**Binding of \( \alpha_2 \)-macroglobulin and limulin: regulation of the plasma haemolytic system of the American horseshoe crab, *Limulus***

Snehasikta SWARNAKAR*,† Rengasamy ASOKAN‡, James P. QUIGLEY§*† and Peter B. ARMSTRONG*†‡

*Department of Pharmacological Sciences, Health Sciences Center, BHS, Level 7, Room 140, State University of New York, Stony Brook, NY 11794, U.S.A., †Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543, U.S.A., ‡Department of Molecular and Cellular Biology, University of California, One Shields Avenue, Davis, CA 95616-8755, U.S.A., §Scripps Research Institute, Department of Vascular Biology/VB 3, 10550 North Torrey Pines Rd, La Jolla, CA 92037-1092, U.S.A.

INTRODUCTION

The mediator of haemolysis in the plasma of the horseshoe crab, *Limulus polyphemus*, is limulin, a sialic acid-binding lectin. The haemolytic activity of limulin is inhibited by thiol ester-reacted forms of *Limulus* \( \alpha_2 \)-macroglobulin, the third-most abundant protein of the plasma. *Limulus* \( \alpha_2 \)-macroglobulin that has experienced cleavage of its internal thiol ester bond, consequent to reaction with proteases, or with the small primary amine, methylamine, reduces the haemolytic activity of limulin when present at molar excesses that approximate the relative concentrations of these two proteins in the plasma. Native, unreacted *Limulus* \( \alpha_2 \)-macroglobulin has no effect on the haemolytic activity of limulin. Limulin binds thiol ester-reacted forms of *Limulus* \( \alpha_2 \)-macroglobulin both in a solid-phase assay and in solution with an avidity 10–25 times higher than native, unreacted *Limulus* \( \alpha_2 \)-macroglobulin. Protease-reacted \( \alpha_2 \)-macroglobulin functions as a marker for the presence of foreign proteases in the blood of *Limulus*, and thus of pathogenic organisms that release proteases as facilitators of invasion and pathogenicity. Modulation of the haemolytic system represents a novel function for \( \alpha_2 \)-macroglobulin.

Key words: cytolysis, innate immunity, lectin, pentraxin, proteinase clearance, thiol ester.

MATERIALS AND METHODS

Purification of \( \alpha_2 \)-macroglobulin and limulin

Recently collected adult *L. polyphemus* were obtained from the Marine Resources Center of the Marine Biological Laboratory (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 [26]. It is important to avoid degranulation of the
blood cells, since this releases proteases [27] and active-site
protease inhibitors [28–30] into the serum. Animals were released
into the ocean unharmed after bleeding. Most of the haemocyanin
was removed from the plasma by ultracentrifugation (141000 g
for 16 h), or by incubation with 3% poly(ethylene glycol)-8000
(PEG) with centrifugation at 30000 g for 30 min. PEG was added
to a final concentration of 10% (w/v), the preparation centri-
fuged as above, and the precipitate recovered and redissolved in
0.1 M citrate, pH 6.7. The remaining traces of the haemocyanin
were precipitated by the addition of zinc acetate to a final
concentration of 0.1 M. The supernatant was then precipitated
with 10% PEG and the precipitate dissolved in buffer A (0.15 M
NaCl, 10 mM CaCl₂, 50 mM Tris (pH 7.4)). This fraction (3
–10% PEG cut) was then depleted of Sepharose-binding proteins
by passage over a column of Sepharose 4B (Pharmacia) equilibriumed with buffer A (0.2 vol. resin/vol. of 3–10% PEG
cut). This was the starting material for purification of α₂-
macroglobulin and limulin.

