Purification and Characterization of Frog α-Macroglobulin: Receptor Recognition of an Amphibian Glycoprotein†

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ABSTRACT: Frog α-macroglobulin was purified to apparent homogeneity by Ni^{2+} chelate affinity chromatography. Frog α-macroglobulin migrated as an α_1-globulin in cellulose acetate electrophoresis. A molecular weight of 730 000 was obtained by equilibrium sedimentation, and in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the protein migrated as a single band of M_r ~360 000 before reduction and M_r ~180 000 after reduction. Treatment with trypsin resulted in subunit cleavage to yield a fragment of M_r ~90 000. After being heated, the protein fragmented, migrating in SDS–PAGE as two bands of M_r ~120 000 and 60 000. This fragmentation was inhibited by prior reaction of the protein with methylamine. In native pore-limit electrophoresis the protein exhibited the characteristic "slow" to "fast" conformational change of protease-treated α-macroglobulins. In contrast, typical "slow" to "fast" conformational change was not observed in native PAGE with this preparation. Moreover, the protein incorporated approximately 2 mol of [^{14}C]methylamine/mol of inhibitor without demonstrating a change in mobility in native PAGE. In circular dichroism studies, the protein exhibited a spectrum similar to that of human α_2M. Reaction with trypsin resulted in a broadening and decrease in the magnitude of the spectrum. Reaction with methylamine resulted in similar changes, but of smaller magnitude. The inhibitor bound approximately 0.7 mol of trypsin in both radiolabeled protease binding and amidolytic titration studies. ^{125}I-Labeled native frog α_1M was removed slowly from the circulation of mice with a $t_{1/2} > 2$ h. After reaction with trypsin, the protein was removed in a first-order reaction with $t_{1/2} < 3$ min. The clearance was significantly inhibited only by coinjection of a combination of human α_2M–methylamine, asialoorosomucoid, and lactoferrin; these results suggest recognition by multiple receptor systems. Native frog α_1M did not inhibit the binding of 0.2 nM ^{125}I-labeled human α_2M–methylamine to mouse peritoneal macrophages in vitro, but frog α_1M–trypsin inhibited the binding by 50% at a concentration of 0.6 nM with a K_i of 0.4 nM.

Human α_2-macroglobulin (α_2M) is a high molecular weight plasma protein (M_r ~720 000) formed by the noncovalent interaction between disulfide bonded pairs of identical subunits (Hall & Roberts, 1978; Harpel, 1973; Swenson & Howard, 1979a). It inhibits endoproteases of each of the four classes by a unique mechanism (Barrett & Starkey, 1973). Proteases cleave the molecule at a highly susceptible site, the "bait" region; a conformational change then "traps" the protease (Barrett & Starkey, 1973). In contrast to other protease inhibitors, human α_2M binds protease without blocking the protease active site, and the complex remains active against small substrates. In native polyacrylamide gel electrophoresis (native PAGE), this conformational change corresponds to an increase in mobility (called "slow" to "fast" change) (Barrett et al., 1979).

Human α_2M contains a thiol ester bond that is susceptible to nucleophilic attack by small amines; cleavage of the thiol esters also results in slow to fast conformational change (Barrett et al., 1979; Swenson & Howard, 1979b; Sottrup-Jensen et al., 1980). After proteases cleave the bait region, protease lysine residues may attack the thiol ester becoming covalently bonded to the α_2M (Salvesen et al., 1981). α_2M subunits are cleaved to fragments of M_r 60 000 and 120 000 when native α_2M is heated (Harpel et al., 1979). This cleavage occurs at the thiol ester and is prevented by prior reaction with methylamine (Sottrup-Jensen et al., 1980).

Human α_2M conformational change results in exposure of a receptor recognition site (Debanne et al., 1975; Ohlsson, 1971) with equivalent recognition by cells of both α_2M–methylamine and α_2M–protease (Imber & Pizzo, 1981; Kaplan et al., 1981; Van Leuven et al., 1981; Feldman et al., 1983). The native molecule is not recognized by the receptor. In vitro binding studies with mammalian cells of many different types demonstrate that the receptor is widely distributed (Van Leuven et al., 1978; Kaplan & Nielson, 1979; Dickson et al., 1981; Imber & Pizzo, 1981; Gliemann et al., 1983; Ney et al., 1984).

α-Macroblobulins have been purified from a number of other mammalian species (Feldman et al., 1984; Gonias et al., 1983), chicken (Nagase et al., 1983; Feldman & Pizzo, 1984a) and fish (Starkey et al., 1982). These proteins exhibit many similarities to human α_2M. The characteristic subunit structure is conserved in mammalian and avian α-macroglobulins, while fish α-macroglobulin is similar to a half-molecule of human α_2M. Slow to fast conformational change and the reactive thiol ester generally are conserved as well. The existence of molecules with the ability to bind protease without blocking the protease active site has been demonstrated in the plasma of many other species of vertebrates as well as the hemolymph of the horseshoe crab (Starkey & Barrett, 1982a,b; Quigley & Armstrong, 1983).

