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α_2M in the Horseshoe Crab

A Structural and Functional Invertebrate Homologue

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INTRODUCTION

Higher animals deploy a variety of defense systems to cope with invading pathogens that are based on components in the blood cells and plasma. These cellular and humoral components function to neutralize and clear the invading pathogens and the molecules introduced by pathogens. Often, the introduced molecules that are most harmful are toxic proteins and degradative enzymes. In many of the cellular (neutrophils, lymphocytes, and monocytes) responses and in a majority of the humoral responses (kinin, complement, and coagulation), a variety of proteolytic enzymes are also generated to act on target proteins or to further amplify the response via cascade

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mechanisms. It is therefore not surprising that animals have evolved regulatory responses for inactivating and clearing both the exogenous and endogenous proteases to limit their potentially degradative side effects. Although many protease inhibitors exist that can carry out the inactivation of specific proteolytic enzymes, the plasma protease inhibitor, alpha-2-macroglobulin (α_2M), is the major endoprotease inhibitor in mammalian blood and has the unique distinction of inactivating almost all known proteases irrespective of their source, structure, and catalytic mechanism.[1,2]

The best-studied animal species of α_2M, human α_2M, is a homotetramer organized as a noncovalently linked dimer of disulfide-linked homodimers of a 180-kDa subunit.[3,4] The disulfide-linked homodimer is thought to be the basic functional unit,[5] but dimers of the noncovalently linked subunits can also bind proteases.[6] Other members of the α_2M family of protease-binding proteins exist as disulfide-linked homodimers.[7,8] The mechanism of protease binding by α_2M is unique. In contrast to the active-site inhibitors of proteases, α_2M selectively inhibits the interaction of the targeted protease with macromolecular substrates without affecting the enzymatically active site. Protease binding to tetrameric human α_2M can involve both covalent[9–12] and noncovalent[11] interactions. The latter involve a physical folding of the α_2M molecule around the protease molecule, so as to "trap" it,[13] with the arms of the α_2M polypeptides forming a steric barrier to prevent contact between the protease and large substrate molecules. Covalent binding involves the establishment of an isopeptide bond with the γ-carboxyl of the glutamyl residue of a reactive intrachain thiol ester.[9–12] Members of the α_2M superfamily, which includes C_3, C_4, and C_5 of the complement cascade, are the only proteins that have been described with an internal thiol ester bond (for review, see reference 14).

It has been difficult to identify the specific physiological roles for α_2M because of the abundance of other protease inhibitors in mammalian plasma. α_2M is presumed to be essential to survival because, in contrast to many other plasma proteins, full genetic deficiency of α_2M in humans has not yet been found, presumably because such deficiency would be lethal. The physiological importance of α_2M also is indicated by its structure and function being conserved throughout the evolution of higher animals.[15] Investigation of structural and functional homologues of α_2M isolated from species evolutionarily distant from mammals and possessing a less complex plasma may contribute to the identification of specific physiological roles for α_2M.

In 1982, we reported the presence of a protease inhibitor in the plasma of the American horseshoe crab, *Limulus polyphemus*, that was proposed to be a functional and molecular homologue to mammalian α_2M.[16–19] Homologues of vertebrate α_2M have now been described from a variety of invertebrates, including chelicerate and mandibulate arthropods[20–24] and mollusks.[25,26] These molecules share numerous functional properties with mammalian α_2M and have remarkable identity at the level of peptide sequence in key functional domains.[27–29] The present report describes our investigations of the structural and functional features of the forms of α_2M found in invertebrates. The presence of homologues of α_2M in an ancient invertebrate such as *Limulus*, whose plasma protein and circulating hemocyte composition is relatively simple, recommends *Limulus* as a model for the elucidation of the physiological function of α_2M as a defense molecule.

TABLE 1. Inhibition of a Variety of Different Proteases by *Limulus* α_2M: Sensitivity of Inhibition to Methylamine Treatment

(A)	Protease Tested	*Limulus* α_2M Addition	Protease Activity (cpm ^{14}C-Casein Hydrolyzed)
	trypsin (0.5 μg)	–	2670
	trypsin (0.5 μg)	+	20
	chymotrypsin (2.0 μg)	–	1544
	chymotrypsin (2.0 μg)	+	76
	elastase (1.0 μg)	–	305
	elastase (1.0 μg)	+	7
	papain (0.2 μg)	–	2250
	papain (0.2 μg)	+	94
	subtilisin (1.0 μg)	–	3187
	subtilisin (1.0 μg)	+	270
	thermolysin (1.0 μg)	–	2541
	thermolysin (1.0 μg)	+	173

(B)	Protease Tested	*Limulus* α_2M Addition	Methylamine-Treated *Limulus* α_2M	Protease Activity (cpm ^{14}C-Casein Hydrolyzed)
	trypsin (0.5 μg)	–	–	2415
	trypsin (0.5 μg)	+	–	140
	trypsin (0.5 μg)	–	+	2350

