Ligand binding, conformational change and plasma elimination of human, mouse and rat α -macroglobulin proteinase inhibitors

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Rat α_1 -macroglobulin (α_1 M), rat α_2 -macroglobulin (α_2 M) and mouse α_2 M migrated as single bands on non-denaturing gels when purified by the methods described. All three proteins demonstrated increased mobility after reaction with trypsin. A single saturable pathway rapidly cleared complexes of trypsin and the α -macroglobulins of mouse, rat and human from the circulation of mice. None of the native α -macroglobulins competed for clearance with the trypsin complexes. [14 C]Methylamine incorporation was 4.1, 3.9, 2.6 and 3.2 mol/mol of proteinase inhibitor for human α_2 M, rat α_1 M, rat α_2 M and mouse α_2 M, respectively. Only rat α_2 M, the acute-phase α -macroglobulin studied, showed no evidence of conformational change when subjected to electrophoresis after reaction with methylamine. The clearance of rat α_2 M–methylamine was comparable with that of the native molecule. The other α -macroglobulin–methylamine complexes cleared faster than the inhibitors that had not reacted. Rat α_2 M and rat α_2 M–methylamine bound equivalent quantities of 125 I-labelled trypsin (1.01 and 0.96 mol/mol respectively). The soya-bean trypsin inhibitor-resistant esterolytic activity of trypsin bound to rat α_2 M–methylamine was approx. 90% suppressed compared with proteinase bound to native rat α_2 M. This suppression was not due to a change in the affinity of soya-bean trypsin inhibitor for the complex. Reaction of rat α_2 M–methylamine with trypsin resulted in a 'slow' to 'fast' electrophoretic conversion of the proteinase inhibitor, and exposure of the signal on the α_2 M that causes the complex to clear from the murine circulation.

Human α_2 M consists of four identical subunits (Harpel, 1973), each containing three separate regions that are critical to function. Peptide bonds susceptible to proteolytic cleavage are found in a well-defined area of the subunit termed the 'bait region' (Barrett & Starkey, 1973). Reaction between α_2 M and a variety of proteinases is initiated at this site. A reactive thioester bond has been identified at a position approx. 250 amino acid residues from the centre of the bait region in the direction of the C-terminus (Sottrup-Jensen *et al.*, 1981*a,b*). Covalent binding of proteinases, incorporation of low-molecular-weight amines and heat-induced fragmentation are all reactions that involve the thioester bond (Harpel *et al.*, 1979; Swenson & Howard, 1979; Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1980). The third functional region, which is involved in receptor recognition, is not as well

characterized. α_2 M undergoes conformational change after reaction with proteinase or amine (Barrett *et al.*, 1979; Gonias *et al.*, 1982). It has been postulated that this conformational change orients or exposes a segment on the α_2 M subunit so that the α_2 M complex is bound by high-affinity receptors on macrophages and fibroblasts (Van Leuven *et al.*, 1979; Imber & Pizzo, 1981; Kaplan *et al.*, 1981). Receptor recognition occurs independently of the proteinase used to form the complex (Debanne *et al.*, 1975) and the thiol groups generated during reaction with proteinase or amine do not contribute directly to clearance (Gonias & Pizzo, 1981*a*; Van Leuven *et al.*, 1982).

α -Macroglobulins homologous with human α_2 M have been identified in a variety of species (Greene *et al.*, 1971; Darcy, 1955; Ohlsson, 1971; Ficard & Heremans, 1963). Rats possess two closely related, but distinguishable, α -macroglobulins (Gordon, 1976). The most significant difference between the two proteins is that the concentration of rat α_2 M

Abbreviations used: α_1 M, α_1 -macroglobulin; α_2 M, α_2 -macroglobulin; NaP_i, sodium phosphate; SBTI, soya-bean trypsin inhibitor.

increases considerably in response to injury or inflammation, whereas that of rat α_1 M increases only minimally (Gordon, 1976; Gauthier & Mouray, 1976). The two α -macroglobulins bind equivalent quantities of proteinase, and the inhibitor–proteinase complexes in both cases are active against low-molecular-weight substrates (Gordon, 1976; Gauthier & Mouray, 1976).

