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α_2-Macroglobulin does not function as a C3 homologue in the plasma hemolytic system of the American horseshoe crab, *Limulus*

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Abstract

A major problem of comparative immunology is the characterization of the internal defense systems that lyse foreign cells, such as bacteria and other microbial pathogens that have gained entry into the body. The plasma cytolytic system of the American horseshoe crab, *Limulus polyphemus*, is sensitive to treatment with methylamine, which inactivates the abundant plasma defense protein α_2-macroglobulin. This has been interpreted to mean that α_2-macroglobulin plays an important role in hemolysis, analogous to the role of complement component C3 of the mammalian complement system (Enghild et al., 1990). Sensitivity to methylamine has been suggested to reflect an evolutionary homology with the plasma cytolytic system of mammals, in which the complement system is inactivated by the reaction of methylamine with complement components C3 and C4. C3, C4 and α_2-macroglobulin contain an internal thiol ester bond linking cysteinyl and glutamic acid residues and methylamine inactivates all three proteins by reaction with the thiol-esterified glutamic acid. However, we have recently shown that the principal effector of hemolysis in *Limulus* is the plasma lectin, limulin (Armstrong et al., 1996). In this article we show that native, unreacted α_2-macroglobulin is not involved directly in hemolysis but instead that methylamine-reacted α_2-macroglobulin inhibits the hemolytic activity of limulin. Thus the thiol ester proteins α_2-macroglobulin and C3 operate very differently in the hemolytic systems of *Limulus* and mammals and are not functionally homologous. *Limulus* α_2-macroglobulin functions indirectly in hemolysis: its inactivation yields an inhibitory molecule for limulin-mediated hemolysis.

Keywords: Limulin; Hemolysis; Lectins; α_2-macroglobulin; *Limulus*

1. Introduction

α_2-Macroglobulin, an abundant protein in the plasma of the horseshoe crab *Limulus polyphemus* (Quigley and Armstrong 1983), is a member of the same protein family as mammalian α_2-macroglobulin and C3, C4 and C5 of the mammalian complement system (Sottrup-Jensen, 1987). A characteristic of C3, C4 and the α_2-macroglobulins is the presence of an internal thiol ester bond (Tack, 1983) that in *Limulus* α_2-macroglobulin links Cys-999 with Glx-1002 (Ikawi et al., 1996). The physiologically important modifications of these thiol ester proteins by proteolysis are followed by the rapid hydrolysis of the thiol ester bond. Small nucleophiles, such as the small primary amine methylamine, can react in the absence of proteolysis with the thiol-esterified glutamic acid, resulting in cleavage of the thiol-ester and functional inactivation of the protein (Barrett et al., 1979; Swenson and Howard, 1979; Sottrup-Jensen et al., 1980; Gonias et al., 1982). Methylamine treatment inactivates the protease-binding activity of mammalian and *Limulus* α_2-macroglobulin (Armstrong and Quigley, 1987) and the cytolytic activities of the mammalian complement system (Tack, 1983). Reaction of α_2-macroglobulin with methylamine or proteases causes a molecular compaction (Barrett et al., 1979; Gonias et al., 1982; Björk and Fish, 1982; Armstrong et al., 1991) and the exposure of new epitopes at the surface of the molecule (Marynen, et al., 1981; Sottrup-Jensen et al., 1986). The molecular compaction produces a form that migrates more rapidly in pore-limit gel electrophoresis (Barrett et al., 1979; Armstrong et al., 1991), which has established the names 'fast-form' for the reacted forms of α_2-macroglobulin and 'slow-form'

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for the unreacted form of the protein (Barrett et al., 1979). α_2-Macroglobulin is the sole protein of *Limulus* plasma that binds [^{14}C]-methylamine, suggesting that it is the only thiol ester protein of this system (Dodds, pers. comm.).

