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Purification and Characterization of a "Half-Molecule" α_2-Macroglobulin from the Southern Grass Frog: Absence of Binding to the Mammalian α_2-Macroglobulin Receptor†

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ABSTRACT: An α-macroglobulin (α_2M), which is a dimer consisting of two non-disulfide-bonded subunits, was identified and purified from frog plasma by Ni^{2+} chelate affinity chromatography. This frog "half-molecule" α-macroglobulin migrated as an α_2-globulin in cellulose–acetate electrophoresis rather than as the previously described frog α_1M, which exists as a tetramer formed by the noncovalent association of disulfide-bonded pairs. A molecular weight of ~380 000 was obtained by gel-filtration high-pressure liquid chromatography, and in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) the protein migrated as a single band of M_r ~180 000 before and after reduction. No evidence was obtained for association of this protein to a higher molecular weight species. After the preparation was heated, additional bands were obtained in SDS–PAGE with M_r ~60 000 and 12 000. The additional bands were not obtained after heating methylamine-treated preparations. The circular dichroic spectrum of frog α_2M exhibits negative ellipticity over the region 205–250 nm with a minimum at 216 nm. After reaction with proteinase, a decrease in the absolute mean residue rotation was obtained. Amino acid analysis demonstrated that frog α_2M and α_1M are similar in composition to avian and mammalian α-macroglobulins; however, there are sufficient differences in the composition of these two amphibian α-macroglobulins to support the conclusion that they are distinct proteins. Frog α_2M bound approximately 0.5 mol of trypsin/mol of inhibitor. This binding was abolished by pretreatment with methylamine. Frog α_2M, in contrast to α_1M, exhibited no specific binding to the mammalian α-macroglobulin receptor in either direct or indirect binding assays. It is concluded that two distinct α-macroglobulins are present in the frog and that naturally occurring half-molecule α-macroglobulins are not recognized by the mammalian α-macroglobulin receptor.

Human α_2-macroglobulin (α_2M)[1] is a high molecular weight plasma proteinase inhibitor (M_r ~720 000) formed by the noncovalent association of disulfide-bonded pairs of identical subunits (Harpel, 1973; Hall & Roberts, 1978; Swenson & Howard, 1979a; Barrett, 1981). By a unique mechanism, termed "trapping", it is capable of inhibiting endopeptidases of each of the four mechanistic classes (Barrett and Starkey, 1973). α_2M contains a region highly susceptible to proteolytic cleavage termed the "bait" region (Barrett & Starkey, 1973). On reaction of a proteinase at this site, a

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[1] Abbreviations: α_1M, α-macroglobulin; α_2M, α_2-macroglobulin; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SBTI, soybean trypsin inhibitor; DEAE, diethylaminoethyl.

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conformational change occurs in the inhibitor that results in irreversible association of the proteinase and the inhibitor (Barrett & Starkey, 1973). The proteinase active site is not blocked in this process. While the activity of the proteinase against small substrates is only marginally affected, the activity against large substrates is greatly reduced. Similarly, small inhibitors such as tosyllysine chloromethyl ketone may inhibit bound proteinase, while large inhibitors such as soybean trypsin inhibitor (SBTI) are poor inhibitors of the complex.

Irreversible association of proteinases to α_2M does not require covalent bond formation; however, a covalent bond between the proteinase and the inhibitor may form by nucleophilic attack of a lysine residue of the proteinase on a specific thiol ester bond of the inhibitor (Swenson & Howard, 1979b; Sottrup-Jensen et al., 1980). Reaction of small amines at this site also results in cleavage of the thiol ester, and a conformational change in the inhibitor occurs, which is similar to that observed upon reaction with proteinase (Barrett et al., 1979). Heat denaturation of α_2M results in autolytic cleavage of the polypeptide chain at the thiol ester site (Harpel et al., 1979; Howard et al., 1980).

The conformational change observed after reaction of α_2M with proteinase or amine results in exposure of a specific receptor recognition site on the inhibitor (Debanne et al., 1975; Imber & Pizzo, 1981; Kaplan et al., 1981). The receptor for the protein is located on numerous cell types (Gliemann et al., 1983; Ney et al., 1984) and is responsible for the clearance of α_2M–proteinase complexes from the circulation (Imber & Pizzo, 1981). The binding of α_2M to its receptor has regulatory activity in some systems (Hoffman et al., 1983).

Human α_2M is capable of binding many proteinases at a 2:1 ratio (Howell et al., 1983), and several studies indicate that the "half-molecule" of α_2M is the functional unit (Pochon et al., 1981; Gonias & Pizzo, 1983a; Bjork et al., 1984; Strickland & Bhattacharya, 1984). Inactivated "half-molecules" of human α_2M may be obtained by mild acid treatment or urea denaturation of the molecule (Barrett et al., 1979). These halves are composed of disulfide-bonded subunit pairs. Functional half-molecules of α_2M, however, have been isolated by limited reduction and alkylation of the parent molecule (Gonias & Pizzo, 1983a). Such a preparation exhibits proteinase binding, with decreased steric hindrance of the proteinase. The stability of the tetrameric form of human α_2M is increased by reaction with proteinase or amine; after reaction with proteinase or methylamine, half-molecules of α_2M formed by reduction and alkylation will reassociate (Gonias & Pizzo, 1983a).