In the present investigation, purified rat α_1 M, rat α_2 M, mouse α_2 M and human α_2 M were allowed to react with trypsin and methylamine. The clearances of the proteins that had reacted and those that had not were compared in mice. The electrophoretic mobility of human α_2 M is increased after reaction with proteinase or amine, despite the more alkaline pI of the complexed inhibitor (Barrett *et al.*, 1979; Van Leuven *et al.*, 1981). Similar changes in electrophoretic mobility are shown here to accompany only some of the homologous reactions with the other three α -macroglobulins. The reaction of methylamine with rat α_2 M is significantly different from the well-described reaction with human α_2 M (Barrett *et al.*, 1979). This difference may be important in the function of rat α_2 M as an acute-phase reactant.

Experimental

Materials

Trypsin was purchased from Worthington. This preparation was 70% active by active-site titration (Chase & Shaw, 1967). Cibacron Blue F3-GA-agarose was purchased from Bio-Rad and DEAE-Trisacryl M was from LKB Instruments. The sources of other reagents are listed elsewhere (Gonias & Pizzo, 1981b).

Methods

Purification of α -macroglobulins. Human α_2 M was purified by using a modification of the procedure described by Kurecki *et al.* (1979). A detailed description of this purification is presented elsewhere (Imber & Pizzo, 1981).

Mouse α_2 M was isolated from systemically heparinized animals. Blood was collected by cardiac puncture. A 10 ml portion of plasma was dialysed into 10 mM-NaP_i, pH 8.0, and transferred to a column (11 cm $\times$ 2.5 cm) of DEAE-Trisacryl M equilibrated in the same buffer. The column was washed with 2 column volumes each of 10 mM-NaP_i and 10 mM-NaP_i/25 mM-NaCl, pH 8.0. Elution was achieved with a linear gradient consisting of 10 mM-NaP_i/25 mM-NaCl (conductivity $\sim$ 5.5 mS) as the leading buffer and 10 mM-NaP_i/37 mM-NaCl (conductivity $\sim$ 12.5 mS) as the limit buffer. Fractions containing α -macroglobulin activity (Ganrot, 1966) were concentrated and applied to a column (100 cm $\times$ 5 cm) of Cibacron Blue F3-GA-agarose

equilibrated in 50 mM-Tris/HCl, pH 8.0. Pure mouse α_2 M eluted in the ascending half of the first protein peak as determined by gel electrophoresis (Wycoff *et al.*, 1977).

Rat α_1 M and α_2 M were prepared from male CD-1 rats injected subcutaneously with 0.5 ml of turpentine/100 g body weight. The animals were heparinized and bled by cardiac puncture 48 h after turpentine administration. A 15 ml portion of plasma was treated with dextran sulphate as described by Okubo *et al.* (1981) and transferred to a column (100 cm $\times$ 2.5 cm) of Bio-Gel 0.5 M equilibrated with 50 mM-Tris/HCl/150 mM-NaCl, pH 8.0. Fractions collected from the leading edge of the first protein peak were combined and dialysed against 10 mM-NaP_i, pH 8.0. DEAE-Trisacryl M column chromatography was then performed as described for the purification of mouse α_2 M. Rat α_2 M eluted at a conductivity of 8 mS; rat α_1 M eluted at 10 mS. Fractions were assayed for purity by gel electrophoresis. α_1 M and α_2 M were concentrated with Millipore CX-30 ultrafilters and stored at -20°C in 20% (v/v) glycerol without loss of activity. Rat α_1 M was purified from normal plasma by the same procedure.

Protein concentrations. Protein concentrations were calculated by using the following constants: human α_2 M, $A_{1\text{cm}}^{1\%} = 8.93$, $M_r = 718\,000$ (Hall & Roberts, 1978); mouse α_2 M, $A_{1\text{cm}}^{1\%} = 7.8$, $M_r = 720\,000$ (W. J. Hubbard, unpublished work); rat α_1 M, $A_{1\text{cm}}^{1\%} = 9.8$, $M_r = 746\,000$ (Gordon, 1976); rat α_2 M, $A_{1\text{cm}}^{1\%} = 9.2$, $M_r = 716\,000$ (Gordon, 1976).