The pentraxin proteins (Limulus C-reactive protein and
limulin) were removed by incubation with phosphoethanolamine–
agarose (PE–agarose) (Sigma), which binds the pentraxins
(0.1 vol. of resin/vol. of plasma). The PE–agarose was washed
with buffer A modified to contain 1 M NaCl and eluted with
0.1 M sodium citrate, pH 6.7 to recover the pentraxin fraction.
Following dialysis with buffer A, the pentraxin fraction was
further fractionated by passage over a column of fetuin–
Sepharose equilibrated with buffer A. The breakthrough fraction
from the fetuin–Sepharose column is Limulus C-reactive protein.
The bound fraction is the lectin limulin. After washing the
fetuin–Sepharose with buffer A containing 1 M NaCl, limulin
was eluted with 0.1 M sodium citrate, pH 6.7. After dialysis with
buffer A, and a second purification on fetuin–Sepharose, pure
limulin free of contaminating proteins was recovered [10].

The unbound fraction from the incubation with PE–agarose
was incubated with PE–agarose to remove all pentraxin
proteins, and was then further purified by chromatography on a
100 × 1.6 cm column of Sephacryl S-300HR gel-filtration resin to
remove aggregated α₂-macroglobulin, and was stored in 0.1 M
citrate, pH 6.7 at 4°C. The activity of α₂-macroglobulin was
determined by the protection of bound trypsin from the macro-
molecular active-site inhibitor, soybean trypsin inhibitor [31],
using trypsin whose fractional activity had been determined by
active-site titration with p-nitrophenyl p'-guanidino benzoxo
dihydrochloride [32].

The thiol ester of Limulus α₂-macroglobulin was activated by
reaction with methyamine as described previously [31], which
resulted in a 96% loss of the trypsin-binding activity. Unreacted
methylamine was removed by dialysis. Proteolytically active
trypsin was purified by affinity chromatography of commercially
available trypsin (Sigma) on benzamidine-Sepharose [33]. To
form the proteinase–α₂-macroglobulin complex, a two-fold
molar excess of trypsin was exposed to α₂-macroglobulin in
50 mM Tris (pH 8.0) for 3 min at room temperature, and the protease–α₂-macroglobulin complex was separated from the
unbound trypsin by gel filtration on Sephadex G-50.

Solution-phase binding
Limulin was labelled with carrier-free Na[¹³¹]I using the clora-
mine T method as described by Erlich et al. [34]. The specific
activity of the labelled limulin was 1 × 10⁶ c.p.m./μg of protein.
For protein cross-linking, ¹³¹I-limulin was incubated with
50 molar excess of Limulus α₂-macroglobulin (native or methyl-
amine-reacted), then 10 mM bis(sulfosuccinimidyl)suberate (BS²)
in 50 mM phosphate buffer (pH 7.4) was added. After 30 min
incubation at room temperature, unreacted BS² was quenched
with 0.17 vol. of 50 mM ethanolamine, pH 7.4 (method described in
[35]). Samples were subjected to gel-filtration chromatography
on a calibrated 95 × 1.6 cm column of Sephacryl S-300 HR. The
radioactivity of the eluted fractions was determined by γ-counting
(LKB-1275 Minigamma; LKB Instruments, Gaithersburg, MD,
U.S.A.).

Ligand blot analysis
Limulin was biotinylated by reaction with N-hydroxysuccinimide-
long-chain biotin (NIH LC-biotin; Pierce, Rockford, IL, U.S.A.)
in a carbonate/bicarbonate buffer (pH 9.1) for 4 h at 4°C
[36], and then separated from free biotin by dialysis against
buffer A. Different forms of Limulus α₂-macroglobulin (native,
methylamine-treated and trypsin treated) were subjected to
electrophoresis under non-denaturing conditions on a 6% con-
tinuous polyacrylamide gel [37]. After electrophoresis, samples
were transferred to nitrocellulose [38]. After blocking the non-
specific protein-binding activity with 5%, BSA in PBS with
0.05% Tween 20 (1 h at room temperature), the blot was
incubated with 10 μg/ml of biotinylated limulin for 1 h at 4°C,
and then with avidin–peroxidase (1:1000 dilution; Boehringer
Mannheim) and the peroxidase was visualized with 4-chloro-1-
naphthol.

