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Author
Armstrong, PB

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\( \alpha_2M \) in the Horseshoe Crab

A Structural and Functional Invertebrate Homologue

PETER B. ARMSTRONG,\textsuperscript{a,b} LARS SOTTRUP-JENSEN,\textsuperscript{c} ATSUSHI IKAI,\textsuperscript{d} SUBITA SRIMAL,\textsuperscript{a,e} AND JAMES P. QUIGLEY\textsuperscript{a,f,g}

\textsuperscript{a}Marine Biological Laboratory
Woods Hole, Massachusetts 02543

\textsuperscript{b}Laboratory for Cell Biology
Department of Zoology
University of California
Davis, California 95616

\textsuperscript{c}Department of Molecular Biology
University of Aarhus
DK-8000 Aarhus, Denmark

\textsuperscript{d}Laboratory of Biodynamics
Tokyo Institute of Technology
Nagatsuta, Midoriku
Yokohama, Japan

\textsuperscript{e}Molecular Biophysics Unit
Indian Institute of Science
Bangalore, 560012 India

\textsuperscript{f}Department of Pathology
Health Sciences Center
State University of New York
Stony Brook, New York 11794

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

\textsuperscript{g}Send all correspondence to J. P. Quigley at the State University of New York at Stony Brook.
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 (α2M), 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.\textsuperscript{1,2}

The best-studied animal species of α2M, human α2M, is a homotetramer organized as a noncovalently linked dimer of disulfide-linked homodimers of a 180-kDa subunit.\textsuperscript{3,4} The disulfide-linked homodimer is thought to be the basic functional unit,\textsuperscript{5} but dimers of the noncovalently linked subunits can also bind proteases.\textsuperscript{6} Other members of the α2M family of protease-binding proteins exist as disulfide-linked homodimers.\textsuperscript{7,4} The mechanism of protease binding by α2M is unique. In contrast to the active-site inhibitors of proteases, α2M selectively inhibits the interaction of the targeted protease with macromolecular substrates without affecting the enzymatically active site. Protease binding to tetrameric human α2M can involve both covalent\textsuperscript{8-12} and noncovalent\textsuperscript{11} interactions. The latter involve a physical folding of the α2M molecule around the protease molecule, so as to "trap" it,\textsuperscript{13} with the arms of the α2M 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.\textsuperscript{9-12} Members of the α2M superfamily, which includes C3, C4, and C5 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 α2M because of the abundance of other protease inhibitors in mammalian plasma. α2M is presumed to be essential to survival because, in contrast to many other plasma proteins, full genetic deficiency of α2M in humans has not yet been found, presumably because such deficiency would be lethal. The physiological importance of α2M also is indicated by its structure and function being conserved throughout the evolution of higher animals.\textsuperscript{15} Investigation of structural and functional homologues of α2M isolated from species evolutionarily distant from mammals and possessing a less complex plasma may contribute to the identification of specific physiological roles for α2M.

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 α2M.\textsuperscript{16-19} Homologues of vertebrate α2M have now been described from a variety of invertebrates, including chelicerate and mandibulate arthropods\textsuperscript{20-24} and mollusks.\textsuperscript{25,26} These molecules share numerous functional properties with mammalian α2M and have remarkable identity at the level of peptide sequence in key functional domains.\textsuperscript{27-29} The present report describes our investigations of the structural and functional features of the forms of α2M found in invertebrates. The presence of homologues of α2M 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 α2M as a defense molecule.
TABLE 1. Inhibition of a Variety of Different Proteases by Limulus α₂M: Sensitivity of Inhibition to Methylamine Treatment

(A) Protease Tested Addition Limulus α₂M Protease Activity (cpm ³⁵C-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 α₂M Methylamine-Treated Limulus α₂M Protease Activity (cpm ³⁵C-Casein Hydrolyzed)

- trypsin (0.5 µg) - - 2415
+ trypsin (0.5 µg) + - 140
- trypsin (0.5 µg) - + 2350

RESULTS

Limulus α₂M: A Functional Homologue of Mammalian α₂M

A preparation of the α₂M homologue isolated from Limulus plasma was tested for its ability to inhibit a variety of proteolytic enzymes (TABLE 1). It is clear that Limulus α₂M 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 α₂M 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 α₂M 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 α₂M is a homologue of mammalian α₂M. α₂M is the only protease inhibitor that we have detected in the plasma of Limulus, although the blood cells of Limulus do secrete active-site protease inhibitors when stimulated to undergo exocytosis.