Higher animals possess a diversity of immune systems to defend against invasive microbial pathogens. One of the important immune defense strategies, found throughout the animal kingdom, is the cytolysis of foreign cells mediated by humoral factors of the plasma or serum (Canicatti, 1990). The principal pathway for foreign cell cytolysis in mammals is the multifactoral complement cascade alluded to above (Law and Reid, 1988). The thiol ester proteins C3 and C4 are key elements in this defense system and the inactivation of C3 by methylamine inactivates complement-mediated cytolysis (Swenson and Howard, 1979; Tack et al., 1980; Tack, 1983). The observation that the hemolytic activity found in the plasma of *Limulus polyphemus* is sensitive to methylamine treatment suggested that α_2-macroglobulin plays a role in the cytolytic destruction of foreign cells similar to that of C3 and C4 in the mammalian complement system (Enghild et al., 1990; Armstrong et al., 1993). Although molecular homologues of C3/C4 have been identified in primitive vertebrates (Dodds and Day, 1993), ascidians (Baish et al., 1997) and echinoderms (Smith et al., 1996; Al-Sharif et al., 1997), this is the first claim for a functional homologue in the protostomata. This notion, which has important implications for the evolution of the complement system, became puzzling when it was demonstrated that the actual effector of the plasma-based hemolytic system in *Limulus* is the sialic acid-binding protein limulin. Purified limulin is hemolytic at 5–10 nM and the removal of limulin from whole plasma eliminates its hemolytic activity (Armstrong et al., 1996a). In the present re-investigation of this problem, we show that unreacted, 'slow-form' α_2-macroglobulin is completely inactive in the hemolytic system of *Limulus* and that thiol ester-reacted, 'fast form' *Limulus* α_2-macroglobulin inhibits hemolysis when present at the high molar excess to limulin that is found in plasma. (Limulin is present in *Limulus* plasma at 30–50 nM (Swarnakar et al., 1995) and α_2-macroglobulin is present at 1–5 μM (Enghild et al., 1990)). Thus the suggestion that α_2-macroglobulin functions as a C3 homologue is incorrect. Instead the inhibition of hemolysis reported previously (Enghild et al., 1990; Armstrong et al., 1993) is attributable to an inhibitory interaction between fast-form α_2-macroglobulin and the actual effector of hemolysis, the protein limulin.

2. Materials and methods

2.1. Purification of α_2-macroglobulin and limulin

Recently collected adult horseshoe crabs were obtained from the Marine Resources Center of the Marine Biological Laboratory in Woods Hole, MA, U.S.A. Blood was collected under sterile, lipopolysaccharide-free conditions by cardiac puncture from pre-chilled animals and the blood cells were removed immediately after bleeding (Armstrong, 1985). It is important to avoid degranulation of the blood cells, since this releases proteases (Iwanaga et al., 1992) and active-site protease inhibitors (Armstrong and Quigley, 1985; Nakamura et al., 1987; Donovan and Laue, 1991) into the serum. Animals were released into the ocean unharmed after bleeding. Most of the hemocyanin was removed from the plasma by ultracentrifugation (141,000 ***g*** for 16 h.) or by incubation with 3% polyethylene glycol-8000 with centrifugation at 30,000 ***g*** for 0.5 h. Residual hemocyanin was precipitated with zinc acetate. Limulin was purified by treatment of the hemocyanin-free plasma with plain Sepharose 4B to remove the Sepharose-binding proteins followed by sequential chromatography on phosphorylethanolamine agarose and fetuin-Sepharose as described previously (Armstrong et al., 1996a). α_2-Macroglobulin was purified from the fraction that failed to bind to phosphorylethanolamine agarose by gel filtration chromatography on Sephadex S-300 HR and ion exchange chromatography on Waters Protein Pak Q 15HR anion exchange resin as described previously (Armstrong et al., 1996b).

