio for trypsin-M-AMG by several active-site titration experiments. The results of one of these studies show that M-AMG and h- α_2 M incorporated 3.9 and 3.2 mol respectively of methylamine/mol of α -macroglobulin (Fig. 1). The percentage of maximal activity appears linear until the trypsin/M-AMG molar

Fig. 2. Inactivation of the trypsin-binding property of M-AMG and h- α_2 M by methylamine

M-AMG (0.55 mg/ml of Tris I buffer, pH 8.0) or h- α_2 M (0.65 mg/ml of Tris I buffer, pH 8.0) was incubated with an equal volume of 50 mM-methylamine (in CaCl₂/Tris buffer, pH 8.0) at 4 °C for various lengths of time. At the appropriate times 200 μ l portions of the treated protein solutions were removed and assayed for the ability to protect trypsin from soya-bean trypsin inhibitor as described in Fig. 1 and in the Experimental section. Symbols: ●, M-AMG; □, h- α_2 M. The best-fit curve for the data has been drawn.

ratio approaches 1.6:1. Beyond this point the percentage of maximal activity begins to decline and reaches a plateau around a 2.0 trypsin/M-AMG molar ratio of 2.0:1. For irreversible binding the data in Fig. 1 should reflect two straight-line segments that intersect at the saturation value for the complex, which in this case is 2.0:1. Thus the results in Fig. 1 indicate that M-AMG, like h- α_2 M, possesses two titratable trypsin-binding sites per molecule, and these sites appear to behave independently. Results from several titration studies with M-AMG of different apparent trypsin-binding activities are shown in Fig. 1 inset. This inset shows the range of trypsin-binding activities for M-AMG, obtained from five separate preparations. In general, freshly purified M-AMG or h- α_2 M had the greatest inhibitory activity towards trypsin.

Effect of methylamine on the ability of M-AMG to protect trypsin from inhibition by soya-bean trypsin inhibitor

Methylamine was used to monitor the time-dependent inactivation of M-AMG in order to determine more

specifically the trypsin-binding similarity of M-AMG and h- α_2 M. Fig. 2 shows the results of one of these studies on M-AMG and h- α_2 M with high trypsin-binding capacity. The logarithm of the esterolytic activity of trypsin on *N*-tosyl-L-arginine methyl ester is plotted versus time. The data indicate that log(activity) is a linear function of time. The slope and ordinate intercept of the 'linear-regression' function for M-AMG are -0.08 and 1.66 respectively. The kinetics of inhibition for h- α_2 M are nearly identical, with a slope and ordinate intercept of -0.08 and 1.63 respectively. This corresponds to a half-life nearly equal to 4.4 h in both cases. This reflects the ability of M-AMG to protect trypsin from soya-bean trypsin inhibitor at 4 °C, but does not measure the incorporation of methylamine into M-AMG, which has been shown to take place at a higher rate (Howard *et al.*, 1983). The results suggest that methylamine affects M-AMG in a manner similar to that found for h- α_2 M.

Active-site labelling of M-AMG with [¹⁴C]methylamine

To address the effect of methylamine on the covalent structure of M-AMG at the molecular level, [¹⁴C]-methylamine was used to label M-AMG specifically. The exchange studies described in the Experimental section were carried out to assess the degree of non-specific labelling. Little or no radioactive label was lost from desalted M-AMG. These results suggest that M-AMG has covalently bound the radioactive label. Similar results were found for [¹⁴C]methylamine-treated h- α_2 M. The degree of labelling with [¹⁴C]methylamine varied from preparation to preparation, and was 1.3–3.9 mol of methylamine/mol of M-AMG (from the results of four preparations) and 2.1–3.2 mol of methylamine/mol of h- α_2 M (two preparations).

In order to investigate structural similarities between methylamine-sensitive sites of M-AMG and h- α_2 M, two-dimensional t.l.c. tryptic-peptide map analyses were performed on [¹⁴C]methylamine-labelled and carboxymethylated M-AMG and h- α_2 M. The results indicate that trypsin-digested M-AMG produces one major labelled peptide, as expected (results not shown), which is consistent with a similar study on h- α_2 M (Swenson & Howard, 1979b).