Further evidence that Limulus α₂M is a functional homologue of mammalian α₂M was provided by the demonstration that trypsin treated with Limulus α₂M, 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). These results provide evidence that Limulus \( \alpha_2 \)M, similar to mammalian \( \alpha_2 \)M, 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 \( \alpha_2 \)M: A Structural Homologue of Mammalian \( \alpha_2 \)M**

When *Limulus* \( \alpha_2 \)M 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 \( \alpha_2 \)M chromatographed under identical conditions. Following SDS-PAGE under nonreducing conditions, the *Limulus* \( \alpha_2 \)M molecule still yields an apparent molecular weight of 500–600 kDa, in sharp contrast to the resulting (350–380)-kDa dimeric human \( \alpha_2 \)M electrophoresed in parallel SDS gels (FIGURE 1B). Under reducing conditions, SDS-PAGE analysis yields a subunit molecular weight for *Limulus* \( \alpha_2 \)M of 185 kDa, nearly identical to the 180-kDa subunit molecular weight of human \( \alpha_2 \)M (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* \( \alpha_2 \)M 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* \( \alpha_2 \)M. Sedimentation equilibrium measurements of *Limulus* \( \alpha_2 \)M gave a value of 366 kDa. Sedimentation velocity experiments indicated that isolated *Limulus* \( \alpha_2 \)M was a homogeneous component with a frictional ratio of 1.41. Thus, the native \( \alpha_2 \)M from *Limulus* appears to be a (350–370)-kDa dimer with an unusually extended conformation.

Visualization of a negatively stained preparation of *Limulus* \( \alpha_2 \)M by transmission electron microscopy (TEM) is indicated in FIGURE 2. Low-power TEM (FIGURE 2A) demonstrates the homogeneity of the *Limulus* \( \alpha_2 \)M preparation and indicates the extended structure. High-magnification views of selected molecules of *Limulus* \( \alpha_2 \)M (FIGURE 2C) illustrate globular subunits joined to form a butterfly-like structure. Chymotrypsin treatment of *Limulus* \( \alpha_2 \)M 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* \( \alpha_2 \)M upon protease treatment was provided by comparative gel filtration chromatography and pore-limit gel electrophoresis. Both of these techniques indicated that *Limulus* \( \alpha_2 \)M dimers undergo a dramatic and rapid compaction upon treatment with chymotrypsin. The magnitude of the structural compaction of *Limulus* \( \alpha_2 \)M upon protease treatment was far greater than that observed for mammalian \( \alpha_2 \)M tetramers and dimers.
FIGURE 1. SDS-PAGE of the isolated *Limulus* α2M and human α2M under reducing (A) and nonreducing (B) conditions. (A) Ten μg of *Limulus* α2M (lane 1) and human α2M (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* α2M subunit was estimated as 185 kDa (dashed line). (B) Ten μg of *Limulus* α2M (lane 1) and human α2M (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* α₂M negatively stained with uranyl acetate. Low-magnification fields of unreacted α₂M and chymotrypsin-reacted α₂M 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) α₂M. The globular subunits are associated as dimers. Bar = 40 nm (A & B); 20 nm (C & D).