α_2M is highly conserved in nature, and homologues have been demonstrated in the plasma of a large number of vertebrates and the invertebrate *Limulus polyphemus* (Starkey & Barrett, 1982; Quigley & Armstrong, 1983). Several of the nonmammalian α_2M homologues have been purified to homogeneity (Starkey et al., 1982; Nagase et al., 1983; Feldman & Pizzo, 1984a, 1985). Generally, the structure of these proteins is similar to that of human α_2M with subtle differences. Fish α_2M homologues, however, exist as half-molecules (Starkey et al., 1982).

Qualitatively, the α_2M homologues exhibit similar proteinase inhibitory activity and reactivity with small amines. After reaction with proteinase, mammalian α_2M homologues and the homologous proteins from the chicken and the frog bind to the mouse α_2M receptor (Feldman et al., 1984; Feldman & Pizzo, 1984a, 1985). The α_2M receptor recognition site is well conserved since the mammalian, chicken, and frog α-macroglobulin homologues exhibit similar affinity for the receptor (Feldman et al., 1984; Feldman & Pizzo, 1984a, 1985). More distant α_2M homologues, such as the naturally occurring half-molecule α_2M of the fish, have not been examined for binding to this receptor.

We have previously described a model of the structure and function of α-macroglobulins (Feldman et al., 1985). The model is generally cylindrical and is composed of two functional "halves", separated by a plane perpendicular to and bisecting the long axis of the cylinder. Proteinases are trapped by movement of two trap arms in each functional half. We have hypothesized that this trap-arm movement exposes a receptor-recognition site located at the base of the arm on the base ring of the functional half. Thus, the dissociation of the halves could result in exposure of the site, a hypothesis that is consistent with published results (Feldman et al., 1985). A further prediction of this model is that a naturally occurring half-molecule α_2-macroglobulin cannot have this same receptor-recognition site as it would be exposed at all times and would clear from the circulation without first reacting with proteinase.

This paper examines a previously undetected α_2M homologue from the plasma of the Southern grass frog (*Rana pipiens*). This protein has the structure of a half-molecule α_2M homologue, as well as characteristic trypsin binding and amine reactivity. Distinctions between this protein and the previously described frog α_1M, containing four identical subunits, are presented. Finally, the interaction of this molecule with the mammalian α_2M receptor is examined.

The frog half-molecule α_2M homologue will be designated as frog α_2M on the basis of its migration in standard protein electrophoresis in order to differentiate it from frog α_1M, a "whole-molecule" α-macroglobulin that migrates as an α_1-globulin (Feldman & Pizzo, 1985).

Materials and Methods

Materials. Outdated human plasma was obtained from the Durham Veterans Administration Medical Center. Frog plasma was obtained diluted 1:5 with 20 mM sodium phosphate, 110 mM NaCl, and 1% heparin, pH 7.0, by cardiac puncture of pithed Southern grass frogs (*Rana pipiens*). Trypsin was obtained from Sigma and methylamine hydrochloride from Aldrich. Thioglycolate-elicited mouse peritoneal macrophages were provided generously by Dr. Dolph O. Adams, Department of Pathology, Duke University Medical Center, and were plated as previously described (Imber et al., 1982). Carrier-free sodium ^{125}I was from New England Nuclear. Lactoperoxidase, coupled to Sepharose, was obtained from P-L Biochemicals.

Isolation of α-Macroglobulin. Human α_2M was purified by the Zn^{2+} chelate affinity chromatography method of Kurecki et al. (1979) as later modified (Imber & Pizzo, 1981). Frog α_1M, the tetrameric homologue of human α_2M was isolated by Ni^{2+} chelate affinity chromatography as previously described (Feldman & Pizzo, 1985). Frog α_2M also was isolated by Ni^{2+} chelate affinity chromatography. Frog plasma, diluted as above, was brought to 12% in poly(ethylene glycol) 8000, 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 and 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 and 0.8 M NaCl, pH 6.5, and of 0.02 M sodium phosphate, 0.15 M NaCl, and 0.02 M EDTA, pH 7.4. After concentration with a Millipore CX-10 ultrafilter, the preparation was applied to an Ultrogel AcA-22 column (2.5 × 70 cm) equilibrated with 0.02 M