Two-dimensional peptide map analysis of M-AMG and h- α_2 M

To ascertain the gross primary structural similarities between M-AMG and h- α_2 M, component bands representing half-molecules (*M_r* 370 000) of M-AMG and h- α_2 M were excised from SDS/polyacrylamide disc gels and subjected to iodination and tryptic digestion. Two-dimensional t.l.c. was performed on these proteins (results not shown). Both proteins contain many tryptic peptides, but little overall similarity exists between the two peptide map patterns. This reflects the overall differences in primary structures and carbohydrate content.

Thermally induced fragmentation of M-AMG

The reaction site responsible for inactivation by methylamine is an activated thioester in h- α_2 M (Howard *et al.*, 1980; Howard, 1981; Sottrup-Jensen *et al.*, 1980, 1981). This activated thioester in h- α_2 M may also participate in the fragmentation of the covalent monomeric structure under thermally denaturing conditions. M-AMG was subjected to thermally denaturing

Fig. 3. Reducing SDS/urea/polyacrylamide-gel electrophoresis of M-AMG after heat treatment

M-AMG (0.60 mg/ml) and h- α_2 M (0.55 mg/ml) in 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0, containing 2% SDS and 14 mM-dithiothreitol were incubated in individual capped containers for various periods of time and at different temperatures. At the end of the incubation period an equal volume of 8 M-urea/1% SDS/5% 2-mercaptoethanol was added to the sample. Protein (5 μ g) from individual samples was applied to the top of an SDS/urea/10%-polyacrylamide slab gel and electrophoresed under constant current. Lanes a, c, e, g, i and k are M-AMG; lanes b, d, f, h, j and l are h- α_2 M. The standards are myosin (M_r 200 000), albumin (M_r 68 000), ovalbumin (M_r 43 000) and chymotrypsinogen (M_r 23 500).

conditions in order to determine if it has fragmentation properties similar to those of h- α_2 M. Fig. 3 shows the results of a representative study. M-AMG and h- α_2 M were incubated in parallel in individually capped containers at the indicated times and temperatures. As the temperature and duration of incubation increases, so does the fragmentation of these large proteins. The major M-AMG polypeptide (M_r 163 000) is converted, in a time-dependent fashion, into 125 000- M_r and 29 000- M_r fragments. The 125 000- M_r fragment further breaks down into 62 000- M_r fragments. This was corroborated by two-dimensional t.l.c. of 125 I-labelled tryptic fragments. There were no clear peptide map homologies between the M-AMG 35 000- M_r polypeptide and the 62 000- M_r fragment. h- α_2 M is found to be thermally fragmented, under reducing conditions, into 125 000- M_r and 62 000- M_r fragments (Harpel *et al.*, 1979). Incubation of both M-AMG and h- α_2 M at temperatures lower than 98 °C for similar periods does not generate as many cleavage products as those found at higher temperatures. Similar fragments are generated after incubation of M-AMG in the presence of SDS and dithiothreitol at 4 °C for 2 weeks. This suggests that both M-AMG and h- α_2 M have heat-susceptible covalent bonds. Specific fragmentation patterns suggest that the heat-susceptible bonds may be similarly located in the two proteins.

Fig. 4. Yield of the first 12 amino acid phenylthiohydantoin derivatives obtained from the M-AMG 163 000- M_r polypeptide

After quantification by amino acid analysis, 2.86 mg (17.6 nmol, based on M_r 163 000) of the 163 000- M_r fragment was applied to the sequencer with 98% initial coupling. The yield of the phenylthiohydantoin derivatives was determined directly by h.p.l.c. analysis or by amino acid analysis of the regenerated amino acids, as indicated in the Experimental section. A repetitive yield of 97% was determined by linear regression in accordance with Smithies *et al.* (1971).