Partial Primary Structure of *Limulus* α₂M: Existence of a Highly Homologous Thiol Ester Domain

Purified *Limulus* α₂M was cleaved with trypsin and 19 of the resulting tryptic peptides were sequenced and compared with sequences of human α₂M. Nine peptides,
FIGURE 3. Sequences of tryptic peptides of Limulus α₂M with significant sequence similarity to stretches of human α₂M. 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 α₂M are shown here. The symbol * indicates sequence identity and † indicates a conservative replacement. The number in parentheses indicates the residue number of human α₂M. The % similarity of Limulus α₂M shown on the right includes the number of identical and conservative replacement residues that align with human α₂M. Covering a total of 186 residues, either did not align with the same region of human α₂M 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 α₂M (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 α₂M exhibited an 83% similarity...
with that of human $\alpha_2$M. A 7-residue stretch in this peptide, bordering the reactive Cys and Glx residues, is identical in Limulus $\alpha_2$M and human $\alpha_2$M, confirming the interpretation of the previously described methylamine treatment studies, which indicated a reactive thiol ester in Limulus $\alpha_2$M.

**Limulus $\alpha_2$M upon Treatment with Trypsin Forms High-Molecular-Weight Isopeptide-Bonded Structures That Do Not Appear to Contain Trypsin**

Treatment of Limulus $\alpha_2$M 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. If the Limulus $\alpha_2$M 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. Human $\alpha_2$M 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 $\alpha_2$M 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. However, the high-molecular-weight complexes of trypsin-treated Limulus $\alpha_2$M showed no evidence of trypsin incorporation. The high-molecular-weight complexes did react with antibody to Limulus $\alpha_2$M. Because methylamine treatment prevented the high-molecular-weight complex formation, the results suggest that the activated thiol ester in Limulus $\alpha_2$M may function to link the resulting proteolytic fragments together directly, excluding the reacting protease. The thiol ester in Limulus $\alpha_2$M may be externally located on the protein (as it is in C3 and C4) away from the "caged" protease, whereas in human $\alpha_2$M 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 $\alpha_2$M, a variety of different nucleophiles were incubated with the Limulus $\alpha_2$M 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 C3), 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 $\alpha_2$M fragments that form the complexes. The results of these studies are illustrated in Figure 4A. Purified Limulus $\alpha_2$M (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* \( \alpha_2 \)M reacts with trypsin. (A) Purified *Limulus* \( \alpha_2 \)M (20 \( \mu \)g) in 0.1 M Tris buffer (pH 8.1) was incubated alone (lane 1) or with 2 \( \mu \)g of trypsin (lanes 2-7) for 2 minutes at 20 °C to yield the formation of high-molecular-weight \( \alpha_2 \)M 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 \( \mu \)g polylysine (lane 5); 40 \( \mu \)g albumin (lane 6); 50 \( \mu \)g coagulagin (lane 7). At the end of the 2-minute reaction, SBTI (4 \( \mu \)g) and TLCK (10 mM) were added to inhibit trypsin and terminate the reaction. SDS sample buffer containing \( \beta \)-mercaptoethanol (reducing conditions) was added and the samples were boiled for 3 minutes and electrophoresed on SDS-polyacrylamide gels (6.5%). Methylamine-treated *Limulus* \( \alpha_2 \)M (20 \( \mu \)g) was also incubated for 2 minutes with trypsin (2 \( \mu \)g) 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* \( \alpha_2 \)M (20 \( \mu \)g) in 0.1 M Tris buffer (pH 8.1) was incubated at 25 °C alone (lane 1) or with 2 \( \mu \)g 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* \( \alpha_2 \)M 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* \( \alpha_2 \)M, a time course of trypsin treatment was carried out (Figure 4B). Two \( \mu \)g of trypsin was added to 20 \( \mu \)g of *Limulus* \( \alpha_2 \)M 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. 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\textsubscript{2}M is involved in complex formation.