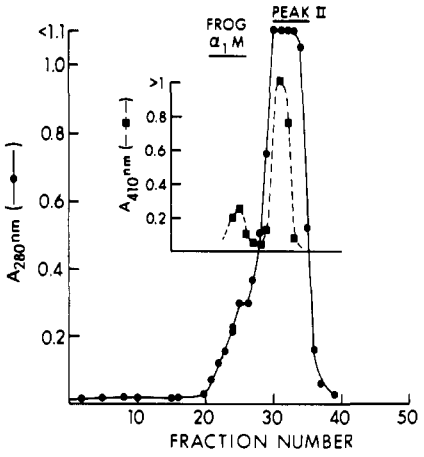

FIGURE 1: Elution profile of the gel-filtration step in the preparation of frog α_2M. After PEG precipitation and Ni^{2+} chelate affinity chromatography, gel filtration was performed on an Ultrogel AcA-22 column (2.5 × 70 cm), equilibrated and eluted as described under Materials and Methods. The inset represents the Ganrot activity assay (Ganrot, 1966) performed on the samples.

sodium phosphate and 0.15 M NaCl, pH 7.4, and was eluted at 10 mL/h. Fractions that eluted at the same volume as human α_2M contained purified frog tetrameric α-macroglobulin (α_1M). A second peak, which also exhibited the ability to protect trypsin from SBTI, was obtained at a greater elution volume (Figure 1). This material was dialyzed against 4 L of 0.02 M sodium phosphate and 0.02 M NaCl, pH 8.0, and was applied to a DEAE-cellulose column (3.5 × 10 cm) equilibrated in the same buffer. The column was washed with 100 mL of this buffer followed by a 150-mL gradient consisting of 75 mL of the above buffer and 75 mL of 0.02 M sodium phosphate and 0.2 M NaCl, pH 8.0. The first peak obtained by this procedure contained the purified frog half-molecule α-macroglobulin (α_2M).

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Wyckoff et al. (1977). Trypsin was inhibited prior to denaturation with phenylmethanesulfonyl fluoride. Standard protein electrophoresis was performed in cellulose–acetate gels in the Duke University Medical Center Coagulation Laboratory.

Gel-Filtration High-Pressure Liquid Chromatography (HPLC). Gel-filtration HPLC was performed on a LKB HPLC equipped with a Model 2150 pump, a Model 2152 controller, and a TSK G3000 SW gel-filtration column (7.5 × 300 mm). The column was equilibrated with 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4. Frog α_2M (30 μg) was applied and eluted in the same buffer. Spectra of the two peaks that eluted were obtained with an LKB Model 2140 rapid spectral detector. An LKB Model 2220 recording integrator was used to quantify the relative absorbances of the two peaks. The molecular weight of frog α_2M was determined by comparison of its retention time (R_t) to those of several standards in a plot of R_t vs. log molecular weight. The following proteins were used as standards: bovine serum albumin, aldolase, catalase, phosphorylase A, ferritin, thyroglobulin, and human α_2M.

Chemical Composition Studies. Amino acid analysis was performed on a Beckman 6300 high-performance amino analyzer. The frog proteins were hydrolyzed in vacuo for 24 h at 110 °C in 6 N HCl prior to analysis.

Protein Radioiodination. Proteins were labeled with ^{125}I by the solid-state lactoperoxidase method of David & Reisfeld (1974).

Gel-Filtration Studies. The apparent size of frog α_2M was compared to human α_2M by filtration on Ultrogel AcA-22. Five microliters of 0.2 g/L ^{125}I-labeled frog α_2M was cochromatographed with 100 μL of 10 g/L human α_2M on a 0.8 × 28 cm column. The column was equilibrated with 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4, and eluted at 2 mL/h, and 0.5-mL fractions were collected. In some studies the ^{125}I-labeled frog α_2M was reacted with a 3-fold molar excess of trypsin before chromatography. ^{125}I-Labeled human α_2M, which had been reacted with 40 mg/mL dithiothreitol at 37 °C for 60 min, was chromatographed on this column to mark the position of half-molecules and "quarter molecules".

Circular Dichroic Spectroscopy. These studies were performed as described previously (Gonias et al., 1982). A mean residue weight of 110.0 obtained from the amino acid composition was used to calculate the spectra.

Methylamine Reactivity. The reaction of frog α_2M with methylamine was performed as previously described (Feldman et al., 1984). Heat fragmentation was effected by heating samples in boiling water for 30 min.

Radiolabeled Proteinase Incorporation. Proteinase incorporation studies were performed as described in detail previously (Feldman & Pizzo, 1985). Briefly, a 3-fold excess of ^{125}I-labeled trypsin to α_2M was used followed by a 2-fold excess of SBTI over trypsin. Gel filtration on Ultrogel AcA-22 was used to separate free proteinase from the α_2M–proteinase complex.

α_2-Macroglobulin Binding Studies. Radioreceptor displacement and direct binding studies were performed. The radioreceptor assay was performed as described previously (Feldman et al., 1984) with 0.2 nM ^{125}I-labeled human α_2M–methylamine as the tracer in binding studies with mouse macrophages at 4 °C. Direct binding studies of ^{125}I-labeled frog α_2M–trypsin were performed as described by Imber & Pizzo (1981) for human α_2M.