Table 1. Comparative amino acid compositions of M-AMG, h- α_2 M and the M-AMG polypeptide components

Purified M-AMG or h- α_2 M (0.2–0.5 mg) was hydrolysed with 200 μ l of 4 M-methanesulphonic acid in 1.0 ml ampoules under vacuum (4 Pa) at 115 °C for 24, 48 or 72 h. The hydrolysate was cooled, partially neutralized with 180 μ l of 4 M-NaOH, and adjusted to pH 2.2 with sample-dilution buffer. Purified polypeptides (M_r 163 000 and 35 000) were hydrolysed in 5.7 M-HCl at 106 °C under vacuum. The N₂-dried residue was resuspended in sample-dilution buffer, pH 2.2, before analysis as described in the Experimental section. The carbohydrate content was not determined.

Amino acid residue	Composition (mol/100 mol of residues)				
	M-AMG	h- α_2 M	h- α_2 M§	163 000- M_r polypeptide	35 000- M_r polypeptide
Asp	9.56	9.64	7.95	8.9	9.8
Thr*	6.28	6.56	6.75	6.2	6.2
Ser*	7.97	7.71	8.35	8.8	13.2
Glu	11.54	11.86	12.25	13.0	13.1
Pro	4.87	5.01	5.25	6.2	3.9
Gly	5.90	6.27	6.20	6.6	11.7
Ala	5.62	5.79	6.35	7.2	6.5
Val	8.34	9.26	9.45	8.6	6.0
Met	1.59	1.64	1.72	1.8	0.0
Ile	5.44	3.91	3.93	4.7	3.8
Leu	9.65	9.64	9.25	9.8	11.0
Tyr	3.19	3.38	3.86	3.3	2.6
Phe	4.22	4.24	4.27	4.1	3.4
His	4.87	5.01	2.69	4.6	1.8
Lys	5.81	5.50	6.14	6.1	5.2
Arg	3.00	2.70	3.17	1.7	2.8
Cys†	1.22	1.16	1.65		
Trp‡	1.03	0.87	0.76		

* Values were extrapolated to time zero of hydrolysis for M-AMG and h- α_2 M.

† Determined as cysteic acid for M-AMG and h- α_2 M.

‡ Determined as 24 h hydrolysis for M-AMG and h- α_2 M.

§ Calculated from Sottrup-Jensen *et al.* (1983).

Amino acid composition of native M-AMG and the 163 000- M_r and 35 000- M_r polypeptides

Parallel amino acid compositional analyses were performed in order to compare M-AMG with h- α_2 M further (Table 1). The compositions of the two proteins are similar, but this similarity may be reflective of their sizes. The amino acid compositions of the 163 000- M_r and 35 000- M_r polypeptides are noticeably different.

N-Terminal sequence analysis of M-AMG

Quantitative automated Edman degradation of the N-terminal residues of the SDS/urea/polyacrylamide-gel-electrophoresis-purified 163 000- M_r polypeptide was undertaken in order to search for subunit similarities in M-AMG and to search for primary structural similarities between M-AMG and h- α_2 M. The M-AMG fraction used in this study was prepared as described in the Experimental section. Fig. 4 indicates the results of the N-terminal sequence analysis and the yields of the first 12 residues of the M-AMG polypeptide. Below is a comparison of this sequence with that found for h- α_2 M (Swenson & Howard, 1979a):

Residue	0	1	2	3	4	5	6	7	8	9	10	11	12
M-AMG		Ala	Thr	Ala	Lys	Pro	Gln	Tyr	Val	Val	Leu	Val	Pro
h- α_2 M	Ser	Val	Ser	Gly					Met				Pro

Particularly noticeable is a one-residue shift in the sequence. There is 90 % homology for residues 4–12 and an overall homology of 67 %. All amino acid changes could represent a one-base change in the coding sequence.

Taken together, the sequence data indicate: (1) primary structural homogeneity among M-AMG 163 000- M_r polypeptides; (2) significant structural similarity between the M-AMG polypeptide (M_r 163 000) and the h- α_2 M polypeptide (M_r 185 000) N-terminal segments that allow the alignment of the major subunit polypeptides of M-AMG.

