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The Properties of Rabbit α_1-Macroglobulin upon Activation Are Distinct from Those of Rabbit and Human α_2-Macroglobulins

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We have characterized native and activated forms of rabbit α_1M and compared them to rabbit and human α_2M. Similar to human α_2M, rabbit α_1M is a tetramer associated *via* disulfide bonds and non-covalent interactions that exhibits autolysis into two fragments when heated. Like human α_2M, rabbit α_1M is cleaved by trypsin at one site; however, rabbit α_1M shares characteristics with rabbit α_2M that are different from the properties of human α_2M. Amine or trypsin treatment of rabbit α-macroglobulins does not result in a significant conformational change or cleavage of four thiolester bonds. Full thiolester cleavage is only observed for rabbit α_1M after exposure to both trypsin and a small amine. Additionally, amine-treated rabbit α-macroglobulins retain trypsin inhibitory potential and do not fully shield bound proteinases. Methylamine and trypsin treatment of rabbit α_1M results in two dissimilar conformations that display differing exposure of the receptor-recognition site. While ammonia- and methylamine-modified rabbit α_1M bind to macrophages with similar affinity to that of human α_2M, trypsin-treated rabbit α_1M exhibits dramatically lower affinity. This suggests that rabbit α_1M may not play the same proteinase-inhibiting physiological role as human α_2M.

Key words: α_2-macroglobulin, α-macroglobulin receptor binding, proteinase inhibitor and bait region, rabbit α_1-macroglobulin, thiolester cleavage.

Abbreviations: α_1M, α_1-macroglobulin; α_2M, α_2-macroglobulin; α_2M*, receptor-recognized form of α_2M; BApNA, benzoyl-L-arginine *p*-nitroanilide; CFA, complete Freund's adjuvant; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); ^{125}I-α_2M, α_2M labeled with iodine-125; LRP, low-density lipoprotein receptor–related protein; NH_2, amino-terminal; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RBP, receptor binding fragment of α_2M; SBTI, soybean trypsin inhibitor; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

The plasma proteinase inhibitor α_2-macroglobulin (α_2M) is a tetramer composed of identical subunits, with the human form exhibiting a molecular weight of approximately 720,000 (*1*, *2*). The tetramer consists of two pairs of subunits associated through interactions, which include disulfide bonds between one subunit pair and non-covalent interactions between the other (*3*). There are two regions per $M_r \sim 180{,}000$ subunit that are the focus of α_2M functional activity. The first consists of a labile thiolester bond, which is formed by a glutamine residue (Glx-952) esterified to a cysteinyl residue (Cys-949), based on human numbering of α_2M (*4*–*7*). The second ~40-residue region (residues 666–706) (*8*, *9*), known as the "bait" region (*10*), is a site of limited proteolysis and contains the primary cleavage sites for serine, cysteine, aspartic, and metallo-proteinases (*9*, *11*, *12*). α_2M is secreted by a range of cells in its native form (*13*, *14*), which is defined by the presence of intact thiolesters, intact bait regions, and an overall structure in an "open" configuration. This native form is not receptor-recognized and is capable of inhibiting various proteinases if they are able to cleave the intact bait region (*15*). Once a proteinase cleaves the bait region, it becomes entrapped in the "cage" of α_2M, and the thiolesters undergo hydrolysis or nucleophilic substitution creating four free thiols per molecule (*4*). As a result, the overall conformation of the human protein is altered to create a more compact structure with an exposed receptor-recognition site on each subunit. This receptor-recognized form of α_2M is designated α_2M*. Additionally, the thiolesters may be chemically modified through direct nucleophilic attack using small primary amines (*16*, *17*). Although this α_2M* (*18*) is not entirely equivalent to the proteinase-bound form, it also contains free thiols, a more compact configuration, and exposed receptor-recognition sites (*19*, *20*).

α_2M has been implicated in the regulation of certain aspects of the immune system through cytokine binding and receptor-mediated antigen delivery (*21*–*23*). More recently, α_2M has shown promise as a potent vaccine adjuvant by creating increased immune reactivity toward specific antigens (*24*, *25*). Naturally occurring human α_2M will be an ideal tool for vaccine development if it is as effective as complete Freund's adjuvant (CFA), which is too toxic for human use. Rabbit models are employed to study the immunological properties of α-macroglobulins, to model receptor-mediated immune responses, and to predict human immune responses (*23*, *26*). These studies are implicitly premised upon the idea that rabbit and human macroglobulins are sufficiently similar. However,

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without a definitive comparison of the properties of rabbit versus human macroglobulins, the validity of such an assumption remains in question.

There are two known rabbit α-macroglobulins: α_1M and α_2M. Although neither has been characterized extensively and their primary sequences are still unknown, more literature is available regarding α_2M. Blood levels of rabbit α_1M, a constitutively expressed protein, are much more analogous to the levels of human α_2M than those of rabbit α_2M, which is an acute-phase protein (*27*). Preparations of rabbit α_2M obtained in previous studies appear to be contaminated with rabbit α_1M, and occasionally rabbit α-macroglobulin was studied without regard to, or specification of, subtype (*26*, *28*). However, many other species express more than one form of α-macroglobulin, each of which has unique characteristics and properties. Therefore, we purified rabbit α_1M and α_2M and compared them to each other and to human α_2M. After detailed characterization of rabbit α_1M, we developed a model of its interaction with amines and proteinases.

EXPERIMENTAL PROCEDURES

Purification of Human α_2M—Human α_2M was purified to apparent homogeneity in its native form from frozen human plasma (American Red Cross, Charlotte, NC) according to a previously published protocol (*23*) without a water dialysis step. Briefly, proteins in human plasma that remained in solution at 4% (w/v) polyethylene glycol (PEG-8000) but precipitated in 16% PEG-8000 were suspended in 0.1 M sodium phosphate (NaPi) containing 0.8 M NaCl (pH 6.5) and loaded on a zinc chelate affinity column. The column was washed extensively with the loading buffer and then with 0.02 M NaPi containing 0.15 NaCl (pH 6.0). Proteins were eluted with 0.01 M sodium acetate containing 0.15 M NaCl (pH 5.0) and sterile filtered. The concentration of α_2M was determined spectrophotometrically, using $A_{280nm}^{(1\%/1cm)} = 8.93$ and a molecular mass of 720 kDa (*29*). Aliquots of α_2M were kept at –20°C until shortly before use.