A number of studies have examined the conservation of receptor recognition in α-macroglobulins derived from various mammalian sources. Rabbit, mouse, rat, bovine, and human α-macroglobulins reacted with protease demonstrate very similar receptor recognition by various mammalian cells

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(Kaplan & Nielson, 1979; Imber & Pizzo, 1981; Gonias et al., 1983; Feldman et al., 1984). Only one published study has examined the question of conservation of the receptor recognition site in a nonmammalian species (Feldman & Pizzo, 1984a).

In the present study, we purified to apparent homogeneity the α-macroglobulin of the southern grass frog (*Rana pipiens*). The molecular weight, subunit structure, protease binding activity, and reaction with methylamine were studied. Circular dichroic spectroscopy and electrophoresis in native PAGE were employed to study conformational change of the frog α-macroglobulin treated with either trypsin or methylamine. Finally, receptor recognition of native frog α-macroglobulin and the α-macroglobulin treated with trypsin or methylamine was studied both in vivo in mice and in vitro with mouse peritoneal macrophages.

Experimental Procedures

Proteins and Reagents. Trypsin was obtained from Millipore Corp. Plasminogen was purified as previously described (Deutsch & Mertz, 1970) and was activated with urokinase (Gonias & Pizzo, 1983b). A 3-fold molar excess was used in reactions with α-macroglobulin. Human lactoferrin (Lf) was purified as previously described (Querinjean et al., 1971). Asialoorosomucoid (ASOR) was prepared by heating orosomucoid (a gift of the American Red Cross) in sulfuric acid (Schmid et al., 1967). Outdated human plasma was obtained from the Durham Veterans Administration Medical Center. Southern grass frog (*Rana pipiens*) plasma was obtained from Henrik Dohlman, Department of Biochemistry, Duke University Medical Center. Mouse peritoneal macrophages were provided by Dr. Dolph O. Adams, Department of Pathology, Duke University Medical Center. The macrophages were plated at a density of 5×10^5 cells per well as described previously (Imber et al., 1982). CD-1 female mice were obtained from the Charles River Laboratories. Carrier free $Na^{125}I$ was from New England Nuclear, and lactoperoxidase, coupled to Sepharose, was obtained from P-L Biochemicals.

Purification of α-Macroglobulins. Human α_2-macroglobulin (α_2M) was purified by the method of Kurecki et al. (1979), as modified by Imber & Pizzo (1981). Human α_2M was acid treated to obtain half-molecules as described by Starkey & Barrett (1982a,b). Frog α-macroglobulin was purified by using Ni^{2+} chelate affinity chromatography. Frog plasma was obtained diluted 1:5 with 10 mM tris(hydroxymethyl)aminomethane (Tris), 110 mM NaCl, and 1% heparin, pH 7.4. One hundred milliliters of diluted plasma was brought to 12% polyethylene glycol 8000 (PEG), and the precipitate was removed by centrifugation at 10000*g* for 15 min. The supernatant was dialyzed against two changes of 6 L of 0.2 M sodium phosphate–0.8 M NaCl, pH 6.5, and was applied to a Ni^{2+} chelate affinity column (3.5 × 10 cm). The column was washed with 5 column volumes each of 0.2 M sodium phosphate–0.8 M NaCl, pH 6.5, and 0.02 M sodium phosphate–0.15 M NaCl, pH 6.0. Protein was eluted with 0.02 M sodium phosphate, 0.15 M NaCl, and 0.02 M ethylenediaminetetraacetic acid (EDTA), pH 7.4, and was concentrated with a Millipore CX-10 ultrafilter. The concentrate was applied to an Ultrogel AcA-22 column (2.5 × 70 cm) equilibrated with 0.02 M sodium phosphate–0.15 M NaCl, pH 7.4. The column was eluted at 10 mL/h, and 10-mL fractions were collected. Fractions that eluted at the same volume as human α_2M were employed in these studies.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Wyckoff et al. (1977) or Weber & Osborn (1969) as noted. Trypsin was inhibited prior to SDS–PAGE with phenylmethanesulfonyl fluoride. Native PAGE was performed as described by Nelles et al. (1980). Pore-limit gel electrophoresis was performed on 3–12% gradient gels obtained from Fisher Scientific employing the buffer system described by Nelles et al. (1980). Standard protein electrophoresis was performed by the cellulose acetate method in the Duke University Medical Center Coagulation Laboratory.