DISCUSSION

This study was done to compare M-AMG and h- α_2 M biochemically and functionally. The similarities between M-AMG and h- α_2 M include native molecular size, number of trypsin molecules bound/molecule, number of methylamine reactive sites/molecule, amino acid composition and N-terminal sequence homology. The differences include number and size of subunit polypeptides, susceptibility to tryptic cleavage, two-dimensional t.l.c. ¹²⁵I-labelled peptide patterns and heat-induced fragmentation patterns. These similarities and differences are described below, and a schematic model based on our accumulated studies of M-AMG is

proposed. As illustrated in Fig. 5, the two proteins share similar native molecular sizes and 'half-molecule' sizes (M_r 370 000) under non-denaturing conditions; however, their subunit structures are strikingly different under

Fig. 5. Proposed model for the structure of M-AMG

This model is described in the Discussion section. (a) Representation of the organization of polypeptide chains within M-AMG as described in the text, along with their relative M_r values. The putative proteolytic event generates the 163 000- M_r and 35 000- M_r polypeptides from the 185 000- M_r polypeptide. (b) Proposed location of tryptic cleavage. (c) Proposed location of the heat-induced fragmentation site and methylamine-binding site.

denaturing and reducing conditions. Whereas h- α_2 M is broken down into one type of polypeptide chain (M_r 185 000), M-AMG is broken down into two major (M_r 163 000 and 35 000) and one minor (M_r 185 000) polypeptide chains. It was noted in 14 separate M-AMG purifications that the minor polypeptide chain was always present in the same concentration relative to the two major polypeptides. We have previously shown by two-dimensional t.l.c. peptide map analysis that the minor polypeptide is a composite of the two major polypeptides and that the multiple polypeptides found in M-AMG are not the result of purification (Hudson & Koo, 1982). In addition, *N*-terminal sequence analysis of intact M-AMG shows two *N*-termini at equal molar ratios (alanine and aspartic acid; N. W. Hudson, J. M. Kehoe & P. H. Koo, unpublished work). Thus the M-AMG 185 000- M_r polypeptide does not spontaneously break down during or after protein isolation. The nature or the significance of the M-AMG 185 000- M_r polypeptide is not understood, but at least two possibilities exist. The large polypeptide could be an isoprotein found at lower concentrations (6–10%) than the two major polypeptides. Alternatively, the large polypeptide subunit may represent an unmodified subunit that has escaped processing (i.e. requiring some proteolytic event, as shown in Fig. 5a) possibly necessary for some physiological function.

Significant differences are also seen in the fragments produced by limited trypsin digestion (as illustrated in Fig. 5b). Trypsin cleaves h- α_2 M into similar-sized components (M_r 85 000), whereas trypsin cleaves M-AMG into a variety of components under conditions where the trypsin/M-AMG molar ratio is less than 2:1 (Hudson and Koo, 1982). The 163 000- M_r polypeptide is cleaved into 86 000- M_r and 63 000- M_r fragments. The 86 000- M_r fragment is then cleaved into 61 000- M_r and 33 000- M_r fragments; however, we cannot document where this cleavage occurs in Fig. 5(b). The 35 000- M_r polypeptides are resistant to trypsin under these conditions. A minimum number of disulphide bonds have been placed in Fig. 5(b) to indicate that trypsin-treated M-AMG, under non-reducing conditions, appears as a single band on electrophoresis in SDS/urea/polyacrylamide gels. The complete disulphide bridge assignment for h- α_2 M has been reported (Jensen & Sottrup-Jensen, 1986).