Purification of Rabbit α_1M and α_2M—Frozen young rabbit plasma (Pel-Freez Biologicals, Rogers, AR) was PEG-precipitated in the same steps used for the purification of human α_2M. The precipitate from the 16% PEG addition was dissolved in 50 mM Tris-HCl (pH 7.6), clarified by centrifugation and applied onto a DEAE–Sepharose™ Fast Flow column (Amersham Pharmacia Biotech, Piscataway, New Jersey) equilibrated with the same buffer. The column was washed with 50 mM Tris (pH 7.6) containing 50 mM NaCl, and an ascending 0.05–1.0 M NaCl gradient was applied. Rabbit α_2M eluted at 50 mM NaCl and rabbit α_1M eluted between 100–150 mM NaCl. Fractions containing rabbit α_1M were concentrated and loaded onto a Sephacryl™ S-400 column (Amersham Pharmacia Biotech) equilibrated with 50 mM HEPES containing 150 mM NaCl (pH 7.4). Active fractions were loaded onto a Resource-Q column in 50 M Tris·HCl (pH 7.6). Pure rabbit α_1M was eluted with a 0–0.3 M NaCl gradient.

Radiolabeling of Human α_2M and Rabbit α_1M—Proteins were radiolabeled with [^{125}I]-Bolton Hunter Reagent (PerkinElmer Life Sciences Boston, MA). The reagent was evaporated in a glass tube to which the macroglobulin in phosphate-buffered saline (PBS) (pH 7.4) was added. The tube was placed on ice overnight. Unreacted Bolton-Hunter reagent was separated from labeled protein on Sephadex G-25 equilibrated in PBS (pH 7.4).

Activation of Human α_2M and Rabbit α_1M with Amines—Native rabbit α_1M or human α_2M was incubated with 0.2 M NH_4HCO_3 (pH 8.0) for 60 min at room temperature. Excess reagent was removed on Sephadex G-25 equilibrated in 0.1 M NaPi containing 0.15 M NaCl (pH 7.4). The efficiency of the conversion was determined by following its electrophoretic mobility on native PAGE (*16*). When treated with methylamine or propylamine, native α_1M was incubated with 0.4 M methylamine or propylamine in 0.2 M Tris·HCl containing 100 mM NaCl (pH 8.5) for 60 min at 37°C. Excess reagent was removed on NAP-5® columns (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris·HCl (pH 7.6).

Preparation of Rabbit α_1M-Trypsin Complexes—Native and methylamine-treated (0.2 M, overnight) rabbit α_1M were added to 0.1 μg of trypsin in 50 mM Tris·HCl containing 100 mM NaCl (pH 8.0). The mixture was incubated at 37°C for 15 min, followed by addition of hide powder azure suspension. After 30 min the residual activity was measured.

Assay Methods—α_2M activity assays were performed using benzoyl-L-arginine *p*-nitroanilide (BApNA) (Sigma Chemical Co., St. Louis, MO) as previously described (*30*). Protein concentrations were determined by measuring the A_{280nm} using an absorbance coefficient [*A*] = 7.6 for rabbit α_1M. Trypsin inhibitory activity of rabbit α_1M and human α_2M was measured using hide powder azure as previously described (*20*, *31*). Trypsin active site titration was performed using *p*-nitrophenyl *p*-quanidinobenzoate (Sigma Chemical Co.) (*32*).

NH_2-Terminal Sequencing Analysis—The NH_2-terminal sequences were determined at the Duke Protein Sequencing Facility on an Applied Biosystems sequencer using Edman degradation.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing—SDS PAGE was performed in precast 4–15% Tris·HCl gels (Bio-Rad, Hercules, CA) using the Laemmli system (*33*). Native PAGE was performed in precast 5% Tris·HCl gels (Bio-Rad) in a Tris/EDTA/boric acid buffer system. All samples for SDS PAGE were denatured and reduced for 5 min at 95°C in buffer containing 2% SDS and 20 μM DTT. The sample buffer also occasionally contained 5 M urea. Samples containing trapped protease were incubated for 5 min at 37°C with 2 mg/ml of serine protease inhibitor Pefabloc™ (Roche Applied Science, Indianapolis, IN) to prevent additional cleavage. All reagents and precast gels for isoelectric focusing were obtained from Bio-Rad and used according to the manufacturer's instructions.

Quantification of Free Thiol Groups—Thiolester cleavage was quantified by reaction of the free cysteine with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Co.) followed by spectrophotometric measurement of the released TNB^- (*31*). TNB^- concentration was determined at λ = 410 nm using an extinction coefficient of 13,500 M^{-1} cm^{-1} (*34*). Measurements were performed using a thermostated multiplate ELISA reader (Molecular Devices, Sunnyville, CA). All reactions were carried out in 0.2 M Tris·HCl (pH 8.5) containing 0.15 M NaCl and 100 μM DTNB for 1 h at 37°C. The concentration of free SH^- groups initially present in the studied samples was

determined based on the time-dependent changes of *A*, using free DTNB in 0.2 M Tris·HCl with 0.15 M NaCl (pH 8.5) as a control. Samples were incubated overnight with 0.4 M amine hydrochloride (pH 8.5) followed by buffer exchange on a NAP-5 column equilibrated with 0.2 M Tris·HCl containing 0.15 M NaCl (pH 8.0) prior to the measurement of free thiol concentration.

TNS Fluorescence Measurements—Kinetic and wavelength (λ) scan fluorescence measurements were performed in 0.3 ml quartz cuvettes in a PTI spectrofluorometer (Monmouth Junction, NJ). For kinetic measurement, the excitation and emission λ = 319 nm and 450 nm with excitation and emission slits of 4 and 12 nm, respectively. Samples in 0.2 M Tris·HCl with 0.15 M NaCl (pH 8.5) were preincubated with 50 μM 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS) (Sigma Chemical Co.) for 30 min at room temperature. Amine or trypsin was added and the change in fluorescence was observed. If amine was added, measurements were initiated after 30 s of preincubation. TNS fluorescence emission spectra were recorded from λ = 360 nm to 550 nm in 1 nm steps with an integration time of 10 s/point with both excitation and emission slits set at 4 nm.

Harvesting of Murine Peritoneal Macrophages—All experiments involving animals were approved by the Duke Institutional Animal Care and Use Committee. Pathogen-free 6-week-old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Peritoneal macrophages were obtained as previously described (*35*). Briefly, thioglycollate-elicited peritoneal macrophages were harvested by peritoneal lavage using ice-cold Hanks' balanced salt solution containing 10 mM HEPES and 3.5 mM $NaHCO_3$ (pH 7.4) (HBSS). The macrophages were pelleted by centrifugation at 4°C at ~800 × *g* for 10 min and resuspended in RPMI 1640 medium supplemented with 25 mM HEPES, 12.5 U/ml penicillin, 6.5 mg/ml streptomycin, and 5% fetal bovine serum. Cell viability was determined by the trypan blue exclusion method and was consistently greater then 95%.