RESULTS

Limulus α_2*M: A Functional Homologue of Mammalian* α_2*M*

A preparation of the α_2M homologue isolated from *Limulus* plasma was tested for its ability to inhibit a variety of proteolytic enzymes (TABLE 1). It is clear that *Limulus* α_2M inhibits by 90-100% the casein-hydrolyzing ability of proteases from animals (trypsin, chymotrypsin, and elastase), plants (papain), and pathogenic microbes (subtilisin). The inhibition by *Limulus* α_2M is substantial whether the protease is a serine protease (trypsin, chymotrypsin, elastase, subtilisin), a cysteine protease (papain), or a metalloprotease (thermolysin). The protease inhibitory property of *Limulus* α_2M is completely abrogated by treatment of the preparation with methylamine (TABLE 1B). This latter property, highly characteristic of thiol ester-containing molecules, provides evidence that *Limulus* α_2M is a homologue of mammalian α_2M. α_2M is the only protease inhibitor that we have detected in the plasma of *Limulus*,[18] although the blood cells of *Limulus* do secrete active-site protease inhibitors when stimulated to undergo exocytosis.[30]

Further evidence that *Limulus* α_2M is a functional homologue of mammalian α_2M was provided by the demonstration that trypsin treated with *Limulus* α_2M, although unable to hydrolyze casein, is capable of hydrolyzing the low-molecular-weight substrate, benzoyl arginine nitroanilide (BAPNA), in the presence of soybean

trypsin inhibitor (SBTI).[19,20] These results provide evidence that *Limulus* α_2M, similar to mammalian α_2M, entraps the target protease in a steric inhibitory "cage" around the protease, restricting access of the protease to macromolecular structures, but leaving the active site of the protease intact and still capable of cleaving low-molecular-weight substrates.

Limulus α_2*M: A Structural Homologue of Mammalian* α_2*M*

When *Limulus* α_2M was purified from horseshoe crab hemolymph by gel filtration chromatography on Sephacryl 300, the native *Limulus* molecule yielded an apparent molecular weight of 500-550 kDa, in contrast to the 720-kDa native molecular weight of tetrameric human α_2M chromatographed under identical conditions.[19] Following SDS-PAGE under nonreducing conditions, the *Limulus* α_2M molecule still yields an apparent molecular weight of 500-600 kDa, in sharp contrast to the resulting (350-380)-kDa dimeric human α_2M electrophoresed in parallel SDS gels (FIGURE 1B). Under reducing conditions, SDS-PAGE analysis yields a subunit molecular weight for *Limulus* α_2M of 185 kDa, nearly identical to the 180-kDa subunit molecular weight of human α_2M (FIGURE 1A). The unexpected results showing that the *Limulus* molecule, composed of 185-kDa subunits, fractionated as a 550-kDa species under two different conditions suggested that *Limulus* α_2M was either a simple trimer or an unusual dimer whose extended structure was maintained under the denaturing conditions of nonreducing SDS-PAGE. Detailed structural studies employing a variety of physical biochemical approaches were undertaken to resolve this dilemma. Scanning transmission electron microscopy (STEM), which allows for the accurate determination of macromolecular mass of native molecules, yielded a mass of 354 kDa for *Limulus* α_2M.[31] Sedimentation equilibrium measurements of *Limulus* α_2M gave a value of 366 kDa.[31] Sedimentation velocity experiments indicated that isolated *Limulus* α_2M was a homogeneous component with a frictional ratio of 1.41.[31] Thus, the native α_2M from *Limulus* appears to be a (350-370)-kDa dimer with an unusually extended conformation.

Visualization of a negatively stained preparation of *Limulus* α_2M by transmission electron microscopy (TEM) is indicated in FIGURE 2. Low-power TEM (FIGURE 2A) demonstrates the homogeneity of the *Limulus* α_2M preparation and indicates the extended structure. High-magnification views of selected molecules of *Limulus* α_2M (FIGURE 2C) illustrate globular subunits joined to form a butterfly-like structure. Chymotrypsin treatment of *Limulus* α_2M results in a significant compaction of the extended molecules (FIGURE 2B) with a dramatic collapse of the butterfly-like structure (FIGURE 2D). Further demonstration of a pronounced alteration in the native structure of *Limulus* α_2M upon protease treatment was provided by comparative gel filtration chromatography and pore-limit gel electrophoresis.[32] Both of these techniques indicated that *Limulus* α_2M dimers undergo a dramatic and rapid compaction upon treatment with chymotrypsin. The magnitude of the structural compaction of *Limulus* α_2M upon protease treatment was far greater than that observed for mammalian α_2M tetramers and dimers.