Sedimentation Equilibria. Sedimentation equilibrium studies were performed in a Spinco Model E ultracentrifuge at 6800 rpm at 18.5 °C with a 24-h equilibration time. Protein concentration was determined by absorbance at 280 nm. A linear plot of the logarithm of absorbance vs. radius squared was obtained. A partial specific volume of 0.73 (Hall & Roberts, 1978) was assumed to calculate the molecular weight.

Protein Radioiodination. Proteins were labeled with ^{125}I by the solid-state lactoperoxidase method (David & Reisfeld, 1974). Labeled protein was separated from free ^{125}I by filtration over Sephadex G-25. Trypsin was labeled at 4 °C to inhibit proteolysis (specific activity 436 cpm/fmol) and was active site titrated with using *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Chase & Shaw, 1967).

Radiolabeled Protease Incorporation. Binding of ^{125}I-trypsin to frog α-macroglobulin was determined by incubating a 3-fold excess of protease (69 nmol) with the frog protein (23 nmol) for 5 min at room temperature. A 2-fold excess of soybean trypsin inhibitor (SBTI) was added to inhibit nonspecific association of protease to the macroglobulin. α-Macroglobulin–protease complexes were isolated from the uncomplexed protease by gel filtration on an Ultrogel AcA-22 column (0.8 × 28 cm). The column was eluted at 2 mL/h, and 0.35-mL fractions were collected. The radioactivity recovered in the first peak eluted from the column, corrected for the total radioactivity recovered, was used to determine the amount of trypsin bound. These studies were performed in duplicate on two preparations. The percentage that was bound covalently was determined by subjecting purified complex to reduced SDS–PAGE. ^{125}I-Trypsin was electrophoresed as a control. After being dried, gel lanes were cut into seven slices. Radioactivity that migrated more slowly than free ^{125}I-trypsin was considered to be covalently bound to the α-macroglobulin. These studies were performed in triplicate.

Trypsin Activity Assay. The amidolytic activity of two preparations of the α-macroglobulin–trypsin complex was determined as described by Ganrot (1966). Experiments were performed in 0.1 M Tris and 0.01 M $CaCl_2$, pH 8.2. Trypsin (10 pmol) was incubated with 0–25 pmol of frog α-macroglobulin in triplicate for 5 min at room temperature before addition of SBTI (30 pmol). Complexes were incubated with 1.5 mM N^{α}-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) at 37 °C for 4–7 h. The reaction was terminated by addition of 100 μL of glacial acetic acid, and the abosrbance at 410 nm was measured.

Methylamine Treatment of Frog α-Macroglobulin and Heat Fragmentation. Frog α-macroglobulin–methylamine was prepared by incubating the protein with 400 mM methylamine for 27 h at pH 8.2 at room temperature. Excess methylamine was removed by dialysis. Heat fragmentation was performed by heating samples in a boiling water bath for 30 min. [^{14}C]Methylamine incorporation was performed by a modification of our previously published procedure (Feldman et al., 1984). Because of the slow reactivity of frog α_1M, the incubation was continued for 300 h with a [^{14}C]methylamine concentration of 25 mM. These studies were performed in triplicate.

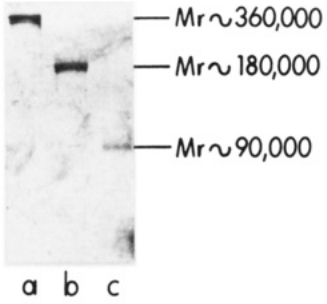

FIGURE 1: SDS–PAGE of frog α-macroglobulin. In each lane 2 μg of frog α_1M was subjected to SDS–PAGE (5% gel) as described by Wyckoff et al. (1977). The sample in lane a is unreduced and in lane b reduced. Lane c is a reduced sample of frog α_1M treated with trypsin. The gel was silver as described by Morrissey (1981).

Circular Dichroism Spectroscopy. A Jobin Yvon Dichrographe III spectropolarimeter was used to measure circular dichroic spectra. A 0.1-cm path length was used, and a mean residue weight of 111.11 was calculated from the amino acid analysis presented here. The ellipticity of trypsin was subtracted from the spectra of the frog α-macroglobulin–trypsin complex as described by Gonias et al. (1982b). Any change in the ellipticity of trypsin occurring in the formation of the complex was considered negligible.

Chemical Composition Studies. Amino acid analysis was performed on a Beckman 6300 high-performance amino acid analyzer. The frog α-macroglobulin was hydrolyzed in vacuo for 24 h at 110 °C in 6 N HCl prior to analysis. Neutral sugar content was determined by the phenol–sulfuric acid method (Dubois et al., 1956) using methyl α-D-galactoside as the standard. Sialic acid content was determined by the thiobarbituric acid assay (Warren, 1959), and galactose content of a hydrolyzed sample was determined by the galactose oxidase method (Amaral et al., 1970). *N*-Acetylneuraminic acid and methyl α-D-galactoside were used as standards in these two assays, respectively.