Receptor Binding Studies—Macrophages were plated in 48-well cell culture plates at 5×10^5 cells per well and incubated for 3 h at 37°C in a humidified 5% CO_2 incubator. The plates were then cooled to 4°C and unbound cells were removed by three consecutive rinses with ice-cold Hanks' balanced salt solution containing 20 mM HEPES and 5% BSA (pH 7.4) (buffer A). As a control for nonspecific binding, some wells were rinsed three times with HBSS, without $CaCl_2$, $MgCl_2$, and $MgSO_4$, containing 20 mM HEPES, 5% BSA, and 5 mM EDTA (pH 7.4) (buffer B), since binding to α_2M receptors is calcium-dependent. To quantify direct binding of the ligand, varying amounts of radiolabeled ligand were added to each well in buffer A or buffer B. Cells were then incubated at 4°C for 12–16 h. Unbound ligand was removed from the wells and the cell monolayer was rinsed three times with ice-cold buffer A or B. Cells were then solubilized with 1 M NaOH at room temperature for >5 h and the contents of the wells were added to polystyrene tubes and counted in an LKB-Wallac, CliniGamma 1272 γ-counter (Piscataway, NJ). Specific binding to cells was determined by subtracting the amount of ^{125}I-ligand bound in the presence of buffer B (nonspecific binding) from the amount of ^{125}I-ligand bound in the presence of buffer A (total binding).

RESULTS

Purification and General Properties of Rabbit α_1M and α_2M—α_1M and α_2M were isolated from rabbit plasma using PEG precipitation and anion-exchange chromatography. The rabbit macroglobulins were identified through comparison of the elution profile with an earlier publication (*36*). Rabbit α_2M eluted at 50 mM NaCl, while rabbit α_1M eluted between 100 and 150 mM NaCl. The eluted fractions of rabbit α_1M were further purified with gel filtration chromatography followed by cation-exchange chromatography. Purity was judged by viewing on native and SDS PAGE. Superdex 200 gel filtration chromatography revealed that both rabbit α-macroglobulins exhibited the same elution profile as human α_2M.

Native rabbit α_1M (Fig. 1A, lane 1, and Fig. 1B, lane 1), native rabbit α_2M (Fig. 1B, lane 2), and native human α_2M (Fig. 1A, lane 4) all migrated as a single band on 5% native PAGE. Rabbit α_1M possessed greater electrophoretic mobility than rabbit α_2M or human α_2M, suggesting that rabbit α_1M may be slightly more compact or more negatively charged than the other two macroglobulins. Under non-reducing conditions, rabbit α_1M incubated in 2% SDS at 95°C produced one large band of approximately 360 kDa. Upon reduction in the presence of SDS and urea, the dimers dissociated into monomers with an apparent molecular mass of ~185 kDa. The presence of urea was necessary in order to fully reduce the rabbit α_1M molecule to individual subunits. From these results it is apparent that rabbit α_1M is approximately the same size and basic shape as rabbit α_2M and human α_2M. These data are consistent with a quaternary structure similar to that of human α_2M in which two non-covalently associated dimers

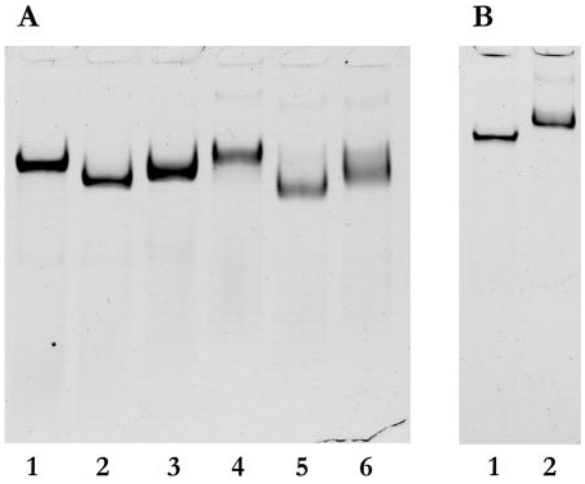

Fig. 1. **Native PAGE analysis of rabbit and human macroglobulins.** (A) Influence of methylamine and trypsin on the electrophoretic mobility of rabbit α_1M and human α_2M. Rabbit α_1M and human α_2M were incubated with 0.4 M methylamine in 0.2 M Tris, 100 mM NaCl (pH 8.5) at 37°C for 1 h followed by buffer exchange to 50 mM Tris (pH 7.6) on NAP-5 columns. Proteins were electrophoresed on 5% Tris·HCl gel using the Tris/EDTA/boric acid buffer system. Lane 1, native rabbit α_1M; lane 2, rabbit α_1M treated with methylamine; lane 3, rabbit α_1M treated with equimolar trypsin; lane 4, native human α_2M; lane 5, human α_2M treated with methylamine; lane 6, human α_2M treated with equimolar trypsin. (B) Comparison of the electrophoretic mobility of rabbit α_1M and rabbit α_2M. Lane 1, native rabbit α_1M; lane 2, native rabbit α_2M.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Human α_2M	Ser	Val	Ser	Gly	Lys	Pro	Gln	Tyr	Met	Val	Leu	Val	Pro	Ser	Leu
Rabbit α_1M			Ser	Gly	Lys	Pro	Asn	Tyr	Met	Val	Leu	Val	Pro	Ser	Leu
Rabbit α_2M			Thr	Asn	Lys	Pro	Gln	Tyr	Ile	Val	Leu	Val	Pro	Ser	Glu

Fig. 2. **NH_2-terminal amino acid sequences of rabbit α_1M and α_2M compared to the sequence of human α_2M.** Rabbit macroglobulin sequences obtained here are compared to the sequence of human α_2M reported by Sottrup-Jensen *et al*. [*J. Biol. Chem.* 259, 8318–8327 (1984)]. Sequence homologies between the rabbit macroglobulins and human α_2M are denoted with double underlines.