Protein Concentrations. The concentrations of human α_2M and frog α_1M were determined spectrophotometrically by using the extinction coefficients $E^{1\%,1cm}$ at 280 nm of 8.93 (Hall & Roberts, 1978) and 8.2 (Feldman & Pizzo, 1985), respectively. The extinction coefficient for frog α_2M was 9.5 as determined by the Lowry protein assay (Lowry et al., 1951) with bovine serum albumin as the standard.

RESULTS

Isolation of Frog α_2M. The frog α_2M half-molecule homologue was isolated from frog plasma by a continuation of the Ni^{2+} chelate affinity chromatography procedure used to isolate frog α_1M (Feldman & Pizzo, 1985). The process involves four steps: PEG precipitation, Ni^{2+} chelate affinity chromatography, Ultrogel AcA-22 gel-filtration chromatography, and DEAE-cellulose ion-exchange chromatography. A yield of 7 mg of protein was obtained from 60 mL of plasma. In standard protein electrophoresis the preparation migrated as an α_2-globulin, hence the designation frog α_2M. This is in contrast to the previously reported frog α-macroglobulin that consists of four subunits and migrates as an α_1-globulin (Feldman & Pizzo, 1985).

Purity of this preparation was determined by SDS–PAGE (Figure 2). Under either nonreducing or reducing conditions, the preparation migrated as a single band with a subunit molecular weight of approximately 180000. The lower molecular weight band seen in Figure 2 probably represents unavoidable proteinase contamination often seen in the preparation of α-macroglobulins. Consistent with this hypothesis, the contaminant was not observed in native PAGE

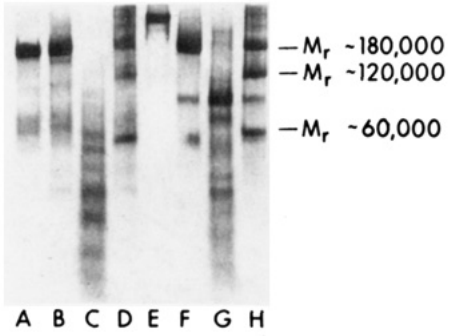

FIGURE 2: SDS–PAGE of frog α_2M (lanes A–D) and human α_2M (lanes E–H). Lanes A and E are unreduced; lanes B and F were reduced with dithiothreitol. Lanes C and G are samples reacted with trypsin, and lanes D and H are preparations that demonstrate heat fragmentation. SDS–PAGE was performed in 5% gels as described by Wyckoff et al. (1977).

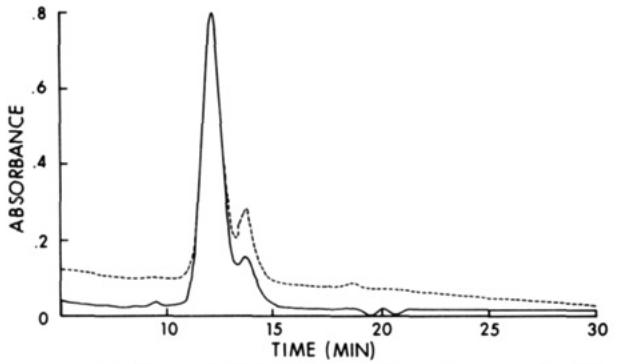

FIGURE 3: Gel-filtration HPLC of frog α_2M. A 30-μg sample of frog α_2M was fractionated on a TSK G3000 SW column (7.5 × 300 nm). The column was equilibrated and eluted with 0.02 and 0.15 M NaCl, pH 7.4. Absorbance was monitored at 205 (—) and 280 nm (---).

(data not shown). The molecular weight of frog α_2M was determined by comparison of its retention time (R_t) in gel-filtration HPLC with several standards for which a plot of R_t vs. log molecular weight was constructed. By this technique a molecular weight of 380 000 was obtained. This molecular weight, equal to two subunits as determined by SDS–PAGE, indicates that the frog α_2M is a half-molecule homologue formed by the noncovalent association of a pair of subunits. Thus, structurally, frog α_2M resembles the functionally active half-molecules of human α_2M, which result from limited reduction and alkylation of the parent molecule (Gonias & Pizzo, 1983a).

During gel-filtration HPLC, a second peak comprising approximately 10% of the total absorbance at 205 nm was obtained at a greater retention time (Figure 3). In order to demonstrate that this did not represent frog α_2M subunits, the spectrum of each of the two peaks was obtained over the interval 190–290 nm. The spectra obtained were not consistent with the hypothesis that the second peak represents single subunits of the material in the first peak (data not shown). In fact, this peak appears to represent a nonproteinaceous contaminant.