2.2. Hemolysis

The hemolytic activity of the plasma was determined in duplicate or triplicate samples using sheep red blood cells (Gee, 1983; Kabat and Mayer, 1961; Sim, 1981). Unactivated sheep erythrocytes in Alsevers solution were obtained from Becton Dickinson and Company, Cockeysville, MD, U.S.A. (reference number 12388). The reaction mixtures contained 3×10^7 washed sheep red cells in a final volume of 800 μl. The buffer system was modified DGVB (0.19 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% glucose, 0.1% gelatin, 2.5 mM sodium barbital, pH 7.3). The samples were incubated at 22–23°C for 4 h, and the reaction was terminated by adding 2 ml of ice-cold phosphate-buffered saline containing 5 mM ethylenediaminetetraacetic acid, followed by centrifugation to remove the red cells. The extent of hemolysis was determined by monitoring released hemoglobin in the supernatant by the optical absorbency at 412 nm and was compared to full hemolysis produced by hypotonic lysis of the red cells by addition of 2 ml of distilled water.

2.3. Hemagglutination

The hemagglutination assay was performed using sheep erythrocytes that had been washed and suspended at 2% (v/v) in buffer A (0.15 M NaCl, 0.01 M $CaCl_2$, 0.05 M Tris, pH 7.3). 25 μl volumes of 2-fold serially-diluted samples dissolved in buffer A were mixed with 25

μl of the erythrocyte suspension in 200 μl round-bottom microtiter wells (Sigma, cat no. M-4029), incubated for 45 min at room temperature and scored for hemagglutination. Hemagglutinated erythrocytes formed a uniform mat covering the entire curved lower surface of the microtiter well ('umbrella formation'); non-agglutinated erythrocytes formed a compact pellet at the very bottom of the well ('button formation') (Sakar et al., 1981). The hemagglutination endpoint was the highest dilution of sample that produced visible agglutination.

2.4. *Generation of fast-form* Limulus α_2-*macroglobulin*

Purified *Limulus* α_2-macroglobulin was treated for 24 h with 0.2 M methylamine in 0.1 M Tris buffer, pH 8.0 at 22°C. Following conversion of α_2-macroglobulin, the methylamine was removed by dialysis. These conditions reduce the trypsin-binding activity of α_2-macroglobulin by 95–98% (Armstrong et al., 1985). Control preparations of α_2-macroglobulin were treated identically with 0.1 M Tris, pH 8.0 alone. Plasma that had been depleted in hemocyanin with 3% polyethylene glycol-8000 was treated similarly: experimental samples were exposed to 0.2 M methylamine, control samples were exposed to buffer alone. Purified α_2-macroglobulin and hemocyanin-depleted plasma were reacted with trypsin in molar excess of the α_2-macroglobulin and then the unbound trypsin was inactivated with soybean trypsin inhibitor (Armstrong et al., 1985). The conditions for treatment with methylamine and trypsin were chosen to convert greater than 95% of the α_2-macroglobulin to the fast form of the protein.

2.5. *Solid-phase binding assay*

Limulin (5 μg/ml, 100 μl/well) was adsorbed to Reactibind maleic anhydride-activated polystyrene plates (Pierce Lot No. 95041069) in PBS, pH 7.3, 4°C, 12 h. Under these conditions approximately 10% of the limulin bound to the microtiter well, as measured by determining the binding of [^{125}I]-limulin of known specific activity. Wells were washed with 0.1% BSA in PBS, 0.05% Tween 20 (PBS-Tween 20) to remove unbound limulin, then were blocked for 1 h at room temperature with 1.0% BSA in PBS-Tween 20. Samples of *Limulus* α_2-macroglobulin (native and methylamine-treated) were incubated in the limulin coated wells for 2 h at room temperature in buffer A. Wells were washed three times with PBS-Tween 20 then incubated with affinity purified rabbit anti-*Limulus* α_2-macroglobulin (1 : 1000) followed by horseradish peroxidase-conjugated goat-anti-rabbit immunoglobulin (1 : 10,000). Horseradish peroxidase was visualized with 0-phenylene diamine (Sigma). The color reaction was stopped by 3 N HCl and the plate was evaluated in an ELISA reader (Bio-Rad) at 490 nm.