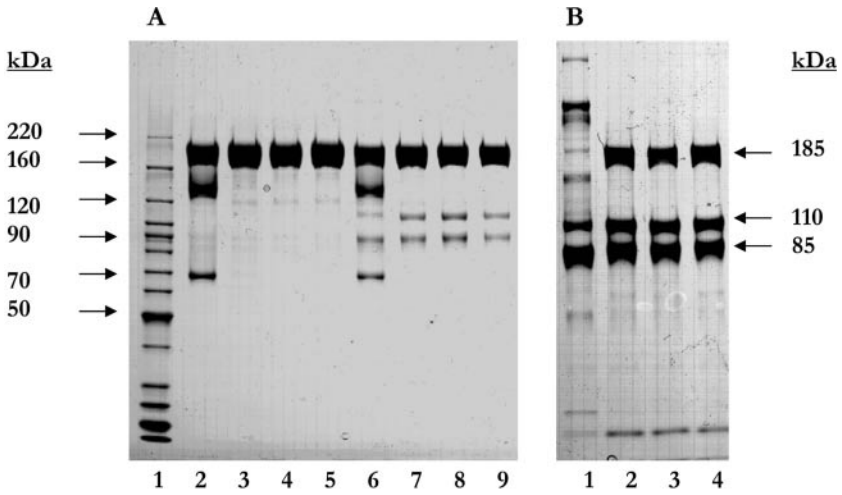

Fig. 3. **SDS PAGE analysis of the reaction of rabbit α_1M with amines and trypsin.** Samples were incubated with different amines for 1 h at 37°C and/or reacted with trypsin for 10 min at room temperature. The serine proteinase inhibitor, Pefabloc, was added before boiling in reducing conditions. (A) Lane 1, broad molecular weight standard (Invitrogen, Carlsbad, CA); lane 2, native rabbit α_1M; lane 3, rabbit α_1M treated with ammonia; lane 4, rabbit α_1M treated with methylamine; lane 5, rabbit α_1M treated with propylamine. Lanes 6–9 represent native, or ammonia-, methylamine-, and propylamine-pretreated rabbit α_1M (respectively) followed by treatment with equimolar trypsin. (B) Lanes 1–4 represent native, or ammonia-, methylamine-, and propylamine-pretreated rabbit α_1M (respectively) followed by treatment with a tenfold molar excess of trypsin.

each contain two subunits joined by disulfide bonds and stabilized by hydrophobic interactions.

NH_2-Terminal Sequence Analysis—The NH_2-terminal sequence was determined for the first fifteen amino acids of both rabbit α_1M and rabbit α_2M (Fig. 2). These data were compared to previously published data in order to determine the identity of rabbit α_1M and α_2M after chromatography. The amino terminus of both rabbit macroglobulins corresponded to the third amino acid of human α_2M. Of the first fifteen amino acids in human α_2M, twelve were identical to the analogous residues in rabbit α_1M, while only nine were identical to the analogous residues in rabbit α_2M. These data suggest that earlier characteristics of rabbit α_2M may have been reported on a mixture of both rabbit α_1M and α_2M (*28*).

Heat Treatment and Reaction of Rabbit α_1M with Amines and Trypsin—Proteins containing thiolester bonds characteristically break into two fragments when heated in the presence of a denaturant and reductant. This is caused by autolysis of the peptide backbone at the glutamate that forms the bond and becomes more pronounced as the time, pH, and temperature are increased (*37*). To determine whether native rabbit α_1M contains a thiolester, it was incubated at 95°C in the presence of SDS, with and without the reductant DTT. A single fragment of 185 kDa resulted from the SDS treatment, whereas two polypeptide fragments of 67 and 125 kDa were generated in the presence of SDS and DTT (Fig. 3A, lane 2). Furthermore, these two smaller fragments were no longer generated under reducing conditions if the α_1M was pretreated with an amine. Rabbit α_1M was treated with three amines of increasing size (ammonia, methylamine, and propylamine) and analyzed by SDS PAGE under reducing conditions (Fig. 3A, lanes 3–5). The heat fragments seen with native α_1M (Fig. 3A, lane 2) decreased dramatically and identical 185-kDa bands were observed in all lanes.

The extent of trypsin cleavage of native rabbit α_1M subunits was also examined by SDS PAGE under reducing conditions (Fig. 3A, lane 6). Incubation of native rabbit α_1M with an equimolar concentration of trypsin resulted in the appearance of five bands: 67, 85, 110, 126, and 185 kDa. These bands can be attributed to heat fragments (67 kDa and 126 kDa), uncleaved monomer (185 kDa), and two cleavage fragments (85 kDa and 110 kDa). These results are similar to what is observed with human α_2M and indicate that, unlike rabbit α_2M, which is cleaved into many small fragments by trypsin (*38*), rabbit α_1M appears to have only one trypsin cleavage site. In the presence of a tenfold molar excess of trypsin, all native rabbit α_1M was cleaved and additional high molecular weight bands were also generated (Fig. 3B, lane 1). These may represent the formation of proteinase-macroglobulin cross-linked species as previously reported in the case of human macroglobulin (*39*).

Pretreatment of rabbit α_1M with ammonia, methylamine, or propylamine prevented the formation of heat fragments (Fig. 3A, lanes 7–9) but did not alter the sizes of the cleavage products by trypsin at a 1:1 ratio of trypsin to rabbit α_1M. At a 10:1 ratio, however, there was a significant amount of uncleaved monomer in the pretreated samples (Fig. 3B, lanes 2–4) as compared to untreated rabbit α_1M (Fig. 3B, lane 1). This suggests that after rabbit α_1M is treated with an amine, the bait region becomes less accessible to trypsin cleavage (Fig. 3B, lane 1 *versus* lanes 2–4).

The electrophoretic mobility of trypsin-treated rabbit α_1M was greater than that of native rabbit α_1M on 5% native PAGE in the Tris-glycine Laemmli buffer system (Fig. 1A, lanes 1 *versus* 3). This difference was more

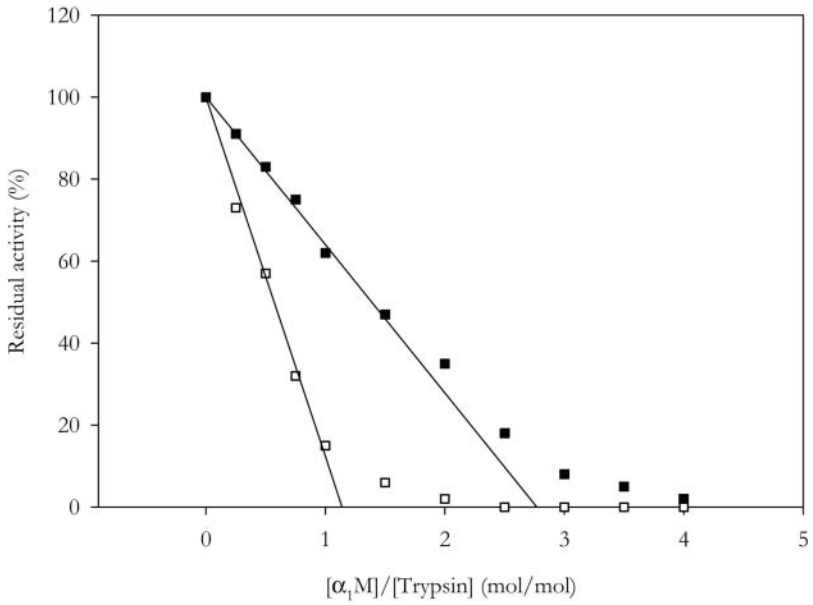

Fig. 4. **Inhibition of trypsin proteolytic activity by native and methylamine-treated rabbit α_1M.** Native and methylamine-treated rabbit α_1M were added to 0.1 μg of trypsin in 50 mM Tris·HCl, 100 mM NaCl (pH 8.0), incubated at 37°C for 15 min, and combined with a hide powder azure suspension. The residual activity was measured after 30 min. Open squares, native rabbit α_1M; solid squares, methylamine-treated rabbit α_1M.

significant when the gels were electrophoresed in the Tris/EDTA/boric acid buffer system as well as when the pH of the sample buffer was raised to 8.5 (data not shown). Similar behavior was observed with amine-treated rabbit α_1M (Fig. 1A, lanes 1 and 2), though the difference between native and amine-treated protein was greater for human α_2M (Fig. 1A, lanes 4 and 5) than for rabbit α_1M. This may indicate that amine treatment results in less compaction or change in surface charge of rabbit α_1M when compared to human α_2M.