Gel-filtration chromatography resolves half-molecules of human α_2M from the native, tetrameric protein (Gonias & Pizzo, 1983a). Frog α_2M eluted from an Ultrogel AcA-22 column as two peaks that were retained longer than human α_2M (Figure 4). These peaks eluted at the same volumes as human α_2M half-molecules and quarter molecules (single subunits). The second of the two peaks represents a highly iodinated contaminant and not dissociated subunits of frog α_2M as assessed by SDS–PAGE and autoradiography of the eluted material (data not shown). Moreover, no evidence was

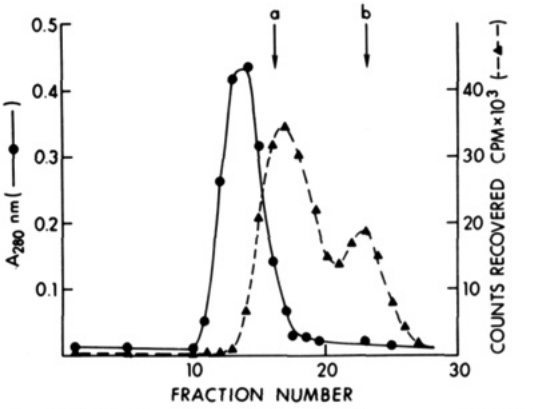

FIGURE 4: Gel-filtration chromatography of human α_2M (●) and ^{125}I-labeled frog α_2M–trypsin (▲). A 1-mg sample of human α_2M was cochromatographed with 1 μg of ^{125}I-labeled frog α_2M–trypsin on an Ultrogel AcA-22 column (0.8 × 28 cm). The column was eluted at 2 mL/h, and 0.5-mL fractions were collected. The elution profile of ^{125}I-labeled frog α_2M was the same as before and after reaction with proteinase. The elution positions of human α_2M half-molecule and quarter molecules are labeled "a" and "b", respectively, in this figure.

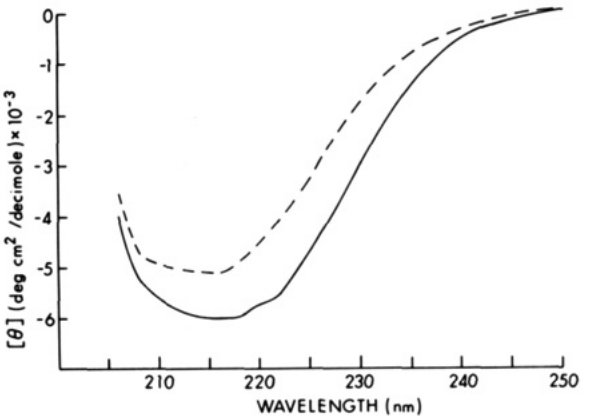

FIGURE 5: Circular dichroic spectra of frog α_2M and frog α_2M–trypsin. The spectrum of frog α_2M is represented by the solid line and of frog α_2M–trypsin by the dashed line.

found for dimerization of frog α_2M even after reaction with proteinase. As with the fish α_2M homologue (Starkey et al., 1982), the frog α_2M half-molecules do not associate after reaction with proteinase.

Circular Dichroic Spectroscopy. Evidence that frog α_2M is to be considered in that class called α-macroglobulins was obtained from circular dichroic spectroscopy (Figure 5). Frog α_2M exhibits a spectrum similar to that of both human and nonhuman α_2M proteins with negative ellipticity over the region 205–250 nm and a minimum at 216 nm (Feldman & Pizzo, 1984b). Moreover, on reaction with proteinase, it undergoes a conformational change in secondary structure similar to the conformational change of other α-macroglobulins: a decrease in the absolute mean residue rotation is obtained with a change in the shape of the spectrum (Feldman & Pizzo, 1984b).

The spectra were analyzed by the methods of Greenfield & Fasman (1969) and Chen et al. (1972), and the results are presented in Table I. Similar to other α-macroglobulins (Feldman & Pizzo, 1984b), frog α_2M exhibits little α-helix, which decreases after reaction with proteinase. Theoretical analysis provided a poor fit to the spectra. The remaining structure was approximately 30% β when analyzed by the method of Greenfield & Fasman (1969). By the method of Chen et al. (1972), the remaining structure was essentially all non-α, non-β. These numerical analyses are presented to

Table I: Numerical Data Obtained by Circular Dichroic Spectroscopy for Frog α_2M

	λ (nm)[a]	$[\theta]$ [(deg-cm^2/dmol) $\times 10^{-3}$]	% α[b]	% α[c]	% β[b]
frog α_2M	216	−6.1	4.5	11	33
frog α_2M with trypsin	216	−5.1	2	6	27

[a] Wavelength and ellipticity are the values at the minimum of the spectrum. [b] Analysis by the method of Greenfield & Fasman (1969). [c] Analysis by the method of Chen et al. (1972).