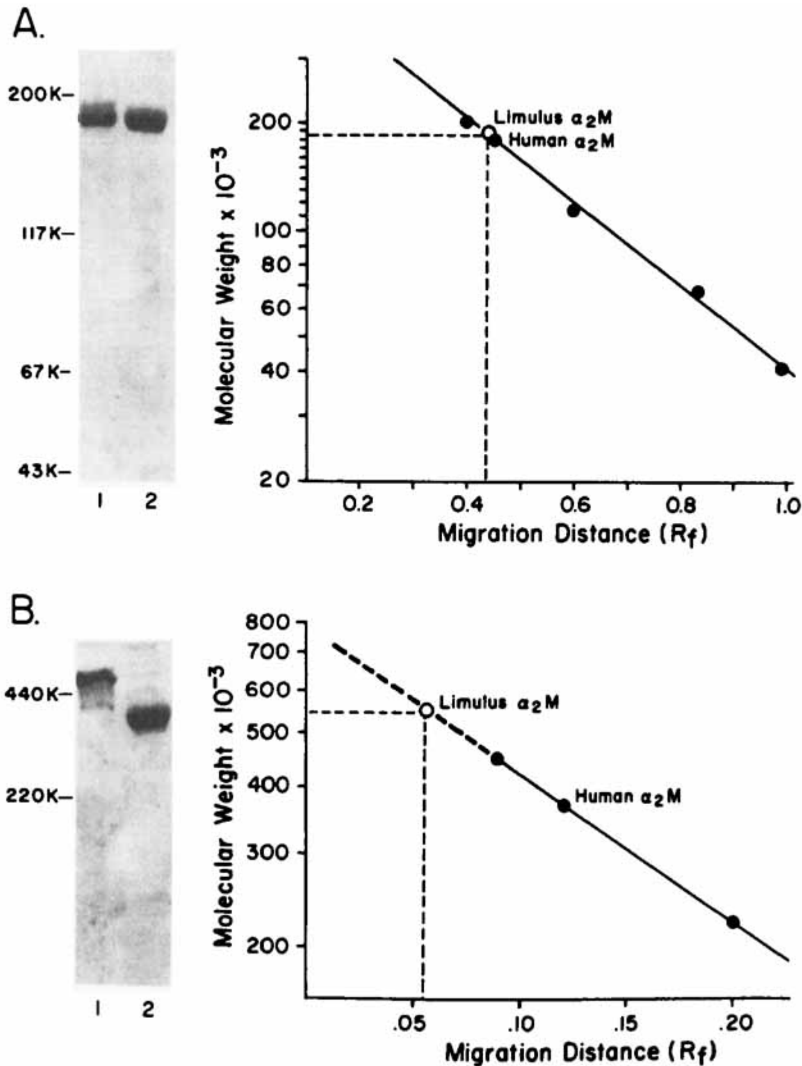

FIGURE 1. SDS-PAGE of the isolated *Limulus* α_2M and human α_2M under reducing (A) and nonreducing (B) conditions. (A) Ten μg of *Limulus* α_2M (lane 1) and human α_2M (lane 2) were reduced with β-mercaptoethanol and were electrophoresed on an SDS-polyacrylamide gel (6.5%). Protein standards, myosin (200 kDa), β-galactosidase (117 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa) were run in parallel lanes. The molecular weight of the *Limulus* α_2M subunit was estimated as 185 kDa (dashed line). (B) Ten μg of *Limulus* α_2M (lane 1) and human α_2M (lane 2) were electrophoresed in the absence of reducing agent on an SDS-polyacrylamide gel (5.5%). Protein standards, dimeric fibronectin (440 kDa), and monomeric fibronectin (220 kDa) were run in parallel lanes. The molecular weight of the unreduced *Limulus* inhibitor was estimated as 550 kDa (dashed line).