Tryptic cleavage is also different for the mouse and human proteins after incubation with methylamine. There seems to be no effect of methylamine on the tryptic-cleavage pattern of M-AMG, whereas methylamine inactivation greatly retards the ability of trypsin to cleave h- α_2 M (Hudson & Koo, 1982). This was one of the most striking differences between the two proteins. According to Howard *et al.* (1983), once h- α_2 M becomes

inactivated by methylamine the molecule is no longer cleaved by trypsin in the usual manner. We have previously shown that M-AMG reacted with 3.9 mol of [^{14}C]methylamine/mol and h- $\alpha_2\text{M}$ reacted with 3.2 mol of [^{14}C]methylamine/mol; the modified proteins were cleaved differently by trypsin. Under these conditions little h- $\alpha_2\text{M}$ was cleaved, whereas M-AMG was completely cleaved in normal fashion by saturating amounts of trypsin. This finding might reflect some conformational difference at the trypsin-binding and/or methylamine-binding sites of these two proteins. From labelling studies and two-dimensional t.l.c. maps it does appear that both M-AMG and h- $\alpha_2\text{M}$ have four methylamine-binding sites per molecule.

h- $\alpha_2\text{M}$ (M_r 185000) is thermally fragmented into 125000- M_r and 62000- M_r fragments whereas M-AMG (M_r 163000) is fragmented into 125000- M_r and 29000- M_r fragments (see Fig. 3). After prolonged heat treatment other fragments are also apparent for both h- $\alpha_2\text{M}$ and M-AMG. This result was not surprising, since Harpel *et al.* (1979) had originally shown these multiple heat-induced fragments in h- $\alpha_2\text{M}$. Of the multiple fragments produced for h- $\alpha_2\text{M}$, only the 125000- M_r and 62000- M_r fragments are thought to result directly from the methylamine-sensitive site. Harpel *et al.* (1979) did not address a mechanism for the generation of these additional fragments; however, Frenoy & Bourrillon (1980) have suggested that the additional fragments produced are the result of trapped proteinases that are active under conditions similar to the ones used in our study. Under identical conditions, M-AMG is also found to be thermally fragmented into multiple fragments, with the two major fragments having M_r 125000 and 29000. Two-dimensional t.l.c. tryptic-peptide mapping studies of M-AMG have shown that the 125000- M_r and 29000- M_r fragments do not overlap, and that a composite map of the 125000- M_r and 29000- M_r fragments strongly resemble the peptide map of 163000- M_r polypeptide (not shown). We believe that these two major fragments (M_r 125000 and 29000) are generated from the methylamine-sensitive site in the 163000- M_r polypeptide. The other fragments seen were probably generated by a mechanism similar to that which acted on h- $\alpha_2\text{M}$.

N-Terminal sequence and amino acid compositional analyses also show similarities between these two proteins, but the two-dimensional t.l.c. peptide map analysis of the ^{125}I -labelled 370000- M_r 'half-molecules' suggests gross differences in their molecular structure. The similarity of the 163000- M_r polypeptide *N*-terminal sequence with the human sequence allows us to place this polypeptide at the *N*-terminal position and the 35000- M_r polypeptide at the *C*-terminal position (Fig. 5), consistent with the work of Starkey (1983).

The 185000- M_r polypeptide of M-AMG can be covalently labelled with [^{14}C]methylamine (Hudson & Koo, 1982), but it was not known if this polypeptide would break down after heat treatment into fragments similar in size to those found for the h- $\alpha_2\text{M}$ 185000- M_r polypeptide. By using two-dimensional t.l.c. tryptic-peptide maps we studied the relationships among the M-AMG 185000- M_r polypeptide and the heat-induced 125000- M_r and 62000- M_r fragments. On the basis of our previous experiences we expected the M-AMG 62000- M_r fragment (Fig. 3) to contain tryptic peptides different from those found in the M-AMG 125000- M_r fragment. Surprisingly, the 125000- M_r fragment contained the