Solid-phase binding assay
Limulin (4 μg/ml and 100 μl/well) was adsorbed to Reacti-bind
maleic anhydride-activated polystyrene plates (Pierce) in PBS
(pH 7.3) for 12 h at 4°C. Under these conditions approx. 10% of
the limulin bound to the microtiter well, as measured by
determining the binding of [¹³¹]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% BSA in PBS-Tween 20.
Samples of Limulus α₂-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 α₂-macroglobulin (1:1000) followed by horseradish
peroxidase (HRP)-conjugated goat-anti-rabbit immunoglobulin
(1:10000). HRP was visualized with o-phenylenediamine (Sigma).
The reaction was stopped with 3 M HCl, and the reaction product
was evaluated with an ELISA reader (Bio-Rad) at 490 nm.

Haemolysis and haemagglutination
The haemolytic activity of the plasma was determined in duplicate
or triplicate samples using sheep red blood cells [7,10]. Unacti-
vated sheep erythrocytes in Alsevers solution were obtained from
Cappel (West Chester, PA, U.S.A.). The buffer system was
modified DGVB buffer (0.19 M NaCl, 0.18 mM CaCl₂, 0.5 mM
MgCl₂, 2.5% glucose, 0.1% gelatin, 2.5 mM sodium barbital,
pH 7.3). The reaction mixtures contained 3 × 10⁶ washed sheep
red cells, the sample to be tested, and the modified DGVB buffer
to a final volume of 800 μl. The reaction mixtures were incubated
with shaking at 22–23°C for 4 h, and the reaction was ter-
minated by adding 2 ml of ice-cold PBS containing 5 mM EDTA,
followed by centrifugation to remove the red cells. The extent of
haemolysis was determined by monitoring released haemoglobin

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in the supernatant by the optical absorbance at 412 nm and was compared with full haemolysis produced by hypotonic lysis of the red cells.

The haemagglutination assay was performed using sheep erythrocytes that had been washed and suspended at 2% (v/v) in buffer A. An array of serial two-fold dilutions of the test sample was prepared in 200 μl round-bottom microtiter wells (Sigma), using buffer A as a diluent, and then mixed with equal volumes of the erythrocyte suspension (50 μl final volume). The array was incubated for 45 min at room temperature, and scored for haemagglutination. Haemagglutinated 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’) [8]. The haemagglutination end point was the highest dilution of sample that produced visible agglutination.

RESULTS AND DISCUSSION

Cytolysis of foreign cells by components of the plasma or serum is an important immune defence strategy for a variety of animals. The cytolytic action of the human complement system, and the cytolytic systems of many invertebrates, have conventionally been studied by the ability to lyse mammalian erythrocytes (haemolysis). In this regard, the erythrocyte, although hardly a pathogen, is a model foreign cell whose lysis is easily quantified. In contrast to the complex, multi-component vertebrate complement system, the cytolytic system of Limulus plasma depends on the action of a single protein, the sialic acid-binding lectin limulin, which can haemolysie red blood cells as the purified protein and whose removal eliminates the haemolytic action of the plasma [10].

The haemolytic action of purified limulin is sensitive to the ionic environment and shows a broad activity-maximum at NaCl concentrations between 0.2 M and 0.35 M (Figure 1A). Although these salt concentrations would be non-physiological for an immune effector from a vertebrate, it is important to note that the blood of the horseshoe crab has the ionic composition of sea water (0.5 M NaCl). At increasing concentrations toward the activity maximum, this dependence on ionic strength correlates inversely with the haemagglutinating action of limulin (Figure 1B). The curve showing the Ca²⁺ dependence for haemolysis is sigmoidal, with a surprisingly sharp rise from zero activity at 0.65 mM to maximal activity at 0.85 mM (Figure 2A). This may reflect the Ca²⁺ dependency of binding of limulin to the red cell, since haemagglutination shows a similar dose-dependence on the concentration of Ca²⁺ (Figure 2B). Under optimized conditions, haemolysis is dependent upon the concentration of limulin (M, 300 × 10⁴) between 2 and 8 nM (Figure 3).