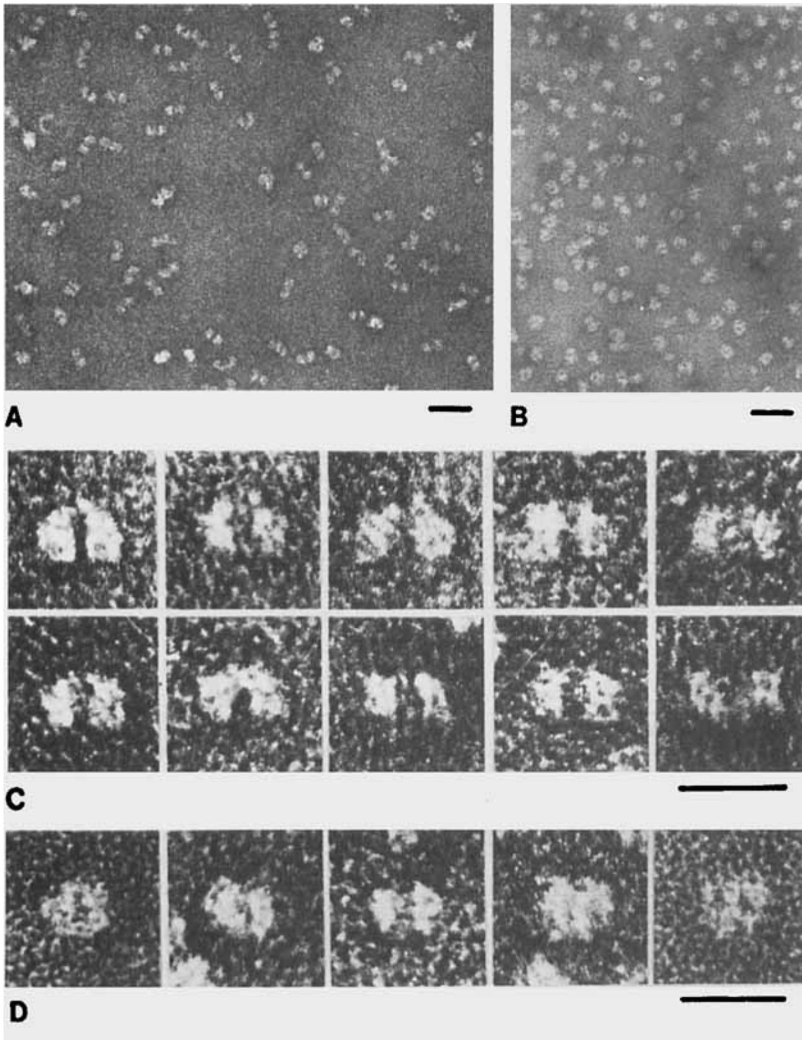

FIGURE 2. Transmission electron microscopy of *Limulus* α_2M negatively stained with uranyl acetate. Low-magnification fields of unreacted α_2M and chymotrypsin-reacted α_2M are shown in panels A and B, respectively. Panels C and D are composites of high-magnification views of individual molecules of unreacted (C) and chymotrypsin-reacted (D) α_2M. The globular subunits are associated as dimers. Bar = 40 nm (A & B); 20 nm (C & D).

Partial Primary Structure of Limulus *α_2M: Existence of a Highly Homologous Thiol Ester Domain*

Purified *Limulus* α_2M was cleaved with trypsin and 19 of the resulting tryptic peptides were sequenced and compared with sequences of human α_2M. Nine peptides,

```
N-terminal Sequence                                                          % Similarity

Limulus α2M:           K S G F I I T A P K S L T P G K
                       *     • • •     *     *                               38%
Human α2M:   S V S G M K P Q Y M V L V P S L L M T E T
             (1)
------------------------------------------------------------------------------------------
Limulus α2M: E Y V L P K
             * • * * * *                                                     100%
Human α2M:   E F V L P K
             (200)
------------------------------------------------------------------------------------------
Limulus α2M: L T A S P H S I C G I G A V D K
             • * *   *   * • *   •   * * *                                   67%
Human α2M:   V T A A P Q S V C A L R A V D Q
             (540)
------------------------------------------------------------------------------------------
Limulus α2M: S L G Q V N L T V Y G T S L P N E A I C G N Q N Y S T V T A R
             * * * • * *   * *         *         • * *         *             42%
Human α2M:   S L G N V N F T V S A E A L E S Q E L C G T E V P S V P E H G
             (841)
------------------------------------------------------------------------------------------
Thiol-ester domain

Limulus α2M: L P T G C G E Q N M I K
             • *   * * * * * * * •                                           83%
Human α2M:   M P Y G C G E Q N M V L
             (945)
------------------------------------------------------------------------------------------
Limulus α2M: G G I S S N D E T P A P L T A Y V L I S L L E A G Y K - K E T V I N Q G V R
             * * •       * *         * • * * •   *   * * *                 * •   •   •      49%
Human α2M:   G G V - - E D E V T - - L S A Y I T I A L L E I P L T V T H P V V R N A L F
             (1070)
------------------------------------------------------------------------------------------
Limulus α2M: S P S S S P Y Y - W G N S I G V E I A G Y A V L T L L Q H G G A S - - - - - - - - - N M A K
               *         *           *     * * •     *   • *     *         *                     * •   *      39%
Human α2M:   A P V G H F Y E P Q A P S A E V E M T S Y V L L A Y L T A Q P A P T S F D L T S A T N I V K
             (1169)
------------------------------------------------------------------------------------------
Limulus α2M: S Y E V D G N Q L N L Y F S E L T D Q K Q C F N F X L E Q D
                 * *     *   •   • * •     • •   *           *   •   * *     47%
Human α2M:   R T E V S S N H V L I Y L D K V S N Q T L S L F F T V L Q D
             (1384)
------------------------------------------------------------------------------------------
Limulus α2M: Q C F N F W L E Q D I E V Q E T K P A T I R
                     *   •   * * •   *   •   * * *   • •                     64%
Human α2M:   L S L F F T V L Q D V P V R D L K P A I V K
             (1404)
------------------------------------------------------------------------------------------
Limulus α2M: L Y D Y Y E L E Q E V V T S Y N I D E N C E K
             • * * * * *   •       •     * *         *   *                   57%
Human α2M:   V Y D Y Y E T D E F A I A E Y N A P - - C S K
             (1426)
```