In Vivo Clearance Studies. In vivo clearance studies were performed in CD-1 female mice at least in duplicate as previously described (Gonias & Pizzo, 1983a). The variation at each time point is generally less than 5% by this protocol (Gonias et al., 1982a).

Radioreceptor Displacement Studies. These studies were performed in duplicate as described previously (Feldman et al., 1984; Feldman & Pizzo, 1984a). Briefly, mouse peritoneal macrophages were incubated at 4 °C with 0.2 nM human ^{125}I-α_2M–methylamine. The incubation mixture also contained dilutions of the macroglobulin being tested. Percent maximal binding was calculated by comparing the amount of labeled ligand bound in the presence of unlabeled ligand to the amount bound in its absence. This was plotted vs. the logarithm of the concentration of unlabeled ligand.

Determination of Protein Concentrations. The concentration of human α_2M was determined by using an $A_{280\ nm}^{1\%,\ 1\ cm}$ of 8.93 (Hall & Roberts, 1978). For frog α_1M, the value 8.2 was determined by a protein assay (Lowry et al., 1951) using bovine serum albumin as the standard.

RESULTS

Purification of Frog α-Macroglobulin. Metal chelate affinity chromatography is an effective method for the purification of α-macroglobulins (Kurecki et al., 1979). While Zn^{2+} chelate affinity chromatography has been used to purify human α_2M, a lower pH is required to elute human α_2M from a Ni^{2+} chelate affinity column as compared to the Zn^{2+} column (data not shown). Ni^{2+} chelate affinity chromatography, in particular, has been used to purify α-macroglobulin from nonhuman species (Feldman et al., 1984; Feldman & Pizzo, 1984a). A three-step process of PEG precipitation, Ni^{2+} chelate affinity chromatography, and gel filtration chromatography was used to purify the frog α-macroglobulin yielding approximatately 5 mg from 20 mL of frog plasma. Purity was assessed by SDS–PAGE (Figure 1). In the absence of reductant, the protein migrated as a single band of M_r ~360000 and, after reduction, as a single band of M_r ~180000. The protein migrated as an α_1-globulin on cellulose acetate protein electrophoresis and, therefore, will be referred to as frog α_1M. A molecular weight of 730000 was calculated from equilibrium sedimentation of the protein. Thus, like human α_2M, frog α_1M is formed by noncovalent association of disulfide bonded pairs. Reaction of the protein with trypsin results in cleavage of the subunit to fragments of M_r ~90000.

Table I: Amino Acid Analysis of Frog α_1M

	% composition		
	frog	chicken[a]	bovine[b]
Asp	10.2	7.9	9.2
Thr	7.3	7.2	7.0
Ser	8.5	9.2	7.9
Glu	12.5	13.2	12.7
Pro	5.8	5.7	6.9
Gly	6.8	6.1	7.2
Ala	6.1	6.7	6.8
Val	6.0	7.0	7.8
Met	1.6	1.1	1.9
Ile	4.6	4.2	3.4
Leu	9.9	10.5	9.4
Tyr	4.1	4.0	3.6
Phe	4.4	4.7	4.4
His	2.4	2.4	2.6
Lys	6.8	6.8	5.3
Arg	3.1	3.6	3.9

[a] Taken from Feldman & Pizzo (1984a). [b] Taken from Feldman et al. (1984) and included for comparison.

Table II: Carbohydrate Composition of Frog α_1M

	frog α_1M	human α_2M
molecular weight	730000	720000[a]
neutral sugar (%)	9	8
sialic acid (%)	2	1.8
galactose (%)	<0.5	1.8

[a] Taken from Hall & Roberts (1978).

Chemical Composition of Frog α_1M. The amino acid composition of frog α_1M is presented in Table I. The composition was typical of α-macroglobulins (Feldman et al., 1984; Feldman & Pizzo, 1984a). Carbohydrate content is presented in Table II. Frog α_1M contained 9% neutral sugar and 2% sialic acid, amounts comparable to that contained in human α_2M. In contrast to the human protein, no galactose was detectable.

Stoichiometry of Trypsin Binding. Trypsin binds to human α_2M at a 2:1 protease to inhibitor molar ratio (Barrett et al., 1979), but α-macroglobulins from several other species bind trypsin at a 1:1 ratio (Gonias et al., 1983; Feldman et al., 1984; Feldman & Pizzo, 1984a). ^{125}I-Labeled trypsin was used to assess the binding of trypsin to frog α_1M. Frog α_1M was incubated with a 3-fold molar excess of ^{125}I-trypsin followed by incubation with a 2-fold excess of SBTI. Bound trypsin was separated from free trypsin by gel filtration chromatography. Different preparations of frog α_1M bound 0.64 ± 0.03 to 0.86 ± 0.02 mol of trypsin/mol of macroglobulin.