Table II: Amino Acid Composition of Frog α_2M

	% composition			
	frog α_2M	frog α_1M[a]	chicken αM[b]	bovine α_2M[c]
Asp	11.0	10.2	7.9	9.2
Thr	4.9	7.3	7.2	7.0
Ser	10.8	8.5	9.2	7.9
Glu	13.2	12.5	13.2	12.7
Pro	4.5	5.8	5.7	6.9
Gly	7.9	6.8	6.1	7.2
Ala	4.3	6.1	6.7	6.8
Val	4.3	6.0	7.0	7.8
Met	1.6	1.6	1.1	1.9
Ile	2.9	4.6	4.2	3.4
Leu	8.5	9.9	10.5	9.4
Tyr	2.8	4.1	4.0	3.6
Phe	3.7	4.4	4.7	4.4
His	2.9	2.4	2.4	2.6
Lys	7.1	6.8	6.8	5.3
Arg	3.7	3.1	3.6	3.9

[a] Feldman & Pizzo, 1985. [b] Feldman & Pizzo, 1984a. [c] Feldman et al., 1984.

quantify the similarity of frog α_2M to other α-macroglobulins; the values obtained may not correspond closely to actual structure.

Chemical Composition Studies. The amino acid composition of frog α_2M is presented in Table II with the compositions of frog α_1M, chicken αM, and bovine α_2M for comparison. The amino acid compositions of frog α_2M and other α-macroglobulins were generally similar. Significant differences, however, were observed between frog α_1M and frog α_2M when the values for Thr, Ser, Ala, Val, Ile, and Tyr were examined, supporting the conclusion that these are distinct proteins.

Methylamine Reactivity and Heat Fragmentation. One characteristic aspect of structure shared by all plasma α-macroglobulins is an internal thiol ester bond. This bond may react with small amines. An intact thiol ester bond is responsible for the heat fragmentation observed in α-macroglobulins. The heat fragmentation of frog α_2M (Figure 6) occurs as with human α_2M and frog α_1M. Notably, the heat-fragmentation pattern of frog α_2M is distinct from that of frog α_1M, providing further evidence that these are different proteins. No change in heat fragmentation is obtained when half-molecules of human α_2M are prepared from the native tetrameric protein (Gonias & Pizzo, 1983a); therefore, the observed behavior of the frog proteins could not result from dissociation of a tetrameric protein to a half-molecule. As with other α-macroglobulins, this fragmentation is prevented by prior reaction of the protein with methylamine.

Stoichiometry of Trypsin Binding. Human α_2M binds 2 mol of trypsin/mol of inhibitor (Barrett et al., 1979; Howell et al., 1983). Frog α_1M bind 0.7 mol of trypsin/mol (Feldman & Pizzo, 1985). The binding of ^{125}I-labeled trypsin to frog α_2M was examined. A value of 0.53 ± 0.03 mol of trypsin/mol of macroglobulin was obtained. This is comparable to the 0.45 mol of proteinases bound per mole of fish α_2M homologue obtained by Starkey et al. (1982) and the 0.50:1 ratio obtained for human α_2M half-molecules (Gonias & Pizzo, 1983a).

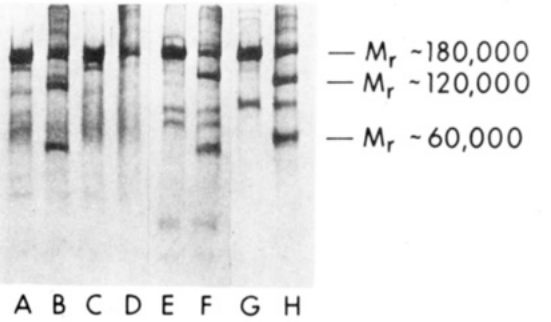

FIGURE 6: Heat fragmentation of frog α_2M. Lanes A and B are reduced frog α_2M, lanes C and D are reduced frog α_2M–methylamine, lanes E and F are reduced frog α_1M, and lanes G and H are reduced human α_2M. Lanes B, D, F, and H have been heat-denatured at 100 °C for 30 min. SDS–PAGE was performed in 5% gels as described by Wyckoff et al. (1977). Low molecular weight fragments (M_r ~90 000) in the frog α_2M preparation probably represent a slight contaminant of the proteinase reacted form.

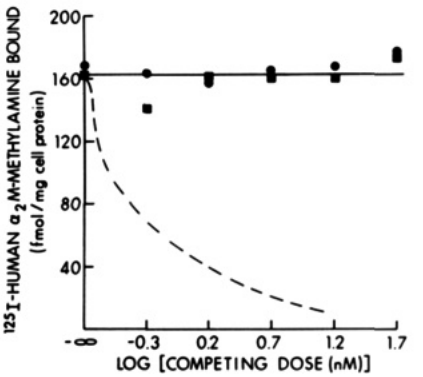

FIGURE 7: Radioreceptor displacement assay of frog α_2M. Cells were incubated with ^{125}I-labeled human α_2M–methylamine and various concentrations of frog α_2M (●) and frog α_2M–trypsin (■). The dashed line represents human α_2M–methylamine in this assay.