FIGURE 3. Sequences of tryptic peptides of *Limulus* α_2M with significant sequence similarity to stretches of human α_2M. The internal tryptic peptides were purified by HPLC and sequenced. In addition to the thiol ester peptide and the N-terminal peptide, 17 internal peptides were chosen at random for partial or complete sequencing. Of these, the 8 that showed significant sequence similarity with stretches of human α_2M are shown here. The symbol $*$ indicates sequence identity and • indicates a conservative replacement. The number in parentheses indicates the residue number of human α_2M. The % similarity of *Limulus* α_2M shown on the right includes the number of identical and conservative replacement residues that align with human α_2M.

covering a total of 186 residues, either did not align with the same region of human α_2M or produced low scores (<20%) of similarity. Ten peptides, including the N-terminal sequence and the thiol ester-containing sequence, could be aligned unambiguously with stretches in human α_2M (FIGURE 3). The percent similarity in these peptides ranged from a low of 38% (N-terminal sequence) to a high of 100%. The 12-residue thiol ester-containing peptide of *Limulus* α_2M exhibited an 83% similarity

with that of human α_2M. A 7-residue stretch in this peptide, bordering the reactive Cys and Glx residues, is identical in *Limulus* α_2M and human α_2M, confirming the interpretation of the previously described methylamine treatment studies, which indicated a reactive thiol ester in *Limulus* α_2M.

Limulus α_2M *upon Treatment with Trypsin Forms High-Molecular-Weight Isopeptide-Bonded Structures That Do Not Appear to Contain Trypsin*

Treatment of *Limulus* α_2M with a twofold molar excess of trypsin yields bait region cleavage products of 100 and 85 kDa and high-molecular-mass products of 200, 250, and 300-350 kDa in reducing SDS-PAGE gels.[32] If the *Limulus* α_2M is pretreated with methylamine to inactivate the thiol ester and similarly treated with trypsin, the 100- and 85-kDa bait region fragments appear along with 125-kDa and 60-kDa fragments, but no high-molecular-weight fragments (>185 kDa) are observed.[32] Human α_2M treated with trypsin undergoes similar bait region fragmentation and formation of high-molecular-weight complexes. The formation of the latter also is eliminated by pretreatment of human α_2M with methylamine. When these reactions are carried out with biotinylated trypsin and the resulting SDS-PAGE gels are blotted and probed with avidin, the human high-molecular-weight complexes are clearly reactive, indicating that the biotinylated trypsin was incorporated into these complexes.[32] However, the high-molecular-weight complexes of trypsin-treated *Limulus* α_2M showed no evidence of trypsin incorporation. The high-molecular-weight complexes did react with antibody to *Limulus* α_2M.[32] Because methylamine treatment prevented the high-molecular-weight complex formation, the results suggest that the activated thiol ester in *Limulus* α_2M may function to link the resulting proteolytic fragments together directly, excluding the reacting protease. The thiol ester in *Limulus* α_2M may be externally located on the protein (as it is in C_3 and C_4) away from the "caged" protease, whereas in human α_2M the activated thiol ester may face or abut the protease entrapped in the cage and reacts with it, forming isopeptide bonds with the available amino groups of the protease.

In order to further analyze the apparently unique generation of high-molecular-weight complexes that form upon trypsin treatment of *Limulus* α_2M, a variety of different nucleophiles were incubated with the *Limulus* α_2M at the time of trypsin addition. The molecules were selected in order to serve as potential competitors or antagonists of the activated thiol ester reaction and to possibly prevent the formation of the isopeptide bonds that give rise to the high-molecular-weight complexes. The molecules included glycerol (a competitor of the thiol ester reaction in C_3),[33] dansyl cadaverine and polylysine (molecules that contain free amino groups that might link with the activated thiol ester), and exogenously added "bystander" proteins that might serve as competitors for the α_2M fragments that form the complexes. The results of these studies are illustrated in FIGURE 4A. Purified *Limulus* α_2M (lane 1) when treated with trypsin for 2 minutes forms the high-molecular-weight complexes (asterisks, lane 2). The presence of glycerol (lane 3), dansyl cadaverine (lane 4), or polylysine (lane 5) does not prevent the formation of the complexes. Bystander