Protein radioiodination. α -Macroglobulins, trypsin and SBTI were radiolabelled by the solid-state lactoperoxidase method (David & Reisfeld, 1974) without loss of activity.

Reactions of α -macroglobulins with [^{14}C]methylamine. Solutions containing 100 pmol of α -macroglobulin and 16 μmol of [^{14}C]methylamine (final concn. 80 mM) were incubated for 4 h at pH 8.0 (ambient temperature). Preparations were dialysed exhaustively and transferred to Aquasol II (New England Nuclear Corp.) for liquid-scintillation counting. This procedure is described in detail elsewhere (Gonias & Pizzo, 1981b).

Trypsin-binding studies. Rat α_2 M and α_2 M-methylamine were allowed to react with a 3-fold molar excess of active ^{125}I -labelled trypsin for 30 min. SBTI at twice the trypsin concentration was then added to each solution for an additional 10 min. α_2 M- ^{125}I -labelled trypsin complex was resolved from trypsin that had not reacted and trypsin-SBTI by gel filtration on a column (23 cm $\times$ 0.8 cm) of Sephadex G-150. Trypsin-binding activity was also measured as described by Ganrot (1966) and modified by Gonias & Pizzo (1981b).

Polyacrylamide-gel electrophoresis. Gel electrophoresis under non-denaturing conditions was performed on 4% and 5% (w/v) slabs by using the

Tris/borate buffer system described by Nelles *et al.* (1980).

Plasma-elimination studies. α -Macroglobulin-trypsin complexes were prepared by incubating the proteinase inhibitors with 3-fold molar excesses of active trypsin for 30 min. Proteinase that had not reacted was removed by gel filtration on a column (23 cm $\times$ 0.8 cm) of Sephadex G-150. Clearance experiments were performed in CD-1 female mice as previously described (Imber & Pizzo, 1981). Experiments were performed in triplicate. Results from each individual studied varied by less than 5% when compared with the mean.

Results

Non-denaturing-gel electrophoresis of α -macroglobulin-trypsin complexes

Rat α_1 M and α_2 M migrated as single distinguishable bands on non-denaturing gels (Fig. 1). The entire protein content of either band was shifted to a position of increased mobility after reaction with trypsin. Purified mouse α_2 M also migrated as a single band under non-denaturing conditions. Reaction with trypsin resulted in an increase in the electrophoretic mobility of the mouse proteinase inhibitor. These electrophoretic 'slow'-to-'fast' transitions suggest that the rat and mouse α -macroglobulins undergo conformational changes after reaction with trypsin that are similar to the change described for human α_2 M.

Fig. 1. Non-denaturing-gel electrophoresis of rat α -macroglobulins

(a) Rat α_1 M that had not reacted; (b) rat α_1 M after reaction with a 3-fold molar excess of trypsin for 30 min; (c) rat α_2 M that had not reacted; (d) rat α_2 M-trypsin (4% slab gel).

Plasma elimination of α -macroglobulin-trypsin complexes

Human α_2 M-trypsin and α_2 M-methylamine clear rapidly from the circulation by the same saturable pathway in mouse (Imber & Pizzo, 1981). α_2 M that has not reacted clears very slowly. Equivalent results are shown in Fig. 2. Significant competition was observed when 125 I-labelled human α_2 M-trypsin was cleared in the presence of a large molar excess of unlabelled mouse α_2 M-trypsin. This result indicates that the pathway responsible for the catabolism of human α_2 M-proteinase complex in mouse is the mouse α_2 M-proteinase pathway. Unlabelled rat α_1 M-trypsin and rat α_2 M-trypsin also prolonged the clearance of radioiodinated human α_2 M-trypsin when included in the injected solution at competitive concentrations. More than a 500-fold molar excess of the four native α -macroglobulins failed to alter the plasma elimination of 125 I-labelled human α_2 M-trypsin.