The proteinase binding activities of human α_2M and rat α_1M are abolished by pretreatment with methylamine (Barrett et al., 1979; Gonias et al., 1983). By contrast, rat α_2M and frog α_1M retain inhibitory activity after reaction with methylamine (Gonias et al., 1983; Feldman & Pizzo, 1985). Frog α_2M, after reaction with methylamine, bound only 0.03 mol of trypsin/mol of inhibitor, providing further evidence that frog α_2M is distinct from frog α_1M.

Radioreceptor Displacement Studies. The binding of frog α_2M to the mammalian α_2M receptor was assessed by the ability of the protein to compete for the receptor binding of a low concentration of human α_2M–methylamine. Studies were performed on plated mouse macrophages at 4 °C using 0.2 nM ^{125}I-labeled human α_2M–methylamine as a probe; frog α_2M and frog α_2M–trypsin were used to compete against the labeled probe. No competition by the frog protein was observed even after reaction of the frog α_2M with trypsin (Figure 7). The highest concentration of frog α_2M used was 50 nM.

Direct Binding Studies. Evidence in the literature suggests that there are four α_2M receptor-recognition sites per α_2M molecule (Marynen et al., 1981; Strickland et al., 1984); thus, a half-molecule of α_2M may have only two such sites. The binding of a ligand with four sites capable of binding to surface receptors may exhibit an off-time, K_{off}, far greater than that predicted from its on-time, K_{on}, and its dissociation constant, K_d, since the presence of multiple interacting sites violates the assumptions of the simple analysis (Reynolds, 1979). Thus,

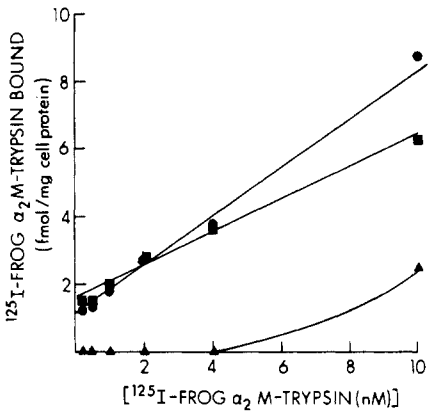

FIGURE 8: Direct binding of ^{125}I-labeled frog α_2M–trypsin to mouse peritoneal macrophages. The binding of ^{125}I-labeled frog α_2M–trypsin to cells was determined over the concentration range 0.2–10 nM. Nonspecific binding was determined in the presence of 100-fold excess unlabeled human α_2M–methylamine. Total binding (●), nonspecific binding (■), and specific binding (▲). No significant specific binding was obtained.

a competition study of a ligand with two binding sites against one that has four sites may not reveal the presence of a receptor-recognition site, even where the recognition site has a similar dissociation constant for the receptor.

Direct binding studies of frog α_2M to mouse macrophages were performed to circumvent this difficulty. The binding of ^{125}I-labeled frog α_2M–trypsin was assessed over the concentration range 0.2–10 nM. Nonspecific binding was determined in the presence of a 100-fold molar excess of human α_2M–methylamine or 5 mM EDTA. A representative study is shown in Figure 8. No significant specific binding was obtained at any concentration. Total binding at 10 nM ^{125}I-labeled frog α_2M–trypsin was only about 5% of the specific binding of human α_2M obtained at 0.2 nM.

Discussion

α-Macroglobulins have been found in all the vertebrates that have been studied (Starkey & Barrett, 1982). Many species have two α-macroglobulins, including the rat and dog (Gordon, 1976; Ohlsson, 1971). In the rat, one of these two α-macroglobulins represents an acute phase reactant (Ganrot, 1973). This paper presents the purification and characterization of a second α-macroglobulin from the Southern grass frog (*Rana pipiens*). Evidence that this protein is distinct from the previously described frog α-macroglobulin (Feldman & Pizzo, 1985) includes differences in electrophoretic behavior (Figure 2), amino acid composition (Table II), changes in proteinase binding on reaction with methylamine, peptide pattern obtained from heat fragmentation (Figure 6), and binding to the mammalian α-macroglobulin receptor (Figures 7 and 8).

The structure of this second α-macroglobulin, frog α_2M, is homologous to the functional half-molecule of human α_2M prepared by limited reduction and alkylation of the parent molecule (Gonias & Pizzo, 1983a). Evidence for this hypothesis includes secondary structural similarities shared by frog α_2M and human α_2M as measured by circular dichroic spectroscopy. Both proteins exhibit negative ellipticity over the range 210–250 nm with minima around 216 nm. Furthermore, the quaternary structure of frog α_2M appears similar to that of human α_2M half-molecules on the basis of SDS–PAGE and gel-filtration HPLC. The two proteins are both composed of two subunits of M_r ~180 000 bound by noncovalent forces.