tryptic peptides found in the 62000- M_r fragment (results not shown). Thus the M-AMG 62000- M_r fragment must have been generated from the 125000- M_r fragment, possibly by the mechanism suggested by Frenoy & Bourrillon (1980). Since the 185000- M_r polypeptide represents 6–10% of the total M-AMG and is related to the 163000- M_r polypeptide, it was expected that a fragment somewhat larger or smaller than M_r 62000 might have been generated from the 185000- M_r polypeptide. Since a similar-sized fragment that could be investigated by two-dimensional t.l.c. was not found, we propose the following postulates: (1) the M-AMG 185000- M_r polypeptide fragment may not be as susceptible to heat-induced cleavage as are the M-AMG 163000- M_r and h- $\alpha_2\text{M}$ 185000- M_r polypeptides; (2) the M-AMG 185000- M_r polypeptide may break down into the 163000- M_r polypeptide before cleavage at the methylamine-sensitive site. Since the M-AMG 185000- M_r polypeptide is rather resistant to thermal fragmentation, we favour the first postulate.

The methylamine-binding site can be localized within the 163000- M_r polypeptide chain, given our accumulated results from limited tryptic digestion, methylamine labelling and heat-induced fragmentation studies. We had previously shown that the methylamine-binding site is located within the 63000- M_r tryptic fragment (Hudson & Koo, 1982). This 63000- M_r tryptic fragment must be placed at the *C*-terminal region of the 163000- M_r polypeptide chain (Fig. 5b), by comparison with the known location of the methylamine-binding site in h- $\alpha_2\text{M}$ (Sottrup-Jensen *et al.*, 1983). The methylamine-binding site in M-AMG, like that in h- $\alpha_2\text{M}$, is believed to be susceptible to heat-induced fragmentation. The production of a heat-induced 29000- M_r fragment in M-AMG indicates that the methylamine-binding site is near the centre of the 63000- M_r tryptic fragment (Fig. 5c). This proposed model is consistent with the work of Starkey (1983).

Both M-AMG and h- $\alpha_2\text{M}$ appear to bind two molecules to trypsin/molecule. This is in contrast with a report (Saito & Sinohara, 1985) that each M-AMG molecule binds only one molecule of trypsin. The method of purification of M-AMG employed by Saito & Sinohara (1985) may have resulted in lower binding. We find that M-AMG is eluted in near pure form from a single Cibacron Blue affinity column in Tris buffer, whereas Saito & Sinohara (1985) utilized three different columns in addition to 10 mM-acetate buffer at pH 5.0. This treatment may have resulted in partial inactivation of M-AMG, since we have found M-AMG to lose trypsin-binding activity at acid pH.

Starkey (1983) has studied the evolution of h- $\alpha_2\text{M}$. All vertebrate species examined appear to contain a papain-binding protein of similar size to h- $\alpha_2\text{M}$, except for fishes, which contain a papain-binding protein of half that molecular size. Among mammals h- $\alpha_2\text{M}$ homologues appear to fall into two distinct molecular types, the type represented by h- $\alpha_2\text{M}$ (type 1) and the other type represented by M-AMG (type 2, described here). The majority of mammals appear to contain type 1 variants.

At least two species, rat and rabbit, contain both types. In rats $\alpha_1\text{M}$ (type 2) is the major constitutive h- $\alpha_2\text{M}$ homologue, but acute-phase $\alpha_2\text{M}$ (type 1) is also found. In rabbits just the opposite is found: type 1 is the constitutive component whereas type 2 is the acute-phase component. At least in rats the two molecular forms

have grossly differing primary structures (Schaeufele & Koo, 1982).

In mouse it is possible that at least two constitutive molecular forms of α_2M exist in serum, the type that contains the 185000- M_r polypeptides and the other type that contains the 163000- M_r and 35000- M_r polypeptides. It might also be possible that hybrid forms exist, given the high degree of primary structural homology between the 185000- M_r polypeptide and the 163000- M_r and 35000- M_r polypeptides. This appears unique to mouse, and may provide a means for study of the structural and functional evolution of h- α_2M homologues.

We thank Dr. Hal E. Broxmeyer, Dr. John Parkinson, Dr. Arun Srivastava and Mr. Michael Schell for proof reading, and Shirley Duke and Stephanie Moore for their secretarial support. This research was supported in part by Grant CA 24337 from the National Institutes of Health and by National Science Foundation Grant BNS-8406944.