Fig. 2. Plasma elimination of human α_2 M and human α_2 M-trypsin in mouse

Radioiodinated human α_2 M-trypsin (5–12 pmol) was cleared alone from the circulation of mice (○), or in the presence of a 150-fold molar excess of unlabelled mouse α_2 M-trypsin (▲), 375-fold molar excess of mouse α_2 M-trypsin (▲), 220-fold molar excess of rat α_2 M-trypsin (◇), and a 375-fold molar excess of rat α_1 M-trypsin (□). Native human α_2 M was cleared alone (●).

Clearance and clearance-competition experiments were performed with radioiodinated mouse and rat α -macroglobulins (Figs. 3 and 4). Complexes formed between the three α -macroglobulins and trypsin cleared rapidly. The inhibitors that had not reacted persisted in the circulation. Competition for clearance was demonstrated by all the unlabelled α -macroglobulin-trypsin complexes and by none of the native proteinase inhibitors. These results indicate that the same receptor recognizes acute- and non-acute-phase α -macroglobulins. Unlabelled ligands competed less extensively for clearance with radioiodinated rat α_1 M than with radioiodinated rat α_2 M, suggesting that the two α -macroglobulins may have different affinities for the receptor or different endocytotic rates.

Reactions with methylamine

[14 C]Methylamine was allowed to react with human α_2 M, rat α_1 M, rat α_2 M, and mouse α_2 M, resulting in the incorporation of 4.1, 3.9, 2.6 and

3.2 mol of methylamine/mol of protein respectively. The incorporation of methylamine into the non-human α -macroglobulins suggests that these proteins also possess labile thioester bonds.

The clearance of rat α_1 M-methylamine was extremely rapid (90% elimination in 3 min) (Fig. 5). Mouse α_2 M-methylamine cleared in at least two phases. More than 75% of the mouse inhibitor was eliminated from the circulation in 10 min; the remaining radioligand did not clear. Approx. 90% of the radioactivity present in the plasma during the second phase of the study was precipitable with 7.5% (w/v) trichloroacetic acid. Only rat α_2 M failed to clear at an increased rate after reaction with methylamine for 1, 2 or 4 h.

The α -macroglobulins were allowed to react with methylamine and subjected to electrophoresis (Fig. 6). Rat α_1 M was completely converted into a form with increased mobility under the described reaction conditions, as was human α_2 M. These two proteins, therefore, react with methylamine homologously in that they both bind approx. 4 mol/mol, undergo conformational change and clear from the circulation rapidly. A fraction of the mouse α_2 M demonstrated increased electrophoretic mobility after exposure to methylamine for 1 h. Further conformational change was not detected after incubation for 2, 4 or 8 h. The electrophoretic mobility of rat α_2 M remained unchanged on non-denaturing gels after reaction with methylamine for 1, 2, 4 and 8 h. This result may explain why methylamine-treated rat α_2 M failed to clear more rapidly than native α_2 M, despite the incorporation of between two and three molecules of amine per protein molecule.

Comparison of rat α_2 M and rat α_2 M-methylamine

Binding experiments were performed with [125 I]-labelled proteinase. Trypsin incorporation into rat α_2 M was 1.01 mol/mol, in good agreement with previous studies with chromogenic assays (Gordon, 1976; Okubo *et al.*, 1981). Rat α_2 M-methylamine incorporated 0.96 mol of trypsin/mol of proteinase inhibitor.

The assay described by Ganrot (1966) measures the esterase activity of trypsin bound to α -macroglobulin in the presence of SBTI. Rat α_2 M and exhaustively dialysed α_2 M-methylamine were allowed to react with a 3-fold molar excess of trypsin for 5, 15, 30 and 60 min before addition of SBTI. The complex formed between rat α_2 M and trypsin demonstrated significant esterase activity. The time of incubation with trypsin affected colour generation by less than 10%. The complex formed between rat α_2 M-methylamine and trypsin hydrolysed the substrate *N* α -benzoyl-DL-arginine *p*-nitroanilide hydrochloride poorly. Ratios of substrate hydrolysis calculated for α_2 M-methylamine/ α_2 M after

Fig. 3. Plasma elimination of mouse α_2 M and mouse α_2 M-trypsin

Radioiodinated mouse α_2 M-trypsin (2–5 pmol) was cleared alone (Δ) or in the presence of a 1300-fold molar excess of unlabelled human α_2 M-trypsin ($\circ$), 1000-fold molar excess of mouse α_2 M-trypsin ($\blacktriangle$), 900-fold molar excess of rat α_1 M-trypsin ($\square$) and 1100-fold molar excess of rat α_2 M-trypsin ($\diamond$). Native mouse α_2 M was cleared alone ($\blacktriangle$).