3. Results and discussion

Treatment of plasma with trypsin or methylamine to cleave the thiol ester and convert the endogenous α_2-macroglobulin to the fast form resulted in a shift of the dose/response hemolysis curve to the right (e.g. higher concentrations of plasma were required to produce hemolysis) (Fig. 1). The inhibitory effect of methylamine treatment is robust and widespread because it was seen in all 18 plasma samples collected from 18 different animals (Table 1). Methylamine is not acting on limulin, the primary hemolytic protein, because the inclusion of 0.2 M methylamine in a hemolysis sample containing only limulin and red cells failed to inhibit hemolysis (data not shown).

The reduction in the hemolytic activity of plasma following methylamine treatment was initially interpreted as reflecting a direct involvement of native, slow-form α_2-macroglobulin in the hemolytic process in *Limulus* (Enghild et al., 1990). However in hemolysis experiments using purified limulin, native slow-form *Limulus* α_2-macroglobulin was inactive, whereas fast-form α_2-macroglobulin, produced by protease or methylamine treatment, depressed the hemolytic activity of limulin (Fig. 2). The interaction is complicated because at low concentrations of limulin and fast-form α_2-macroglobulin

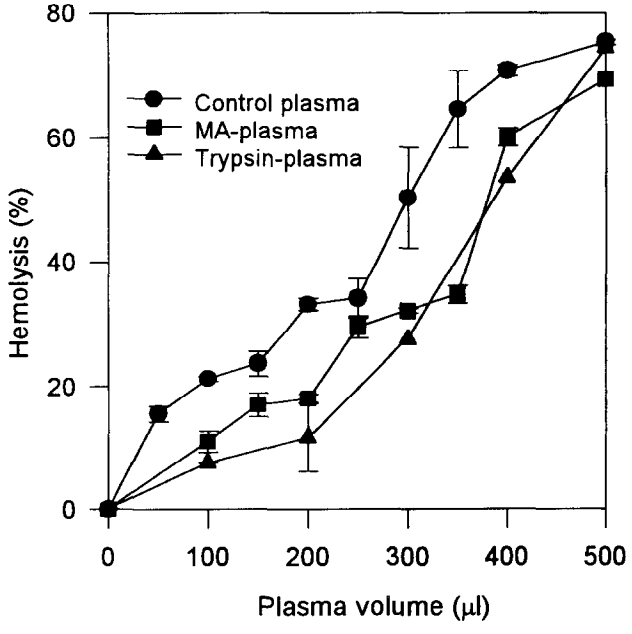

Fig. 1. Treatment of hemocyanin-depleted *Limulus* plasma with methylamine or trypsin, agents that cause a conversion of the endogenous α_2-macroglobulin to the fast form of the protein, diminishes the hemolytic activity of the plasma. Hemocyanin was removed from plasma with 3% polyethylene glycol and the supernatant was assayed for hemolytic activity as described in Materials and methods. Conversion of the endogenous α_2-macroglobulin to fast-form protein shifted the hemolytic dose-response curve to the right with higher concentrations of plasma required to produce a given amount of hemolysis.

Table 1
Reduction of the hemolytic activity of hemocyanin-depleted *Limulus* plasma[a] by treatment with methylamine

Animal	Hemolytic activity (%)	
	Untreated plasma[b]	Methylamine-treated plasma[b]
1	58	15
2	29	2
3	24	3
4	54	4
5	51	18
6	29	0
7	46	4
8	51	5
9	41	6
10	34	6
11	68	23
12	65	27
13	74	11
14	23	0
15	63	11
16	44	5
17	25	8
18	57	8

[a] Samples of plasma from each of 18 horseshoe crabs were depleted of hemocyanin with 3% polyethylene glycol-8000 and 0.2 ml aliquots were tested for hemolytic activity with sheep erythrocytes in the standard 0.8 ml assay.
[b] Samples of plasma were treated for 1 day at 22 °C with 0.2 M methylamine with 0.1 M Tris, pH 8.0 (experimental samples) or with 0.1 M Tris, pH 8.0 alone (control samples).