Isoelectric focusing analysis demonstrated that the trypsin- and methylamine-treated rabbit α_1M were more acidic than the native molecule (data not shown). Comparison of human α_2M on the same gel showed a negligible difference between the pI values of native and methylamine-treated proteins, while the trypsin-treated human α_2M migrated at a much more alkaline pI. This leads to the conclusion that human α_2M is more compact after methylamine treatment, since the pI values are nearly identical yet they migrate differently on a native gel (Fig. 1). Likewise, the trypsin-treated, relatively alkaline human α_2M is more compact as seen on native PAGE. However, all forms of rabbit α_1M (native and treated) were more acidic than human α_2M, with the treated versions exhibiting the lowest pI values. This suggests that the electrophoretic mobility changes of rabbit α_1M observed on native PAGE after proteinase or methylamine treatment (Fig. 1) are a function of surface electrostatic potential changes of this molecule and are not necessarily accompanied by a transformation to a more compact conformation.

Effect of Rabbit α_1M on the Proteolytic Activity of Trypsin—The ability of rabbit α_1M to inhibit the proteolytic activity of trypsin was assessed using hide powder azure as a substrate (Fig. 4). As the molar ratio of α_1M to trypsin increased, the proteolytic activity of trypsin decreased linearly. The linear portion of the curve was extrapolated to the *x*-axis to determine the ratio of α_1M to trypsin at complete inhibition. The intersection with the *x*-axis was at approximately 1, suggesting that a tetrameric molecule of rabbit α_1M bound an average of one molecule of trypsin (equimolar binding). Methylamine-treated rabbit α_1M still retained inhibitory activity against trypsin. However, as previously seen with rabbit α_2M (*38*), the slope of the linear portion of the data decreased and complete inhibition was reached at a ratio of methylamine-treated α_1M to trypsin of 3:2, indicating a weaker inhibitory ability.

Table 1. **Number of thiol groups exposed per tetramer of rabbit α_1M based on type of treatment before reaction with DTNB.**

Type of treatment	Thiol groups exposed per tetramer
No treatment (native molecule)	0.56 ± 0.02
Ammonia	2.2 ± 0.04
Methylamine	1.4 ± 0.07
Trypsin (tenfold molar excess/10×)	1.2 ± 0.05
Methylamine + trypsin (10×)	4.1 ± 0.1

Thiol Group Exposure upon Treatment with Amines and Trypsin—The number of free thiol (-SH) groups after rabbit α_1M was reacted with ammonia, methylamine, or trypsin (1 h) was determined by subsequent reaction with DTNB. Trypsin and methylamine liberated approximately one thiol group per rabbit α_1M tetramer, while ammonia released approximately two groups. Reaction with both methylamine and trypsin released all four thiol groups (Table 1). This result implies that after one thiolester is cleaved, access to the others is limited. Furthermore, the exposure of all four thiol groups upon addition of both trypsin and methylamine may be a result of synergy between the two. In particular, the cleavage by trypsin in the bait region could allow methylamine to access previously protected thiolester bonds.

Effect of SBTI on the Amidolytic Activity of Trypsin Bound to Rabbit α_1M—To study the steric-shielding potential of rabbit α_1M toward entrapped proteinases, the effect of soybean trypsin inhibitor (SBTI) on the esterolytic activity of trypsin bound to rabbit α_1M was measured and compared to that of human α_2M. As the molar ratio of SBTI to

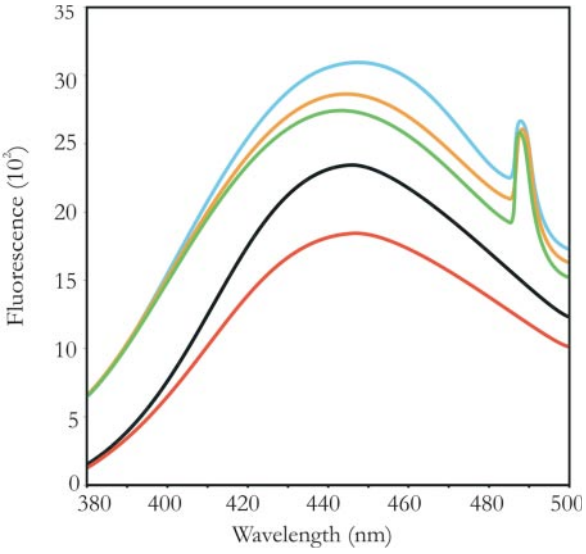

Fig. 5. **Uncorrected emission spectra of TNS induced by binding of rabbit α_1M treated with amines and trypsin.** Rabbit α_1M in 0.2 M Tris·HCl, 0.15 M NaCl (pH 8.5) was preincubated with 0.4 M amine for 1 h or trypsin for 10 min at 37°C. The excitation wavelength was 319 nm and both excitation and emission slits were at 4 nm. Black line, native rabbit α_1M; rabbit α_1M incubated with: blue line, ammonia; orange line, methylamine; green line, propylamine; red line, equimolar trypsin.