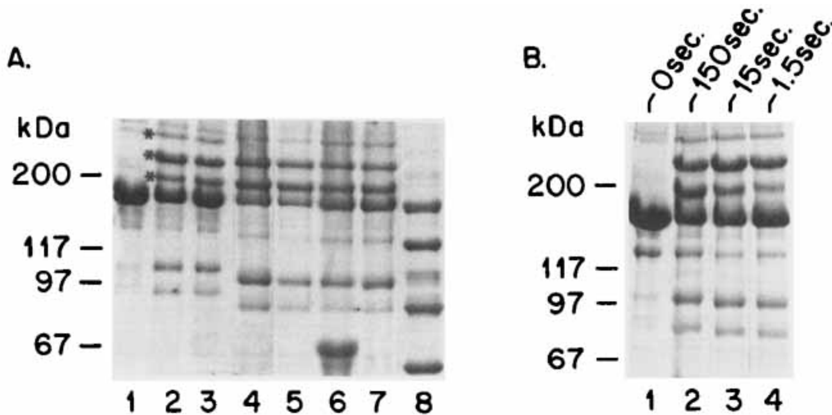

FIGURE 4. Formation of high-molecular-weight complexes when *Limulus* α_2M reacts with trypsin. (A) Purified *Limulus* α_2M (20 μg) in 0.1 M Tris buffer (pH 8.1) was incubated alone (lane 1) or with 2 μg of trypsin (lanes 2–7) for 2 minutes at 20 °C to yield the formation of high-molecular-weight α_2M complexes. Prior to the addition of trypsin, the following components were added into the reaction mixture: buffer (lane 2); 100 mM glycerol (lane 3); 7.5 mM dansyl cadaverine (lane 4); 100 μg polylysine (lane 5); 40 μg albumin (lane 6); 50 μg coagulagin (lane 7). At the end of the 2-minute reaction, SBTI (4 μg) and TLCK (10 mM) were added to inhibit trypsin and terminate the reaction. SDS sample buffer containing β-mercaptoethanol (reducing conditions) was added and the samples were boiled for 3 minutes and electrophoresed on SDS-polyacrylamide gels (6.5%). Methylamine-treated *Limulus* α_2M (20 μg) was also incubated for 2 minutes with trypsin (2 μg) and similarly analyzed (lane 8). The formation of the 200-kDa, 250-kDa, and (300–350)-kDa complexes (asterisks, lane 2) was not significantly diminished by any of the components. The added albumin is visible as a 67-kDa band in lane 6. (B) Purified *Limulus* α_2M (20 μg) in 0.1 M Tris buffer (pH 8.1) was incubated at 25 °C alone (lane 1) or with 2 μg of trypsin for 150 seconds (lane 2), 15 seconds (lane 3), and 1.5 seconds (lane 4). At the end of the incubation period, SBTI and TLCK were added and the samples were analyzed as described in part A on an SDS-polyacrylamide gel (6.0%). The formation of high-molecular-weight complexes occurred rapidly, within 1.5 seconds.

proteins such as albumin (lane 6) and coagulagin (a natural *Limulus* protein) (lane 7) also do not prevent or even diminish the generation of the high-molecular-weight complexes. When methylamine-treated *Limulus* α_2M is incubated with trypsin, no high-molecular-weight complexes are formed (lane 8), demonstrating that an activated thiol ester is required for complex formation.

In order to determine how rapidly the complexes form upon addition of protease to *Limulus* α_2M, a time course of trypsin treatment was carried out (FIGURE 4B). Two μg of trypsin was added to 20 μg of *Limulus* α_2M and, after 150 seconds (lane 2), 15 seconds (lane 3), or 1.5 seconds (lane 4) of incubation at 25 °C, an excess of SBTI and tosyl lysine chloromethyl ketone (TLCK) was immediately added to inhibit the trypsin and terminate the reaction. SBTI and TLCK when added prior to trypsin addition completely prevented the formation of high-molecular-weight complexes (data not shown). The results (FIGURE 4B) demonstrate that high-molecular-weight complex formation occurs within 1.5 seconds, the minimum time it takes to add the