The activity of proteases toward small substrates is only moderately inhibited by most α-macroglobulins. In contrast, marked inhibition is obtained against large protein substrates.

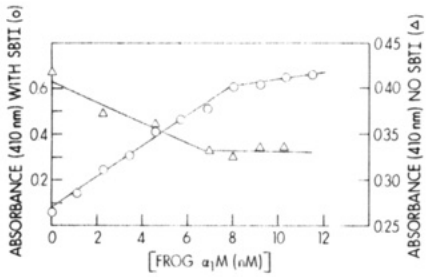

FIGURE 2: Amidolytic activity titration of frog α_1-macroglobulin. Various concentrations of frog α_1M (0–25 pmol) were incubated with trypsin (10 pmol), and 1.5 nM BAPNA was added in the presence (O) or absence (Δ) of SBTI. Reaction conditions are described under Experimental Procedures.

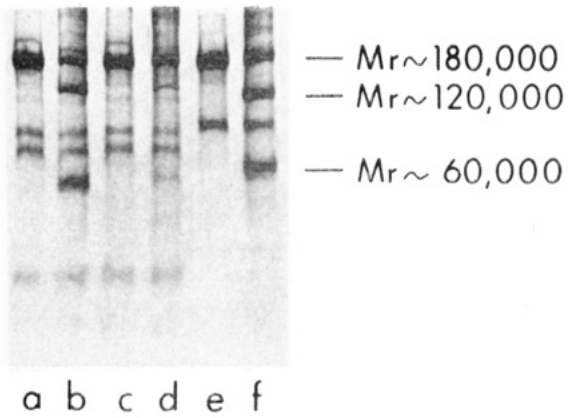

FIGURE 3: Heat fragmentation of frog α_1-macroglobulin. Frog α_1M and human α_2M were heated in a boiling water bath for 30 min. Lanes a and c are frog α_1M before heating and lanes b and d after heating. Lane e is human α_2M before heating and lane f after heating. Lanes c and d are samples reacted with 400 mM methylamine at pH 8.2 for 27 h.

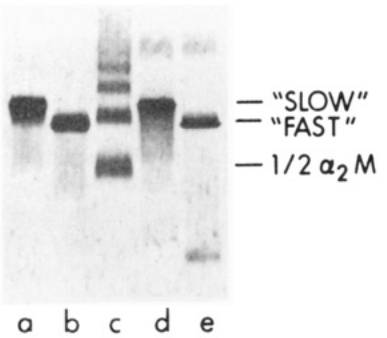

FIGURE 4: Pore-limit electrophoresis. Human α_2M and frog α_1M were electrophoresed in 3–12% polyacrylamide gradient gels. Lane a, human α_2M; lane b, human α_2M and trypsin; lane c, acid-treated human α2M; lane d, frog α_1M; lane e, frog α_1M plus trypsin. Each lane contains 10 μg of protein.

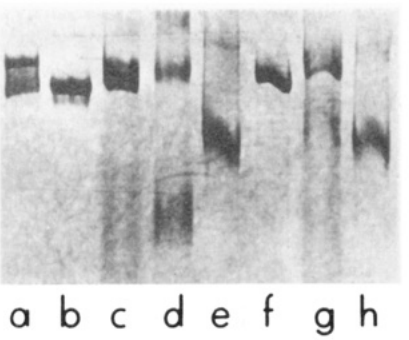

FIGURE 5: Native PAGE of α-macroglobulins. Lane a is native human α_2M, lane b is reacted with trypsin, lane c is reacted with plasmin, and lane d is acid treated (half-molecules). Lane e is native frog α_1M, lane f is reacted with trypsin, lane g is reacted with plasmin, and lane h is reacted with methylamine. Ten micrograms of α-macroglobulin was electrophoresed in each lane as described by Nelles et al. (1980) in 5% gels.

Similarly, large protease inhibitors such as SBTI are poor inhibitors of α-macroglobulin–protease complexes (Barrett & Starkey, 1973). These properties were used to provide an independent assessment of the stoichiometry of protease binding by frog α_1M. A fixed quantity of trypsin was incubated with increasing quantities of frog α_1M in the presence or absence of SBTI. BAPNA amidolytic activity was plotted as a function of frog α_1M concentration (Figure 2). The equivalence point of the reaction of protease with inhibitor was obtained from the intersection of the two linear portions of each of the curves. In the absence of SBTI, values of 0.7 ± 0.07 to 0.85 ± 0.08 mol of protease/mol of frog α_1M were calculated for two preparations of frog α_1M. In the presence of SBTI, 0.80 ± 0.08 mol of protease was bound per mole of frog α_1M.