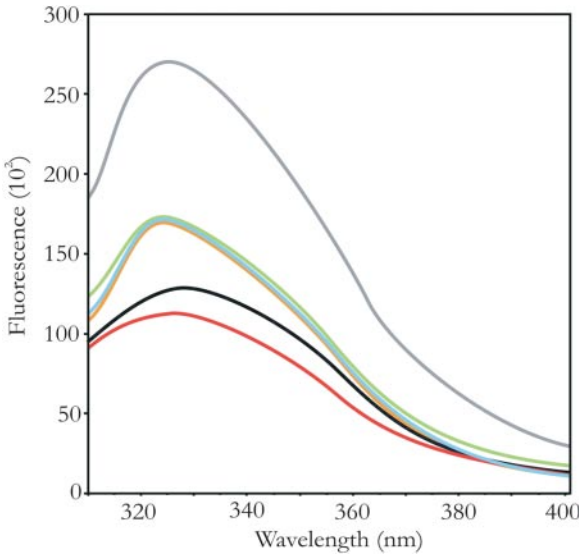

Fig. 6. **Intrinsic fluorescence emission spectra of rabbit α_1M after amine and trypsin treatment.** Rabbit α_1M in 0.2 M Tris·HCl, 0.15 M NaCl (pH 8.5) was preincubated with 0.4 M amine for 1 h or with trypsin for 10 min at 37°C. The excitation wavelength was 290 nm and both excitation and emission slits were at 4 nm. Black line, native rabbit α_1M; rabbit α_1M incubated with: blue line, ammonia; orange line, methylamine; green line, propylamine; red line, equimolar trypsin; grey line, tenfold molar excess of trypsin.

trypsin increased, the extent of inhibition of trypsin bound to rabbit α_1M also increased, reaching a plateau at around 80% of the control value at a SBTI to trypsin ratio of 5:1 (data not shown). Under the same conditions, trypsin bound to human α_2M was almost completely protected from SBTI inhibition, in agreement with previous results (*40*).

Changes in TNS Florescence Induced by Amine and Trypsin Treatment—Previous studies show that the conformational changes occurring in α-macroglobulins as a result of amine or proteinase treatment can be monitored by measuring the fluorescence changes of TNS (*41*, *42*). We therefore recorded the emission spectra of fluorescence for rabbit α_1M after reaction with 0.4 M ammonia, methylamine, propylamine, and with trypsin (equimolar with α_1M) (Fig. 5). Upon incubation with native rabbit α_1M, the wavelength of maximal emission of TNS shifted from 490 to 445 nm. This maximal wavelength was fairly uniform over all of the rabbit α_1M samples. The intensity of fluorescence increased 16%, 20%, and 30% after interaction of rabbit α_1M with propylamine, methylamine, and ammonia, respectively. Treatment with an equimolar amount of trypsin resulted in a 25% decrease in fluorescence intensity. This change in fluorescence was not accompanied by a significant shift in the wavelength of maximal emission for any of the samples. The increase and decrease in TNS fluorescence observed when native rabbit α_1M interacted with methylamine and trypsin, respectively, was in similar proportion to that observed with native rabbit α_2M. In contrast, native human α_2M exhibited a marked increase in TNS fluorescence after both methylamine and trypsin treatment (*41*, *43*). This suggests that unlike human α_2M, the conformations of trypsin-modified and amine-modified rabbit α_1M are significantly different.

Changes in Intrinsic Florescence Induced by Amine and Trypsin Treatment—Measurement of intrinsic tryptophan fluorescence is another method for evaluating the reaction of human α_2M with amines and proteinases. In the presence of these reactants, the change in the fluorescence intensity of α_2M correlates with the structural and functional changes within the molecule (*44*). Compared to the maximum intensity caused by native rabbit α_1M, the intensity of tryptophan fluorescence increased by approximately 36% after the α_1M was treated with ammonia, methylamine, or propylamine (Fig. 6). The increase in fluorescence was not accompanied by a shift in the wavelength of maximal emission. Conversely, treatment with an equimolar amount of trypsin resulted in a 15% decrease in intrinsic fluorescence. However, a 125% increase in intrinsic fluorescence, without any changes in the maximum of fluorescence emission, was observed when rabbit α_1M was treated with a tenfold molar excess of trypsin.

The identical changes in intrinsic fluorescence between untreated native rabbit α_1M and each rabbit protein treated with ammonia, methylamine, and propylamine suggests that the structural changes in the microenvironment surrounding the aromatic amino acids induced by amines of various sizes are identical. Moreover, contrary to the data obtained for human α_2M (*41*, *45*), an equimolar trypsin treatment of rabbit α_1M did not trigger the increases in intrinsic and TNS fluorescence that were observed after methylamine treatment of the rabbit protein. The results indicate that trypsin-treated rabbit α_1M exists in more than one conformation, depending on the trypsin concentration. The different conformations may be due to differences in the number of bait regions cleaved or

the number of bound trypsin molecules. Complete cleavage by trypsin, which occurs at the higher trypsin to rabbit α_1M ratio, has been shown to create not only the same cleavage products as the lower ratio but also the larger, cross-linked species viewed in lane 1 of Fig. 3B.

Cellular Binding of Rabbit α_1M to Lipoprotein-Receptor Related Protein—To determine the affinity of the various forms of rabbit α_1M for cellular receptors, we compared their binding to thioglycollate-elicited murine peritoneal macrophages to that of human α_2M* (treated with ammonium bicarbonate). Murine macrophages are widely used for in vitro binding studies on various forms of α-macroglobulins, and inhibitors such as bovine and rat macroglobulins bind to the murine peritoneal macrophage α-macroglobulin receptor with similar affinity to that of human α_2M* ($K_d \sim 1$ nM) (*46*). Radiolabeled native rabbit α_1M, human α_2M*, and ammonia-, methylamine-, and trypsin-modified rabbit α_1M were incubated at concentrations of 160 pM to 20 nM with macrophages (5×10^6/well) at 4°C (data not shown). Scatchard analysis of the binding of human α_2M* revealed a binding site with a dissociation constant of approximately $K_d = 0.6$ nM, consistent with previously published reports (*47*). As expected, native rabbit α_1M did not bind, and ammonia- or methylamine-treated rabbit α_1M bound to peritoneal macrophages with a similar affinity ($K_d \sim 5$–10 nM) to that of the human α_2M*. Finally, trypsin-treated rabbit α_1M bound with such a low affinity that the K_d could not be determined.

DISCUSSION

The biochemical and biophysical characteristics of rabbit α_2M have been reported (*26–28*, *38*), though two of the studies (*26*, *28*) employed a mixture of rabbit α_1M and α_2M. Tamamizu *et al*. (*38*) provided a comprehensive report on the characteristics of rabbit α_2M. However, according to Versavel *et al*. (*27*), the homolog to human α_2M is more likely rabbit α_1M, based on the observed relative levels of the macroglobulins in serum. But a complete listing of the biochemical and biophysical properties, which would offer a more direct comparison, has not been reported. In Table 2, we have summarized the properties of rabbit α_1M and compared them to those of rabbit α_2M and human α_2M. The main characteristics provided in this table include size, effects of primary amines, and trypsin-binding properties.