Haemolysis at the level of the individual red cell appears to be an all-or-nothing condition, because the fractional haemolysis, as measured by the appearance of haemoglobin in solution, is paralleled by the decrease in the number of red cells present, as determined by haemocytometer counts (results not shown). Partial haemolysis is not the consequence of all cells releasing some of their haemoglobin, but is the result of a few cells haemolysing completely and liberating all of their haemoglobin. The macromolecular osmolytes dextran-8 (M, 8-12 × 10⁶) and, to a lesser extent, dextran-4 (M, 4-6 × 10⁴) block haemolysis (Figure 4). Protection is reversible because red cells treated with limulin plus dextran show partial lysis when washed into limulin- and dextran-free buffer (results not shown). This suggests that limulin causes haemolysis by insertion into the plasma membrane to generate hydrophilic channels that allow water to flow into the cell in response to the high internal concentration of macromolecular osmolytes, principally the protein haemoglobin. Protection is imagined to result from the presence of osmolytes (e.g. the dextrans) larger than the channel pore size in the external milieu at concentrations sufficient to balance the osmotic pressure of haemoglobin in the cell, thereby protecting the cell from osmotic rupture [39]. Dextran-8 fails to block haemagglutination at low Ca²⁺ concentrations (results not shown) and exerts the same protective effect against haemolysis at standard (0.85 mM) and high (10 mM) Ca²⁺ concentrations (Figure 4). The activity of dextran, therefore, does not involve Ca²⁺ sequestration. The
Figure 2 Dependence of limulin-mediated haemolysis and haemagglutination on Ca²⁺

The haemagglutination titer (B) again is expressed as the minimum concentration of limulin that showed positive haemagglutination. Haemolysis (A) and haemagglutination (B) showed a dependence on the concentration of Ca²⁺ between 0.6 and 0.85 mM. A similar precipitous rise in haemolysis, this time between 0.43 and 0.49 mM CaCl₂, was found using a 0.17 M NaCl, 2.3 mM barbital buffer system (results not shown).

molecular size of dextran-4 is approx. 1.7 nm [40], indicating an effective pore size for membrane-associated limulin approximating this value.

Proteinases are essential virulence factors for a broad range of microbial and multicellular parasites [41–44], and an important function of the proteinase inhibitors of potential hosts for these parasites is to inactivate and clear these foreign proteinases as a strategy of immune defence. The multifunctional proteinase inhibitor, α₂-macroglobulin, is found in a diverse array of higher animals and is the third most abundant protein of the plasma of Limulus [45]. Its ability to bind and mediate the clearance of proteinases of every catalytic mechanism makes it a key element in the clearance of foreign proteinases, and its presence in the

Figure 3 Haemolysis by limulin at optimized salts

Haemolysis by purified limulin conducted at 0.25 M NaCl, 10 mM Tris, 0.85 mM CaCl₂, showed a concentration dependence between 2–8 nM limulin (Mᵣ 300).

Figure 4 Protection by high-molecular-mass osmolites

Haemolysis was conducted under optimal conditions (7 nM limulin, 0.25 M NaCl, 0.85 mM CaCl₂) in the presence of 30 mM carbohydrate osmolites of different molecular weights as indicated. The high-molecular-mass osmolites dextran-8 and dextran-4 protected the limulin-treated red cell against haemolysis. Smaller osmolites exert a reduced protecting effect. Protection is not due to sequestration of Ca²⁺ by the dextrans since protection occurs at high concentrations of Ca²⁺ (sample 6).

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A high-molecular mass of 300 kDa (Figure 7A), consistent with it being chromatography. Purified "#& reacted macroglobulin. Solution-phase binding of limulin to thiol ester-reacted forms of Limulus a2-macroglobulin to inhibit limulin-mediated haemolysis. Treatment of methylamine-reacted a2-macroglobulin, estimated of the peak was consistent with a size larger than that of thyroglobulin (M, 660 × 10^3), and equal or smaller than that of proteins excluded from the internal compartment of the resin (M, = 1.5 × 10^6).