Reaction of Frog α_1M with Methylamine. α-Macroglobulins contain reactive internal thiol ester bonds that are labile to spontaneous autolysis at high temperatue resulting in fragmentation of the macroglobulin polypeptide chain (Harpel et al., 1979; Howard et al., 1980). Heat fragmentation of α-macroglobulins may be prevented by prior reaction with methylamine. A nucleophilic attack on the thiol ester bond takes place opening the ring to form an *N*-methylglutamine residue and a free sulfhydryl group (Sottrup-Jensen et al., 1980). Frog α_1M was heated in a boiling water bath for 30 min and was then subjected to SDS–PAGE. Heat fragmentation was observed, which yielded fragments of M_r ~120 000 and 60 000 (Figure 3). Reaction with 200 mM methylamine at pH 8.0 for 2 h at room temperature is sufficient for complete reaction with human α_2M (Gonias et al., 1982b). Reaction of frog α_1M with 400 mM methylamine at pH 8.2 for 27 h at room temperature did not completely inhibit the heat fragmentation; however, significant inhibition was achieved under these conditions (Figure 3). The incorporation of methylamine was studied with [^{14}C]methylamine. Human α_2M which binds 4 mol of methylamine/mol (Sottrup-Jensen et al., 1980; Swenson & Howard, 1979b) served as a control. The frog inhibitor incorporated 1.8 ± 0.2 mol of labeled methylamine/mol of protein.

Reaction of different α-macroglobulins with methylamine inhibits protease binding to varying degrees (Feldman et al., 1984; Gonias et al., 1983). Frog α_1M was incubated with ^{125}I-trypsin after reaction with methylamine as described above, and bound protease was separated from free protease by gel filtration chromatography. Frog α_1M–methylamine bound 0.7 ± 0.1 mol of protease/mol of inhibitor, approximately the same stoichiometry as frog α_1M not treated with methylamine. However, the amount of protease that was bound covalently to frog α_1M–methylamine was decreased up to 70%. The SBTI-resistant amidolytic activity of trypsin bound to frog α_1M toward the substrate BAPNA was 30 ± 2% that of human α_2M. The activity of trypsin bound to frog α_1M–methylamine was only 18 ± 2% that of human α_2M.

Conformational Change after Reaction of Frog α_1M with Protease or Methylamine. Reaction of human α_2M with protease or methylamine results in a species that migrates faster in pore-limit electrophoresis under nondenaturing conditions, hence, the term slow to fast conformational change (Barrett et al., 1979). Under similar conditions, frog α_1M reacted with trypsin also migrates as a species with increased mobility (Figure 4). By contrast, frog α_1M subjected to native

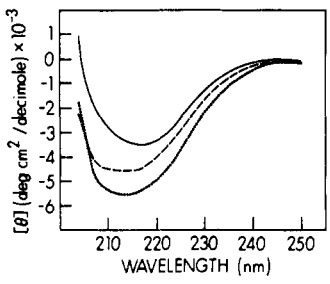

FIGURE 6: Circular dichroism spectra of frog α_1-macroglobulin. The circular dichroism spectrum of frog α_1M was examined over the region 205–250 nm. Frog α_1M (···), frog α_1M–methylamine (– – –), and frog α_1M–trypsin (—) were compared.

PAGE (5% gel) migrated significantly faster than human α_2M (Figure 5). After reaction with protease, the migration was comparable to that of human α_2M. The most likely explanation is that under electrophoretic conditions in this native 5% gel system, the frog α_1M has some tendency to migrate as half-molecules. This is not due solely to the pH of the gel system in view of the data obtained with pore-limit gels which are electorphoresed under similar buffer conditions. Moreover, ^{125}I-labeled frog α_1M coeluted with human α_2M tetramers in gel filtration studies on Utrogel AcA-22 (28 × 0.8 cm) performed at alkaline pH. We have previously demonstrated that chromatography under similar conditions resolves human half-molecules and whole molecules (Gonias & Pizzo, 1983a,b).

Reaction of frog α_1M with protease appears to stabilize the interaction between the disulfide bonded pairs. Barrett et al. (1979) found that protease- or methylamine-treated human α_2M is more stable to dissociation of the subunits than the native molecule. Furthermore, human α_2M half-molecules prepared by reduction and alkylation of the native molecule reassociate after reaction with protease or methylamine (Gonias & Pizzo, 1983a). Plasmin-treated halves do not reassociate to a great extent, however, apparently because of steric hindrance (Gonias & Pizzo, 1983b). Frog α_1M reacted with plasmin or methylamine was subjected to native PAGE. The frog α_1M treated with plasmin migrated as tetramers; frog α_1M reacted with methylamine continued to migrate as an apparent half-molecule. Thus, unlike human α_2M, the reaction of methylamine with frog α_1M does not appear to result in stabilization of the tetrameric form of the molecule.