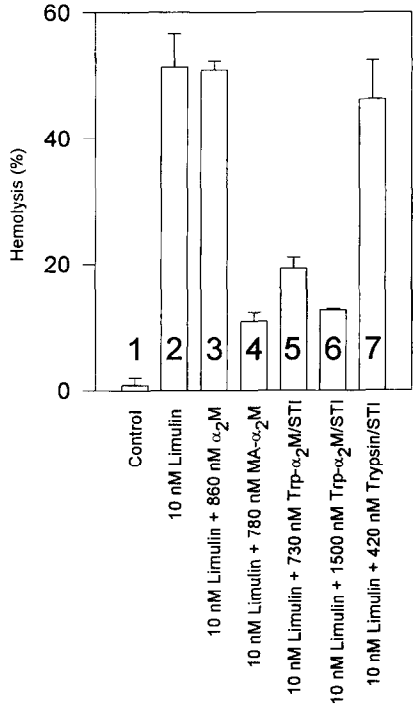

Fig. 2. The hemolytic activity of purified limulin (sample 2) is reduced by a large molar excess of fast-form *Limulus* α_2-macroglobulin generated by incubation of α_2-macroglobulin with methylamine (MA-α_2M; sample 4) or trypsin (Trp-α_2M; samples 5 and 6). Native, slow-form *Limulus* α_2-macroglobulin (α_2M; sample 3) is without effect. Trypsin inactivated by soybean trypsin inhibitor (STI; sample 7) is not inhibitory.

a potentiation of the hemolytic action of sub-threshold concentrations of limulin was observed, whereas at higher concentrations of limulin and at molar ratios of the two proteins that resemble those of plasma, the above-reported inhibition of hemolysis was found (Fig. 3). In plasma, the concentration of limulin is approximately 30–50 nM (Swarnakar et al., 1995) and α_2-macroglobulin is present at 1–5 μM (Enghild et al., 1990).

Fast-form α_2-macroglobulin binds to substrate-immobilized limulin with a 10-fold higher avidity than native α_2-macroglobulin (Table 2, lines 1 and 2), suggesting that a direct interaction of these two molecules underlies the effects of fast-form *Limulus* α_2-macroglobulin on the hemolytic actions of limulin. Consistent with this explanation is the observation that fast-form α_2-macroglobulin selectively depressed the hemagglutinating action of limulin (Table 3). It is suggested that binding of fast-form α_2-macroglobulin to limulin reduces limulin's ability to interact with sialoglycoconjugates on the test red cells, resulting in a reduction both in hemolysis and hemagglutination.

Native, unreacted *Limulus* α_2-macroglobulin can partially reverse the inhibition of hemolysis by methylamine treatment of plasma (Enghild et al., 1990, Armstrong et al., 1993). This can be reproduced using purified reagents (Fig. 4). Purified *Limulus* α_2-macroglobulin restored hemolysis in a dose-dependent fashion to limulin depressed by methylamine-reacted α_2-macroglobulin (Fig. 4, samples 7 and 8). This effect appears to result from reduced binding of fast-form α_2-macroglobulin to substrate-immobilized limulin (Table 2, lines 3, 4) but does not involve direct competition of native and fast-form α_2-macroglobulin for limulin because only fast-form α_2-macroglobulin is capable of binding (Table 2, line 1). At present, we have no data that identifies the molecular mechanism responsible for the restorative powers of native, slow-form α_2-macroglobulin. In the absence of limulin, neither slow-form nor fast-form α_2-macroglobulin has detectable hemolytic activity (Fig. 4, samples 9 and 10).