In ligand blot studies, limulin bound to thiol ester-reacted Limulus a2-macroglobulin electroblotted from a pore-limit electrophorogram to nitrocellulose (Figure 8), but failed to bind to native a2-macroglobulin. Thiol ester-reacted Limulus a2-macroglobulin also bound to immobilized limulin in a solid-phase assay. The binding of limulin to thiol ester-reacted a2-macroglobulin showed a 10–25 times greater avidity than to native a2-macroglobulin in the solid-phase assay (Table 1). Unlike the solution-phase binding assay, chemical cross-linking was not

proteinase-reacted form is a potential indicator of parasitic invasion [19]. Thiol ester-reacted forms of Limulus a2-macroglobulin, generated by reaction with proteinases or methylamine, inhibited the haemolytic activity of purified limulin (Figure 5). Inhibition of limulin-mediated cytolsis was specific for the thiol ester-reacted form of a2-macroglobulin; native, unreacted a2-macroglobulin had no effect on limulin-mediated haemolysis (Figure 5).

Cleavage of the thiol ester bond of Limulus a2-macroglobulin generates a new free thiol at cysteine-999 [21]. This is the only free thiol of the protein [20,46] and appears to be important for the ability of thiol ester-reacted a2-macroglobulin to inhibit limulin-mediated haemolysis. Treatment of methylamine-reacted Limulus a2-macroglobulin with the thiol alkylating agent iodoacetamide abolished the ability of a2-macroglobulin to inhibit haemolysis (Figure 6). Alkylation of the thiol of methylamine-reacted a2-macroglobulin also abolished its ability to reduce the haemagglutinating activity of limulin (results not shown).

The inhibition of haemolysis correlates with a direct interaction of limulin preferentially with the thiol ester-reacted form of a2-macroglobulin. Solution-phase binding of limulin to thiol ester-reacted a2-macroglobulin could be demonstrated by gel-filtration chromatography. Purified [125I]limulin eluted at an apparent molecular mass of 300 kDa (Figure 7A), consistent with it being a duodecamer of 25–30 kDa subunits [10,11]. A high-molecular-mass peak of [125I]limulin appeared during chromatography on Sephacryl S-300 HR resin when limulin and methylamine-reacted a2-macroglobulin were co-chromatogrammed (Figure 7B). This peak was absent when 125I-limulin was chromatographed alone (Figure 7A), and was much reduced in the presence of native a2-macroglobulin (Figure 7B). The shoulder of 125I-limulin, displaced to a high molecular mass in the presence of native a2-macroglobulin, is probably due to a minor contamination of this preparation with thiol ester-reacted a2-macroglobulin, estimated by the trypsin binding assay [31] to be approx. 10%. The binding in solution appears to be of relatively low affinity because the limulin-a2-macroglobulin complex required stabilization with the protein cross-linker BS3 (Pierce). The position of the peak was consistent with a size larger than that of thyroglobulin (M, 660 × 10^3), and equal or smaller than that of proteins excluded from the internal compartment of the resin (M, = 1.5 × 10^6).

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required to detect binding in the solid-phase assay. This may reflect an increase in binding avidity resulting from the association of single $\alpha_2$-macroglobulin molecules to multiple limulin molecules immobilized on the solid surface.

$\alpha_2$-Macroglobulin was initially identified in Limulus and vertebrates as a protease inhibitor [15,16], and as the carrier molecule responsible for the clearance of proteases from the blood [17]. In mammals, $\alpha_2$-macroglobulin also functions as the principal carrier for peptide growth factors in the plasma [47]. Here we report a novel function for this protein. Inhibition of limulin-mediated cytolysis is restricted to the thiol ester-reacted form of Limulus $\alpha_2$-macroglobulin. The selective binding of limulin to thiol ester-reacted forms of Limulus $\alpha_2$-macroglobulin suggests that direct binding of the two proteins is responsible for the modulation of haemolysis (Figure 5) by thiol ester-reacted Limulus $\alpha_2$-macroglobulin. The role of this binding in immunity is not clear from these studies, but might function in the potentiation of the proteinase clearance functions of $\alpha_2$-macroglobulin [17].

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Cooperative interaction of limulin and fast-form α₅-macroglobulin


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