Circular dichroism spectroscopy has been used to examine conformational change in human α_2M (Gonias et al., 1982b; Bjork & Fish, 1982). Human α_2M exhibits negative ellipticity over the region 205–250 nm with a minimum at 216 nm. Reaction with trypsin results in a decrease in absolute mean residue rotation and a shape change in the spectrum. Reaction with methylamine results in an indistinguishable change. The circular dichroic spectrum of frog α_1M is presented in Figure 6. Negative ellipticity was observed in the region 205–245 nm with a minimum at 214 nm. Reaction with trypsin resulted in a significant decrease in the absolute mean residue rotation. Reaction with methylamine resulted in a change in the same direction, but of smaller magnitude.

In Vivo Clearance in Mice. The in vivo clearance of ^{125}I-labeled frog α_1M was studied in CD-1 female mice as described previously (Figure 7) (Gonias & Pizzo, 1983a). Frog α_1M cleared slowly from the circulation of the mouse ($t_{1/2}$ > 2 h). This suggests that no determinant that may be recognized by any of the major plasma clearance receptors in a mammalian species is exposed on the frog protein. After reaction with trypsin the complex cleared rapidly ($t_{1/2}$ < 3 min). Competition studies were performed to identify those

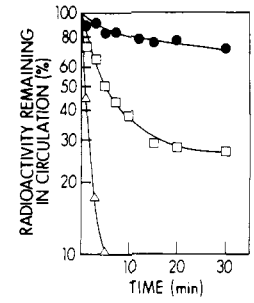

FIGURE 7: In vivo clearance of frog α_1-macroglobulin in mice. Native frog α_1M (●), frog α_1M reacted with trypsin (△), and frog α_1M reacted with trypsin in the presence of the competing ligands human α_2M–methylamine, ASOR, and Lf (□).

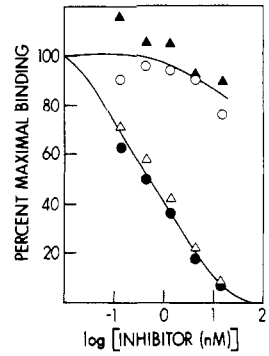

FIGURE 8: Radioreceptor displacement study. Mouse peritoneal macrophages were incubated with the probe ^{125}I-labeled human α_2M–methylamine in the presence of dilutions of frog α_1M (●), frog α_1M–methylamine (○), frog α_1M reacted with trypsin (△), and frog α_1M–methylamine reacted with trypsin (▲).

Table III: Clearance of ^{125}I-Labeled Frog α_1M in Mice

	competing ligand	half-life (min)
frog α_1M		>60
frog α_1M + trypsin		1
frog α_1M + trypsin +	ASOR + Lf	1
+	human α_2M–methylamine	2
+	ASOR + human α_2M–methylamine	2
+	Lf + human α_2M–methylamine	2
+	Lf + ASOR + human α_2M–methylamine	5

receptors that were involved in this clearance phenomenon (Table III). Optimal competition was achieved only in the presence of three competing ligands: human α_2M–methylamine, which binds to the α-macroglobulin receptor; ASOR, which binds to the galactose receptor; lactoferrin, which binds to a receptor which has not been well-defined. The interaction is at least, in part, carbohydrate dependent (Prieels et al., 1978). Human α_2M is recognized by the α-macroglobulin receptor only after reaction with protease. Interestingly, frog α_1M exhibited this pattern for α-macroglobulin receptor recognition and for carbohydrate recognition.

In Vitro Binding Studies to Mouse Peritoneal Macrophages. While in vivo clearance studies detect several recognition determinants, the binding to the mammalian α-macroglobulin receptor can be isolated in an in vitro system. The radioreceptor displacement assay detects binding only to those receptors that can bind the labeled probe, which in this case is ^{125}I-labeled human α_2M–methylamine. Thus, the in vitro binding of frog α_1M to the mouse peritoneal macrophage α-macroglobulin receptor was assessed by inhibition of cell binding of 0.2 nM ^{125}I-labeled human α_2M–methylamine (Figure 8). Native frog α_1M did not inhibit the binding of

the labeled probe significantly. Frog α_1M–methylamine also failed to inhibit the cell binding. Frog α_1M, or frog α_1M-methylamine, reacted with trypsin inhibited the cell binding of ^{125}I-labeled human α_2M–methylamine by 50% at a concentration of 0.6 nM. By use of the equation $I_{50} = K_i(1 + [L]/K_1)$ (Cheng & Prusoff, 1973), a K_i of 0.4 nM was calculated. This value is comparable to the K_d of other α-macroglobulins binding to the mouse macrophage α-macroglobulin receptor (Feldman et al., 1983, 1984; Feldman & Pizzo, 1984a).

Discussion

α-macroglobulins have been found in the plasma of all vertebrates studied as well as in the invertebrate *Limulus polyphemus*. The existence of an α-macroglobulin in the frog has been demonstrated previously (Starkey et al., 1982). In the present study frog α-macroglobulin was purified to apparent homogeneity and characterized. The protein was examined to determine which characteristics of α-macroglobulins are conserved in the amphibian macroglobulin. In particular, data on receptor recognition determinants of this amphibian glycoprotein were obtained.