Frog α_2M binds trypsin in a molar ratio similar to that for both naturally occurring half-molecules and those prepared by reduction of human α_2M. While α-macroglobulins characteristically undergo a conformational change on binding proteinases, the fish α_2M homologue does not exhibit a conformational change after binding proteinase as measured by pore-limit gel electrophoresis (Starkey et al., 1982). Frog α_2M also does not exhibit a conformational change in pore-limit gels after reaction with proteinase (data not shown). It was shown previously that circular dichroic spectroscopy is a more sensitive technique for the study of a conformational change in α-macroglobulins than gel electrophoresis (Feldman & Pizzo, 1984b). By this technique, frog α_2M exhibits a change similar to that for other α-macroglobulins after reaction with proteinase: a decrease in the absolute mean residue rotation is obtained with a shape change of the spectrum.

Frog α_2M also exhibits the characteristic reactions of a molecule that contains a thiol ester bond: methylamine reactivity and heat fragmentation. Further evidence of reactivity of frog α_2M with methylamine included loss of proteinase binding activity and heat fragmentation of frog α_2M on reaction with the amine.

The stability of the noncovalent intersubunit interaction must be considered as well. Frog α_2M exhibited a tendency to dissociate in both 5% native and pore-limit gel electrophoresis (data not shown). Frog α_1M tends to migrate as a half-molecule in 5% native PAGE, though in gel-filtration studies, pore-limit PAGE, and sedimentation equilibria it exhibits the characteristics of whole molecules (Feldman & Pizzo, 1985). In mammalian α-macroglobulins, no tendency to dissociate is observed except under denaturing conditions.

An exception to these last remarks must be made on the basis of the recent presentation of an α-macroglobulin from the mouse that appears to be a quarter molecule or single subunit (Saito & Sinohara, 1985). This protein, termed murinoglobulin, exhibits the characteristic proteinase and amine reactivity of α-macroglobulins and a molecular weight of about 180 000 in vitro. Some evidence for association to higher molecular weight species was obtained by gel electrophoresis, however. In view of the evidence that two 180 000-dalton subunits are required for functional activity (Gonias & Pizzo, 1983a,b) and the tendency of some of the α-macroglobulins to dissociate (Feldman & Pizzo, 1985), it is tempting to hypothesize that murinoglobulin functions in a half-molecule form in vivo.

The frog α_2M is the first example of a naturally occurring plasma α-macroglobulin that is not recognized by the mammalian α_2M receptor. The clearance of this protein from the frog circulation remains to be defined, but studies in the mouse suggest that the protein does not clear by a mechanism where competition by human α_2M–methylamine occurs (unpublished observations).

A model of the structure of α-macroglobulin is presented in Figure 9. We have hypothesized that the receptor-recognition site is located at the base of the functional half, masked at the interface between the halves and exposed with trap-arm movement (Feldman et al., 1985). This site is present within a functional half, since half-molecules clear rapidly from the circulation without association to the tetrameric protein (Gonias & Pizzo, 1983b). We have predicted that naturally occurring half-molecules could not have this receptor-recognition site as it would be exposed constantly within the circulation, resulting in rapid clearance of the protein.

The existence of a frog half-molecule α-macroglobulin is a fortuitous one as it allows this prediction to be tested. The mammalian α-macroglobulin receptor recognizes frog whole-molecule α-macroglobulin with an affinity similar to

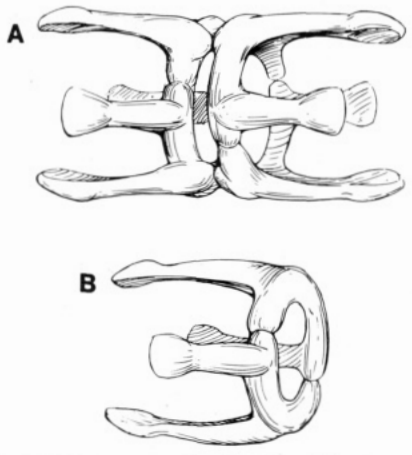

FIGURE 9: A model of macroglobulin. Panel A represents the tetrameric protein such as human or bovine α_2M and frog α_2M (whole molecule). Panel B represents a half-molecule either produced chemically from human α_2M or naturally occurring such as fish or frog α_2M.

that of mammalian α-macroglobulins. Thus, absence of recognition of the frog half-molecule by the mammalian receptor is not due to simple evolutionary distance between amphibians and mammals. We speculate that the development of this receptor–recognition site may have occurred with the change involved in creating an interface of association of subunit pairs (half-molecules) to form a tetrameric protein. We therefore conclude that future investigation should discover no naturally occurring plasma half-molecule homologues that bind to the mammalian α-macroglobulin receptor.

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