Previous studies have shown that limulin is responsible for hemolysis (Armstrong et al., 1996a). The observation that conversion of the endogenous α_2-macroglobulin of *Limulus* plasma to fast-form protein by treatment of plasma with protease or methylamine reduces its hemolytic activity (Enghild et al., 1990; Armstrong et al., 1993) was interpreted as indicative of a direct involvement of α_2-macroglobulin in the hemolytic process. The obvious precedent for this suggestion is the mammalian complement system where inactivation of the thiol ester of C3 by methylamine inactivates the hemolytic activity

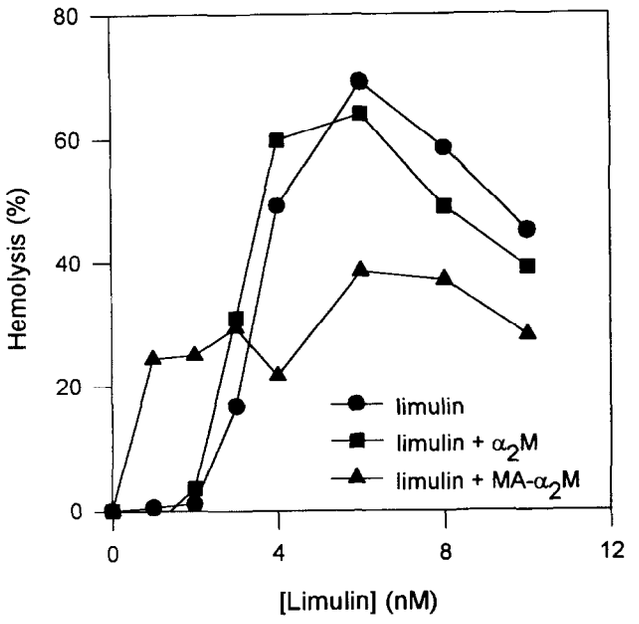

Fig. 3. Effects of α_2-macroglobulin on the hemolytic activity of limulin. *Limulus* α_2-macroglobulin was present at 100 molar excess of limulin for all of the data points. Hemolysis of sheep red blood cells by limulin (—●—) is unaffected by native *Limulus* α_2-macroglobulin (native α_2M) (—■—). At low concentrations of protein (0.1–0.3 μM of methylamine-reacted *Limulus* α_2-macroglobulin (MA-α_2M), 1–3 nM of limulin), hemolysis is potentiated. Hemolysis is depressed at higher concentrations of the proteins (0.4–1.0 μM of methylamine-reacted α_2-macroglobulin, 4–10 nM limulin) (—▲—). The situation at the higher concentrations of limulin and α_2-macroglobulin approaches the condition of plasma, which contains 1–5 μM of α_2-macroglobulin and 30–50 nM of limulin. Two additional trials of the effects of α_2-macroglobulin on hemolysis gave similar results.

Table 2
Binding of *Limulus* α_2-macroglobulin to substrate-immobilized limulin[a]

Addition to limulin-coated microtiter well	Optical absorbency (490 nm)[b]
1.4 nM Native *Limulus* α_2-macroglobulin	0.026
1.4 nM Methylamine (MA)-treated α_2-macroglobulin	0.239
1.4 nM MA-α_2-macroglobulin + native α_2-macroglobulin (1 : 1)	0.026
1.4 nM MA-α_2-macroglobulin + native α_2-macroglobulin (1 : 10)	0.025

[a] Malic anhydride-activated microtiter wells were coated with 50 ng of limulin/well and then were incubated with α_2-macroglobulin in buffer A, 2 h, 22°C. The α_2-macroglobulin that remained bound to the plate following extensive washing was detected with an affinity-purified anti-*Limulus* α_2-macroglobulin antibody and the amount of antibody bound was quantified with a horseradish peroxidase-linked second antibody system.

[b] Horseradish peroxidase was visualized with 0-phenylene diamine (Sigma). The color reaction was stopped by 3 N HCl and the plate was evaluated in an ELISA reader (Bio-Rad) at 490 nm.

Table 3
Effect of *Limulus* α_2-macroglobulin (α_2M) on the hemagglutination activity of purified limulin

Sample	Endpoint[a]
Control	0
10 nM Limulin	64
10 nM Limulin + 120 nM native *Limulus* α_2M	32
10 nM Limulin + 100 nM methylamine (MA)-treated *Limulus* α_2M	4
120 nM native *Limulus* α_2M	0
100 nM MA-*Limulus* α_2M	0

[a] The hemagglutination endpoint was the highest dilution of sample that produced visible agglutination.