Compared to rabbit α_2M, the size, heat fragmentation pattern, and trypsin cleavage pattern for rabbit α_1M are all more similar to those of human α_2M. In addition, as shown in Fig. 2, the NH_2-terminal sequences of rabbit α_1M and human α_2M have a higher identity (twelve of fifteen) than those of rabbit α_2M and human α_2M (nine of fifteen). The rabbit α_2M tetramer, moreover, is larger, lacks the intrachain disulfide bridge connecting the heat-cleaved fragments, and is cleaved at multiple sites by trypsin. Although these differences suggest that rabbit α_1M is more similar to the human protein, rabbit α_1M also exhibits certain characteristics that make it distinct from the other macroglobulins. For example, although rabbit α_1M is similar to human α_2M with respect to both gross size and shape, it has a more negative surface charge. The subunit structures of the three macroglobulins are also similar, but the monomers that make up rabbit α_1M form dimers through strong hydrophobic interactions, which do not appear to play the same role in either of the α_2M molecules. In addition, the specific interactions of rabbit α_1M with methylamine and trypsin are identical to neither rabbit α_2M nor human α_2M. These subtle variations are clear when comparing the properties listed in the columns at the right-hand side of Table 2.

The native form of rabbit α_1M, which contains intact thiolester linkages and the open cage conformation, is relatively similar to native human α_2M. In addition to size and shape similarities, native rabbit α_1M and human α_2M are more tightly packed than rabbit α_2M for two principal reasons. First, heat treatment of rabbit α_1M under reducing conditions led to two fragments, as it did with human α_2M. Only one fragment appeared when reductant was not added, suggesting that, like human α_2M, the two heat fragments are connected by disulfide bonds. Rabbit α_2M, in contrast, lacks an intrachain disulfide bridge connecting the two heat fragments and releases a peptide fragment upon heat treatment of the loosely packed molecules (*38*). Second, both rabbit α_1M and human α_2M were only cleaved by trypsin at one site, the bait region, and were cleaved into two fragments. No additional proteolysis was observed, even after pretreatment with methylamine (*6*, *37*). In contrast to both of these macroglobulins, trypsin cleaved rabbit α_2M at many sites (*38*). Pretreatment with methylamine resulted in more extensive fragmentation.

Once the three macroglobulins were treated with an amine or proteinase, the characteristics of each became more variable. The compaction of human α_2M after amine treatment results in a total loss of activity, as proteinases can no longer access the bait region (*16*). Furthermore, human α_2M appears to enclose bound proteinases and significantly compact upon bait region cleavage, as seen from its ability to fully shield bound trypsin (*38*, *40*, unpublished data). In both types of activation, amine and proteinase, human α_2M exhibits all four exposed thiols

Table 2. **Summary of human and rabbit macroglobulin characteristics.**

Protein	Size (kDa)	Intra-chain S-S bond?	Thiols exposed by MA[a]	Tryp[b] cleavage/ thiols exposed	Tryp-bound shielding	αM* inhibits tryp?	ΔE-FL[d] from native αM		ΔI-FL[e] from native αM		RBP[f] exposure	
							MA	Tryp (1:1)	MA	Tryp (1:1)	MA	Tryp
Human α_2M	720	yes	all equally	bait[c]/all thiols	complete	no	+	+	+	+	strong	strong
Rabbit α_1M	720	yes	hindered	bait/1 thiol	partial	partial	+	–	+	–	strong	weak
Rabbit α_2M	880	no	hindered	≫1	partial	partial	+	–	+	+	NR[g]	NR

[a]MA = methylamine, [b]Tryp = trypsin, [c]bait = bait region, [d]ΔE-FL = change in extrinsic fluorescence from native macroglobulin; '+' increase in fluorescence; '–' decrease in fluorescence, [e]ΔI-FL = change in intrinsic fluorescence from native macroglobulin; '+' increase in fluorescence; '–' decrease in fluorescence, [f]RBP = receptor binding site, [g]NR = not reported.

per molecule. However, two main effects occurred upon activation of the rabbit macroglobulins that made them distinct from human α_2M activation: 1) the rabbit macroglobulins did not compact after methylamine or trypsin treatment in the same manner as human α_2M, and 2) amine and trypsin treatment did not fully expose the thiol groups of the rabbit proteins, in contrast to the full exposure that occurred with the human protein. The first effect was not obvious when simply comparing the elecrophoretic migration rates of native and activated rabbit α_1M (Fig. 1), since their pI values were different. However, the effect did become detectable from the fact that methylamine-modified rabbit α_1M retained partial ability to inhibit trypsin. This property is shared with rabbit α_2M (*38*), as well as with macroglobulins from other species such as the bovine protein (*48*). There was also a clear difference in compaction between rabbit macroglobulins and human α_2M when the ability of each to sterically shield complexed proteinases was measured (Table 2) (*38*). The second effect for both rabbit macroglobulins is shown in the results listed in Table 1 and the study by Tamamizu *et al*. (*38*), which exhibited incomplete exposure of thiol groups by methylamine or trypsin.

The hypothesis that the three macroglobulins undergo distinct conformational changes upon activation is further supported by the fluorescence data. As illustrated in Figs. 5 and 6 and listed in Table 2, the changes in both extrinsic and intrinsic fluorescence values after treatment with an amine or trypsin were variable among the three macroglobulins. Unlike human α_2M, in which the structures of methylamine-treated α_2M and trypsin-treated α_2M are almost indistinguishable, notable differences were observed between methylamine- and trypsin-treated rabbit α-macroglobulins. The intensity of fluorescence emissions of TNS bound to human α_2M increased after both methylamine and trypsin treatment. This change was also accompanied by a blue shift in the maxima of the TNS fluorescence emission. Methylamine treatment of each rabbit macroglobulin, however, increased the TNS fluorescence, while trypsin treatment decreased it and did not change the fluorescence emission maximum (*38*). The intrinsic fluorescence changes in rabbit α_1M after treatment with methylamine were similar to those of rabbit α_2M, but treatment with an equimolar amount of trypsin caused a rise in the intrinsic fluorescence of rabbit α_2M and a decrease in the fluorescence of rabbit α_1M. In contrast, treatment of rabbit α_1M with a saturating concentration of trypsin resulted in an intrinsic fluorescence change similar to that of equimolar trypsin-treated rabbit α_2M. This disparity could indicate that the bait regions in rabbit α_1M are more difficult for trypsin to access than those of rabbit α_2M. This may be due to a more restrictive structure, a hypothesis supported by the heat-treatment and trypsin-cleavage data.

Further evidence suggesting significant differences in the conformational changes upon amine or trypsin activation of rabbit α_1M compared to human α_2M is apparent from the receptor binding results. In many species, cleavage of the thiolester bonds results in exposure of the receptor-binding region (*45*, *49*, *50*). This is generally thought to be a binary process, although this is not the case with rabbit α_1M. The binding of trypsin-modified rabbit α_1M was of a much lower affinity than that of methylamine-modified rabbit α_1M. Rabbit α_1M modified by methylamine exhibited a similar binding affinity to human α_2M, which is constant whether treated with methylamine or trypsin (*51*, *52*). This suggests that methylamine and trypsin treatment result in different exposure of the receptor recognition sites in rabbit α_1M, with methylamine resulting in more optimal exposure.