Fig. 4. Plasma elimination of rat α -macroglobulins in mouse

(a) Radioiodinated rat α_1 M-trypsin (2-5 pmol) was cleared alone ($\square$) or in the presence of a 500-fold molar excess of unlabelled rat α_1 M-trypsin ($\blacksquare$), a 500-fold ($\circ$) and a 1300-fold ($\bullet$) molar excess of human α_2 M-trypsin, a 1000-fold molar excess of rat α_2 M-trypsin ($\diamond$) and a 1600-fold molar excess of mouse α_2 M-trypsin ($\triangle$). Native rat α_1 M was cleared alone ($\blacksquare$). (b) Radioiodinated rat α_2 M-trypsin (2-5 pmol) was cleared alone ($\diamond$), or in the presence of a 500-fold molar excess of unlabelled rat α_2 M-trypsin ($\blacklozenge$), a 770-fold molar excess of human α_2 M-trypsin ($\circ$), a 600-fold molar excess of mouse α_2 M-trypsin ($\triangle$), and a 400-fold molar excess of rat α_1 M-trypsin ($\square$). Native rat α_2 M was cleared alone ($\blacklozenge$).

Fig. 5. Plasma elimination of α -macroglobulins after reaction with methylamine

Radioiodinated α -macroglobulins were allowed to react with 200 mM-methylamine for 1 h at pH 8.0 and cleared from the circulation of mice. $\circ$, Human α_2 M; $\triangle$, mouse α_2 M; $\square$, rat α_1 M; $\diamond$, rat α_2 M.

Fig. 6. Electrophoresis of α -macroglobulin-methylamine complexes

The α -macroglobulins were allowed to react with 200 mM-methylamine for 1 h at pH 8.0 and were compared with the native proteins that had not reacted. (a) Native human α_2 M; (b) human α_2 M-methylamine; (c) native mouse α_2 M; (d) mouse α_2 M-methylamine; (e) native rat α_1 M; (f) rat α_1 M-methylamine; (g) native rat α_2 M; (h) rat α_2 M-methylamine. A 5% (w/v)-acrylamide slab gel was used under non-denaturing conditions.

reaction with trypsin for 5, 15, 30 and 60 min were 0.10, 0.13, 0.09 and 0.10 respectively. One explanation for these data is that methylamine slightly alters the proteinase-binding site in rat α_2 M so that the steric hindrance of the trypsin-SBTI interaction is lessened. To test this hypothesis, rat α_2 M and α_2 M-methylamine were allowed to react with trypsin and then with 125 I labelled SBTI. Gel filtration was performed on Sephadex G-150 to resolve 125 I-labelled SBTI bound to α_2 M complex from the remaining radioligand. Rat α_2 M-trypsin bound 0.14 mol of 125 I-labelled SBTI/mol of proteinase inhibitor when incubated with SBTI at 4-fold molar excess for 10 min. Equivalent incubation conditions resulted in the binding of 0.17 mol of 125 I-labelled SBTI to each mol of α_2 M-methylamine-trypsin.