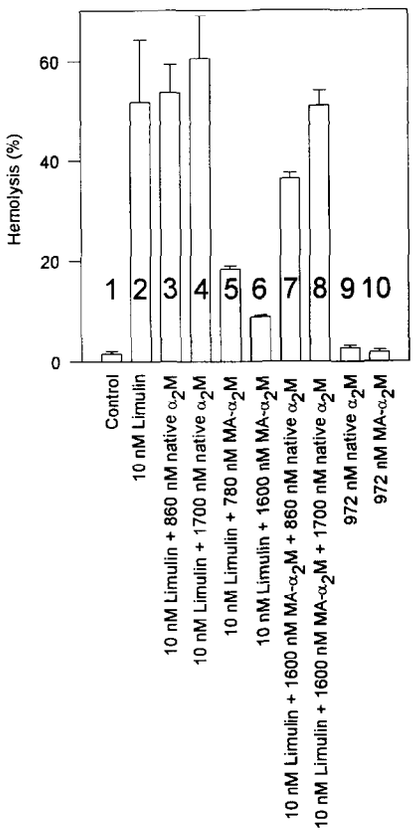

Fig. 4. Native *Limulus* α_2-macroglobulin (native α_2M) can reverse the inhibitory actions of methylamine-reacted α_2-macroglobulin (MA-α_2M) on limulin-mediated hemolysis in a dose-dependent manner (samples 7 and 8). Native, slow-form α_2-macroglobulin is not inhibitory (samples 3 and 4) whereas fast-form α_2-macroglobulin does inhibit limulin-dependent hemolysis (samples 5 and 6). Neither native α_2-macroglobulin (sample 9) nor methylamine-reacted α_2-macroglobulin (sample 10) are hemolytic in the absence of limulin.

of mammalian plasma (Tack, 1983). On the basis of this interpretation, Enghild et al. (1990) suggested that *Limulus* α_2-macroglobulin be named 'limac' for *Limulus* α-macroglobulin complement-like protein. The present study shows that this analysis is not correct and indicates

that the name 'limac' should not be used to refer to *Limulus* α_2-macroglobulin. One of the key determinants of the differences in molecular reactivity between the α_2-macroglobulins and the complement components C3 and C4B is residue Asp-1064 (human α_2-macroglobulin numbering). In C3 and the C4B isomer, where this residue is His, the thiol ester-reacted protein shows reactivity with hydroxyl groups whereas if the residue is Asp, Asn or Ala, the principal reactivity is with primary amines (Dodds et al., 1996). In *Limulus* α_2-macroglobulin the equivalent residue is Asn-1113 (Ikawi et al., 1996), consistent with the α_2-macroglobulin pattern of reactivity and not the complement component pattern.

In the horseshoe crab, limulin is sufficient for hemolysis (Armstrong et al., 1996a). Native α_2-macroglobulin is not involved in hemolysis whereas fast-form α_2-macroglobulin is inhibitory at molar ratios of α_2-macroglobulin : limulin approaching the 30–170-fold molar excess that would be expected upon conversion of the 1 5 μM α_2-macroglobulin present in whole plasma to fast form protein. The reactivity of protease-reacted *Limulus* α_2-macroglobulin with the limulin-mediated hemolytic system may be physiologically important for the modulation of foreign cell cytolysis in situations of immune defense in vivo. The presence of foreign proteases, and thus of protease-reacted α_2-macroglobulin, can be expected to be an indicator of the invasion of the tissue spaces by bacteria and other potential pathogens. In *Limulus*, α_2-macroglobulin is the only known protease-binding agent in the plasma (Quigley and Armstrong, 1983) and is the principal mediator of the clearance of proteases from the blood (Melchior et al., 1995). The present documentation of an additional modulatory role of α_2-macroglobulin for the plasma cytolytic defense system represents a novel function for α_2-macroglobulin.

Acknowledgments

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