REFERENCES

- Bender, M. L., Bergue-Canton, M. L., Blakeley, R. L. & Stoops, J. K. (1966) *J. Am. Chem. Soc.* **88**, 5890-5913
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427
- Frenoy, J. P. & Bourrillon, R. (1980) *Biochimie* **62**, 497-502
- Graham, D. D. (1975) *Trans. Am. Microsc. Soc.* **94**, 375
- Greene, N. D., Damian, R. T. & Hubbard, W. J. (1971) *Biochim. Biophys. Acta* **236**, 659-663
- Hanna, M. G., Nettesheim, P., Fisher, W. D., Peters, L. C. & Francis, M. (1967) *Science* **157**, 1458-1461
- Harpel, P. C. (1973) *J. Exp. Med.* **138**, 508-521
- Harpel, P. C., Hayes, M. B. & Hugli, T. E. (1979) *J. Biol. Chem.* **254**, 8669-8678
- Heimburger, N. (1975) *Cold Spring Harbor Conf. Cell Proliferation* **2**, 367-386
- Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2235-2239
- Howard, J. B., Vermeulen, M. & Swenson, R. P. (1980) *J. Biol. Chem.* **255**, 3820-3823
- Howard, J. B., Swenson, R. & Eccleston, E. (1983) *Ann. N.Y. Acad. Sci.* **421**, 160-166
- Hudson, N. W. & Koo, P. H. (1982) *Biochim. Biophys. Acta* **704**, 290-303
- James, K. (1980) *Trends Biochem. Sci.* **5**, 43-47
- Jensen, P. E. H. & Sottrup-Jensen, L. (1986) *J. Biol. Chem.* **261**, 15863-15869
- Kaplan, R., Li, S. S.-L. & Kehoe, J. M. (1977) *Biochemistry* **16**, 4297-4303
- Koo, P. H. (1982) *Cancer Res.* **42**, 1788-1797
- Koo, P. H. (1983) *Ann. N.Y. Acad. Sci.* **421**, 388-390
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mendez, E. & Lai, L.-Y. (1975) *Anal. Biochem.* **68**, 47-53
- Nettesheim, P., Hanna, M. G. & Fisher, W. D. (1968) *Radiat. Res.* **35**, 378-389
- Olinescu, A., Popescu, D. E., Dumitrescu, A. & Ganea, D. (1976) *Arch. Roum. Pathol. Exp. Microbiol.* **35**, 317-321
- Olinescu, A., Dumitrescu, A., Babes, S. & Sandulescu, T. (1980) *Arch. Roum. Pathol. Exp. Microbiol.* **39**, 121-125
- Saito, A. & Sinohara, H. (1985) *J. Biol. Chem.* **260**, 775-781
- Schaeufele, J. T. & Koo, P. H. (1982) *Biochem. Biophys. Res. Commun.* **100**, 1-7
- Simpson, R. J., Neuberger, M. R. & Liu, T.-Y. (1976) *J. Biol. Chem.* **251**, 1936-1940
- Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G. & Ballantyne, D. L. (1971) *Biochemistry* **10**, 4912-4921
- Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1980) *FEBS Lett.* **121**, 275-279
- Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E. & Magnusson, S. (1981) *FEBS Lett.* **123**, 145-148
- Sottrup-Jensen, L., Stepanik, T. M., Weirzbicki, D. M. & Magnusson, S. (1983) *Ann. N.Y. Acad. Sci.* **421**, 41-60
- Starkey, P. M. (1983) *Ann. N.Y. Acad. Sci.* **421**, 112-118
- Swenson, R. P. & Howard, J. B. (1979a) *J. Biol. Chem.* **254**, 4452-4456
- Swenson, R. P. & Howard, J. B. (1979b) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4313-4316
- Vincent, A. & Scherrer, K. (1979) *Mol. Biol. Rep.* **5**, 209-214
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412

Received 4 December 1985/8 July 1987; accepted 26 August 1987