**Involvement of Limulus α\textsubscript{2}M and Its Thiol Ester in the Hemolytic Activity of Limulus Plasma**

The function of α\textsubscript{2}M 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\textsubscript{2}M homologue suggest a role for this molecule in animal defense mechanisms. Further indication of this role was provided by Enghild et al., who observed that Limulus α\textsubscript{2}M is a component of the plasma-based cytolytic system of Limulus. We have confirmed these observations and have demonstrated 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\textsubscript{2}M (as assayed by the ability of α\textsubscript{2}M 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 α2M. 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 α2M (LAM) (●).

Stored by the addition of purified Limulus α2M (FIGURE 6). Methylamine-treated Limulus α2M was unable to restore the hemolytic activity (data not shown). Purified Limulus α2M, 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. Limulus CRP, purified by phosphorylethanolamine Sepharose affinity chromatography, was hemolytic by itself in the absence of other plasma components including α2M. 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 α2-macroglobulin. It shows several of the functional activities of α2-macroglobulin, including the binding of proteases in a manner that prevents their subsequent interaction with protein substrates and with macromolecular active-site inhibitors, without affecting the activity of the bound protease against low-molecular-mass amide substrates. Limulus α2M possesses an internal thiol ester that is required for activity and undergoes a profound structural compaction following interaction with proteases and reaction of
the thiol ester.\textsuperscript{31} \(\alpha_2\)M from humans and \textit{Limulus} show significant identity at the level of peptide sequence (FIGURE 3), especially at the thiol ester domain.\textsuperscript{29} A number of structural and functional differences, however, occur between human and \textit{Limulus} \(\alpha_2\)M, such as the unusually extended structure of \textit{Limulus} \(\alpha_2\)M (FIGURE 2), the large compaction that it undergoes upon protease treatment,\textsuperscript{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 \textit{Limulus} \(\alpha_2\)M and the large magnitude compaction or collapse that it undergoes make \textit{Limulus} \(\alpha_2\)M 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 \textit{Limulus} \(\alpha_2\)M upon protease treatment, which appear not to contain the reacting protease, clearly differentiate the invertebrate molecule from mammalian \(\alpha_2\)M. It was originally thought\textsuperscript{32} that \textit{Limulus} \(\alpha_2\)M's thiol ester might be located externally on the structure of \(\alpha_2\)M away from the internal hydrophilic pocket that becomes the ultimate domicile for the entrapped protease, as is the case with the complement components \(C_1\) and \(C_4\). If this were the case, this would allow \textit{Limulus} \(\alpha_2\)M's reactive thiol ester to form covalent complexes with external targets, such as cell membrane proteins, as happens with complement component \(C_3\).\textsuperscript{14,37} However, the inability of either glycerol (a competitor for macromolecules for \(C_1\)'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 \textit{Limulus} \(\alpha_2\)M to \(C_3\) may not be accurate. It has been suggested that the efficient covalent binding of proteases to human \(\alpha_2\)M 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.\textsuperscript{38} Possibly, the exclusively intra-\(\alpha_2\)M isopeptide bonding observed in protease-reacted \textit{Limulus} \(\alpha_2\)M may serve the same ends, but by strengthening the \(\alpha_2\)M cage rather than by restraining the mobility of the entrapped protease molecule. The cloning and sequencing of the \textit{Limulus} \(\alpha_2\)M cDNA and the elucidation of the higher-order structure of native and reacted \textit{Limulus} \(\alpha_2\)M 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 \textit{Limulus} \(\alpha_2\)M in the cytolytic activity of \textit{Limulus} plasma and the dependence of this involvement on the thiol ester (FIGURES 5 and 6) suggest that \(\alpha_2\)M functions in a cytolytic defense mechanism of this ancient invertebrate. However, important differences exist between the involvement of \(\alpha_2\)M in the plasma cytolytic system of \textit{Limulus} and the role of \(C_1\) in the vertebrate complement-mediated cytolytic system. In the complement system, \(C_1\) is absolutely required, whereas in \textit{Limulus} the thiol ester-containing molecule, \(\alpha_2\)M, is dispensable. It appears in \textit{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,M plays in hemolysis and its relationship and possible synergy with the CRP-related lectin remain unresolved.

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