Not only is the receptor binding region exposure different for each macroglobulin, but the interior thiol exposure upon activation is also variable (see Tables 1 and 2). Upon treatment with excess amounts of methylamine, only one thiolester bond is cleaved in rabbit α_1M. This is in marked contrast to the effect of methylamine on human α_2M, which is to cleave all four thiolester bonds (*41*, *53*). It is also in contrast to the effect of methylamine on rabbit α_1M, which is hindered but still creates an average of 3.5 free thiols (*38*). It appears that cleavage of one thiolester bond by an amine blocks cleavage of the others. This may result from an overall increase in hydrophobicity around the other thiolester sites resulting from the conformational change that occurred after the first thiolester was cleaved or from a steric blockage of the thiolester sites caused by the conformational change. This increase in hydrophobicity is supported by the TNS fluorescence data. Interestingly, ammonia exposure results in the cleavage of two thiolester bonds in rabbit α_1M, suggesting that after one thiolester bond is cleaved, the remaining thiolester bond in the dimer may be slightly more accessible than the two uncleaved bonds on the other dimer. Ammonia, due to its smaller size can access this remaining bond in the dimer, while methylamine can not. Furthermore, trypsin exposure (tenfold molar excess) results in the cleavage of one thiolester bond, most likely the one on the same monomer on which the bait region was cleaved. This effect is different from the other macroglobulins, since interaction of human α_2M with an excess of trypsin results in the binding of one to two trypsin molecules and cleavage of all four thiolester bonds (*4*, *54*, *55*) and rabbit α_2M is cleaved more than once by trypsin (*38*). Two possible explanations for the limited cleavage in rabbit α_1M are: (1) after bait region cleavage, the subsequent conformational change may physically prevent other trypsin molecules from entering α_1M and interacting with the remaining bait regions, and (2) the trypsin molecule itself may block access to the other bait regions. Since a mixture of methylamine and trypsin causes full thiol exposure in rabbit α_1M, the former explanation may be the more likely reason. Trypsin cleavage may create a conformational change, particularly near the thiolester bonds, that blocks other large molecules while still allowing space for small structures such as methylamine. The model illustrated in Fig. 7 summarizes these likely effects of amines and proteinases on rabbit α_1M.

In summary, the results listed in Table 2 not only suggest significant differences in the conformational effects by activators on the macroglobulins of rabbit versus human, but also raise question as to the primary role of rabbit α_1M. Human α_2M is clearly an effective proteinase inhibitor, and the receptor binding results suggest that this could be an important physiological function for the protein. However, the results here show that, in contrast to human α_2M, rabbit α_1M is not an effective proteinase inhibitor, since it cannot fully shield bound trypsin and the

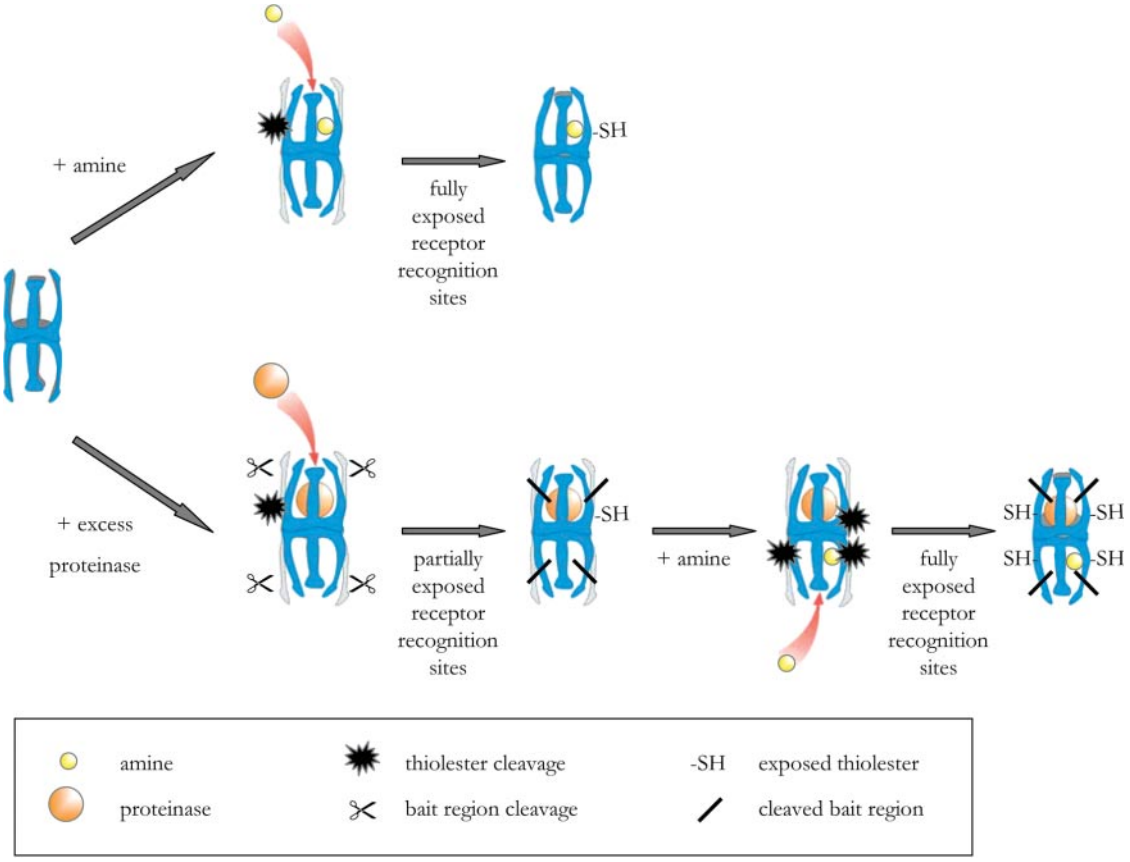

Fig. 7. **Model of observed effects by amines and proteinases on rabbit α_1M.**

methylamine-treated form can still inhibit trypsin activity. Furthermore, interaction with a proteinase does not result in optimal exposure of the receptor-binding region of rabbit α_1M, an action necessary for the clearance of the spent molecule. A further difference occurs with rabbit α_2M, which not only is unable to effectively act as a proteinase inhibitor, but is extensively cleaved into fragments instead. These observations strongly suggest that neither of the rabbit macroglobulins plays the same type of inhibitory role in plasma as the human protein does, leaving their true primary functions yet to be determined.

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