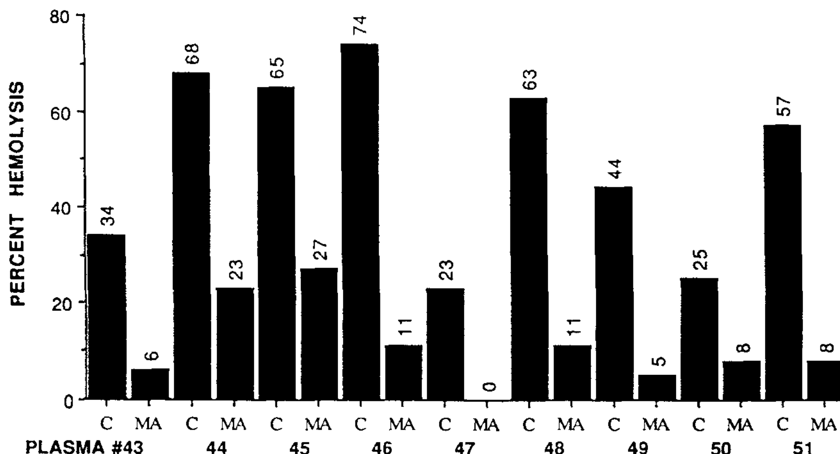

FIGURE 5. Effect of methylamine treatment on *Limulus* plasma-mediated hemolysis. Samples (0.2 mL) of plasma from each of nine individual horseshoe crabs were depleted of hemocyanin with 3% polyethylene glycol-8000 and were tested for hemolytic activity with sheep erythrocytes in the standard 0.8-mL assay.[35] Control samples (C) contained 0.1 M Tris (pH 8.1). Methylamine-treated samples (MA) contained 0.1 M Tris (pH 8.1) and 0.2 M methylamine. Control and methylamine-treated samples were incubated for 24 h at 22 °C prior to the assay of their hemolytic activity. The numbers at the top of each bar report the fraction of total hemoglobin released during the hemolytic assay. The plasma from every animal tested showed a reduction of its hemolytic activity following treatment with methylamine.

trypsin and immediately add the SBTI and TLCK inhibitor mixture. Thus, high-molecular-weight complex formation occurs rapidly (within seconds) and is not prevented by excess hydroxyl groups (glycerol), amino groups (polylysine and dansyl cadaverine), or an excess of bystander proteins. The nature of the complexes and the nature of the biochemical reaction(s) leading to their formation remain unresolved, except it is clear that the thiol ester of *Limulus* α_2M is involved in complex formation.

Involvement of Limulus *α_2M and Its Thiol Ester in the Hemolytic Activity of* Limulus *Plasma*

The function of α_2M as an efficient protease-binding and protease-clearing molecule and its structural conservation through 500 million years of evolution as evidenced by the *Limulus* α_2M homologue suggest a role for this molecule in animal defense mechanisms. Further indication of this role was provided by Enghild *et al.*,[34] who observed that *Limulus* α_2M is a component of the plasma-based cytolytic system of *Limulus*. We have confirmed these observations and have demonstrated[35] that the dose-dependent hemolytic activity of *Limulus* plasma is sensitive to methylamine treatment. The methylamine treatment, which eliminated greater than 95% of the activity of *Limulus* α_2M (as assayed by the ability of α_2M to bind trypsin), substantially reduced the hemolytic activity of *Limulus* plasma isolated from a number of different animals (FIGURE 5). The hemolytic activity of methylamine-treated plasma was re-

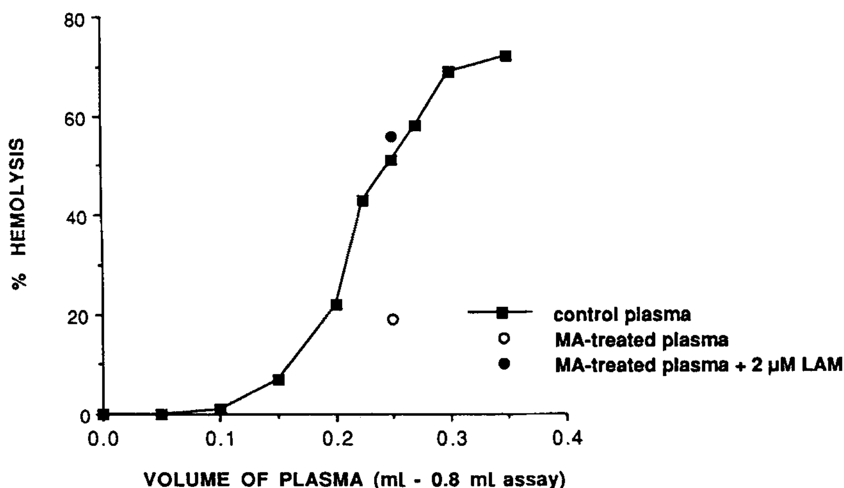

FIGURE 6. Hemolytic activity of *Limulus* plasma and the involvement of *Limulus* α_2M. Increasing amounts of *Limulus* plasma (■) show a dose-dependent increased level of hemolysis. Methylamine-treated plasma (○) shows reduced hemolytic activity, which can be restored by the addition of 2 μM purified *Limulus* α_2M (LAM) (●).

stored by the addition of purified *Limulus* α_2M (FIGURE 6). Methylamine-treated *Limulus* α_2M was unable to restore the hemolytic activity (data not shown). Purified *Limulus* α_2M, however, was not hemolytic by itself and thus *Limulus* plasma contains other factors that contribute to the observed hemolysis.