Properties of rat α_2 M-methylamine-trypsin

The results presented thus far suggest that rat α_2 M-methylamine binds trypsin readily and inhibits the activity of the proteinase more completely than does the native inhibitor. Rat α_2 M-methylamine was allowed to react with trypsin and compared with the

Table 1. Summary of data comparing rat α_2 M and rat α_2 M-methylamine

	Rat α_2 M	Rat α_2 M-methylamine
[14 C]Methylamine incorporated*	—	2.6
125 I-labelled trypsin binding capacity*	1.01	0.96
125 I-labelled SBTI bound by trypsin complex*	0.14	0.17
Relative activity of trypsin complex towards substrate Bz-Arg-Nan-HCl†	1.0	0.1
Electrophoretic conformation	Slow	Slow
Amount cleared in 10 min (%)	6	7
Electrophoretic conformation after reaction with trypsin	Fast	Fast
Amount of trypsin complex cleared in 10 min (%)	84	86

* Binding data are expressed as mol/mol of proteinase inhibitor.

† Abbreviation: Bz-Arg-Nan-HCl, N^α -benzoyl-DL-arginine *p*-nitroanilide hydrochloride.

Fig. 7. Plasma elimination of rat α_2 M-methylamine-trypsin in mice

Rat α_2 M was allowed to react with 200 mM-methylamine for 4 h, dialysed to remove free amine, and allowed to react with a 3-fold molar excess of trypsin for 30 min. The clearance of rat α_2 M-methylamine is also shown (----) for comparison.

protein that had not reacted by non-denaturing-gel electrophoresis. The α_2 M-methylamine was converted into a fast form indistinguishable from α_2 M-trypsin.

Radiolabelled rat α_2 M-methylamine was allowed to react with trypsin and cleared from the circulation of mice (Fig. 7). More than 85% of the radioligand was eliminated from the circulation in 10 min. Rat α_2 M-methylamine is therefore capable of clearing bound proteinases as rapidly as the native molecule. A summary of data comparing rat α_2 M and rat α_2 M-methylamine is presented in Table 1.

Discussion

Human α_2 M that has not reacted with proteinase or low-molecular-weight amine migrates as a single band on non-denaturing gels. α_2 M that has reacted migrates as a second band with increased mobility (Barrett *et al.*, 1979). Non-denaturing-gel electrophoresis is therefore a useful tool for detecting inactive components in samples of human α_2 M. Rat α_1 M, rat α_2 M and mouse α_2 M, when purified by the methods described here, are present as single electrophoretic bands on non-denaturing gels, indicating that the α -macroglobulins are contaminated with little or no inactive protein. This result is contrasted with previously published purifications that yielded two bands for one α -macroglobulin (Gauthier & Mouray, 1976; Hudig & Sell, 1979). All three proteinase inhibitors demonstrated 'slow'-to-'fast' electrophoretic transitions after reaction with trypsin. These transitions most likely indicate

that conformational change is a conserved property of acute- and non-acute-phase mammalian α -macroglobulins.

Experiments performed with all four α -macroglobulins consistently demonstrated a correlation between inhibitor clearance rate and conformation as determined with electrophoresis on non-denaturing gels. Only some of the structural alterations that occur during conformational change may be critical for receptor recognition (Van Leuven *et al.*, 1982); however, it seems probable that there is little variability in the recognized site on different acute- and non-acute-phase α -macroglobulins.

All four α -macroglobulins incorporated [14 C]-methylamine to a significant extent. Rat α_2 M bound less amine than did the other three proteins; however, further experiments showed that rat α_2 M-methylamine is not cleared from the circulation. Significant levels of amine may, therefore, already be bound to α_2 M isolated from the plasma of rats undergoing an acute-phase reaction with its attendant tissue damage.

Under the appropriate conditions, the function of the acute-phase α -macroglobulin from rat differs considerably from the non-acute-phase α -macroglobulin. The proteinase-binding activity of rat α_2 M is insensitive to amines. The enzymic activity of trypsin bound to rat α_2 M is suppressed more strongly if the α_2 M has already incorporated amine. The accumulation of proteinase at sites of severe necrotic inflammation, where clearance may not occur as rapidly as in the plasma, would make these acute-phase-inhibitor properties desirable.

Mouse α_2 M behaved as a single population of molecules when allowed to react with trypsin and studied by electrophoresis and clearance experiments. Heterogeneity was detected by using the same experimental techniques after reaction with methylamine. These results parallel those obtained comparing the two rat α -macroglobulins and suggest that two slightly different α_2 -macroglobulins may be present in the plasma of mice.

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