Frog α_1M was purified by Ni^{2+} chelate affinity chromatography, a method that has proven useful for the purification of other α-macroglobulins. The subunit structure and molecular weight are similar to those of human α_2M. It is interesting, however, that the characteristic slow to fast conformational change of α-macroglobulins was observed for frog α_1M in pore-limit gel electrophoresis but not in standard 5% native PAGE. It was observed that frog α_1M migrates much faster in native PAGE than expected, though not as fast as human α_2M half-molecules prepared by heat denaturation. The mobility in native 5% gel is affected by both charge and size; but, in view of the gross change in mobility on binding different proteases, a charge effect alone probably does not account for the observed migration. It is likely that the molecule is exhibiting a tendency to disassociate in the 5% gel, a tendency not observed in mammalian α-macroglobulins. This may represent weaker noncovalent interaction between disulfide bonded pairs, though other interpretations cannot be ruled out. If this interpretation is correct, the weak interaction between frog subunit pairs may represent a transition from the absence of interaction in fish α-macroglobulin to the strong interaction in mammalian α-macroglobulins.

Trypsin binds to human α_2M in a 2:1 molar ratio (Barrett et al., 1979) and to other mammalian α-macroglobulins at a 1:1 molar ratio (Gonias et al., 1983; Feldman et al., 1984; Feldman & Pizzo, 1984a). A ratio of 0.6–0.8:1 was obtained for trypsin with frog α_1M. That the ratio was not 1:1 may result from several factors including the presence of inactive inhibitor, an incorrect extinction coefficient, or poor efficiency of trypsin trapping. Starkey et al. (1982) found that fish α-macroglobulin bound only 0.45 mol of trypsin/mol of inhibitor. Thus, α-macroglobulins from more primitive species may have a generally lower trypsin binding efficiency.

Frog α_1M exhibits the characteristic heat fragmentation of α-macroglobulins, and the fragmentation is inhibited by prior reaction with methylamine. The kinetics of reaction of frog α_1M with methylamine are slow compared to the reaction in other macroglobulins (Gonias et al., 1982b; Feldman et al., 1984). Thus, the value of 1.8 ± 0.2 mol of amine incorporated per mole of inhibitor may underestimate the true number of thiol esters. Slow kinetics may be protective in amphibians because byproducts of amino acid metabolism in amphibians are present in the blood and other body fluids as reactive ammonia and not inactive urea. Slow reaction kinetics may explain why only some investigators have found a methylamine-sensitive site in the *Limulus* macroglobulin (Starkey & Barrett, 1982a,b; Quigley & Armstrong, 1983). Unlike human α_2M, reaction of frog α_1M with methylamine does not result in a change in mobility in native PAGE or exposure of the receptor recognition site. This pattern of reaction with methylamine (as demonstrated by loss of heat fragmentation, decreased covalent protease binding, and [^{14}C]methylamine incorporation) without slow to fast conformational change has been recognized previously for bovine α_2M (Feldman et al., 1984; Feldman & Pizzo, 1984b) and fish α-macroglobulin (Starkey & Barrett, 1982b). A change in the conformation of the polypeptide backbone does occur, however, as measured by a change in the circular dichroic spectrum.

Frog α_1M–methylamine binds trypsin at the same ratio as the native inhibitor. This is distinct from human and bovine α_2M which lose trypsin binding capacity on reaction with methylamine (Barrett et al., 1979; Feldman et al., 1984). The amidolytic activity of the frog α_1M–methylamine–trypsin complex is reduced by 40% compared to that of frog α_1M–trypsin. This is comparable to rat α_2M which on reaction with methylamine continues to bind trypsin with decreased ability of the protease to hydrolyze low molecular weight substrates (Gonias et al., 1983).

In vivo clearance studies in mice demonstrated that frog α_1M cleared by multiple mechanisms involving the α-macroglobulin receptor and carbohydrate recognition. All these mechanisms exhibited a requirement for protease-treated α_1M, and this is the first example of carbohydrate clearance that is conformation dependent.

While the existence of α-macroglobulin homologues has been documented in many vertebrates, conservation of the α-macroglobulin receptor recognition site has been documented only in mammalian and avian species. In this study, the K_i for the binding of frog α_1M–protease complex to the mammalian α-macroglobulin receptor was the same as that of human α_2M–trypsin binding to this receptor. Recognition in mouse of frog α_1M–trypsin by carbohydrate receptors was unexpected. This raises the question of whether conformation-dependent exposure of carbohydrate may be responsible for clearance in more distant species rather than a specific pathway for α-macroglobulins.

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Registry No. Methylamine, 74-89-5; trypsin, 9002-07-7.

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