Recently, we have demonstrated that a C-Reactive Protein (CRP) homologue present in *Limulus* plasma is an essential component of the hemolytic system of *Limulus*.[35] *Limulus* CRP, purified by phosphorylethanolamine Sepharose affinity chromatography, was hemolytic by itself in the absence of other plasma components including α_2M.[35] A minor fraction of the CRP preparation, which functions as a lectin with sialyl-recognition specificity and is identical in molecular weight to CRP, appears to be the hemolytic protein. It represents less than 1% of the total CRP protein population and evinces the total sialyl-binding activity of *Limulus* CRP.

DISCUSSION

The sole protease inhibitor in the plasma of the ancient arthropod, *Limulus polyphemus*, clearly is a molecular and functional homologue of human α_2-macroglobulin. It shows several of the functional activities of α_2-macroglobulin, including the binding of proteases in a manner that prevents their subsequent interaction with protein substrates[16–19] and with macromolecular active-site inhibitors,[20] without affecting the activity of the bound protease against low-molecular-mass amide substrates.[18] *Limulus* α_2M possesses an internal thiol ester that is required for activity[36] and undergoes a profound structural compaction following interaction with proteases and reaction of

the thiol ester.[31] α_2M from humans and *Limulus* show significant identity at the level of peptide sequence (FIGURE 3), especially at the thiol ester domain.[29] A number of structural and functional differences, however, occur between human and *Limulus* α_2M, such as the unusually extended structure of *Limulus* α_2M (FIGURE 2), the large compaction that it undergoes upon protease treatment,[32] and the failure to incorporate the reacting protease molecule in the covalently linked high-molecular-weight complexes that are formed after proteolysis (FIGURE 4). The butterfly-like extended structure of *Limulus* α_2M and the large magnitude compaction or collapse that it undergoes make *Limulus* α_2M an interesting model protein for studying the biophysics of dramatic protein structural changes. These studies are now under way using stop-flow kinetic analysis to determine the rates of proteolytic cleavage of the bait region and activation of the thiol ester bond, in conjunction with small-angle X-ray scattering analysis to analyze both the kinetics and character of the structural alterations that follow upon protease binding. We hope that these studies will provide a more detailed analysis of the nature of the conformational change.

The covalent, high-molecular-weight complexes that form with *Limulus* α_2M upon protease treatment, which appear not to contain the reacting protease, clearly differentiate the invertebrate molecule from mammalian α_2M. It was originally thought[32] that *Limulus* α_2M's thiol ester might be located externally on the structure of α_2M away from the internal hydrophilic pocket that becomes the ultimate domicile for the entrapped protease, as is the case with the complement components C_3 and C_4. If this were the case, this would allow *Limulus* α_2M's reactive thiol ester to form covalent complexes with external targets, such as cell membrane proteins, as happens with complement component C_3.[14,37] However, the inability of either glycerol (a competitor for macromolecules for C_3's thiol ester reaction) or polylysine or bystander proteins to prevent or even diminish high-molecular-weight complex formation indicates that this proposed structural analogy of *Limulus* α_2M to C_3 may not be accurate. It has been suggested that the efficient covalent binding of proteases to human α_2M may function to immobilize the protease molecule and thereby limit the damage that the entrapped protease can eventually wreak upon the polypeptide bars of its cage.[38] Possibly, the exclusively intra-α_2M isopeptide bonding observed in protease-reacted *Limulus* α_2M may serve the same ends, but by strengthening the α_2M cage rather than by restraining the mobility of the entrapped protease molecule. The cloning and sequencing of the *Limulus* α_2M cDNA and the elucidation of the higher-order structure of native and reacted *Limulus* α_2M now under way should elucidate the orientation of the thiol ester and might suggest the mechanism of the unusual high-molecular-weight complex formation.

The participation of *Limulus* α_2M in the cytolytic activity of *Limulus* plasma and the dependence of this involvement on the thiol ester (FIGURES 5 and 6) suggest that α_2M functions in a cytolytic defense mechanism of this ancient invertebrate. However, important differences exist between the involvement of α_2M in the plasma cytolytic system of *Limulus* and the role of C_3 in the vertebrate complement-mediated cytolytic system. In the complement system, C_3 is absolutely required, whereas in *Limulus* the thiol ester-containing molecule, α_2M, is dispensable. It appears in *Limulus* plasma that a CRP-related lectin with sialyl-reactive selectivity plays the central role in cytolysis. Highly purified preparations of the CRP-related lectin are hemolytic in the

absence of other plasma proteins. The role that *Limulus* α_2M plays in hemolysis and its relationship and possible synergy with the CRP-related lectin remain unresolved.

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