



## Membrane activity of a C-reactive protein

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### ABSTRACT

**C-reactive protein (CRP) from the American horseshoe crab, *Limulus polyphemus*, exhibits complex membrane activities. Here, we describe the behavior of protein and lipid as CRP interacts with model liposomes and bacterial membranes. *Limulus* C-reactive protein (L-CRP) forms extended fibrillar structures that encapsulate liposomes in the presence of  $\text{Ca}^{2+}$ . We have observed structures consistent in size and shape with these fibers bound to the surface of Gram-negative bacteria. The membranes of *Limulus* CRP-treated bacteria exhibit significantly different mechano-elastic properties than those of untreated bacteria. In vitro, bilayer lipids undergo a rigidification and reorganization of small domains. We suggest that these interactions reflect the protein's role as a primary defense molecule, functioning in the entrapment and killing of potential pathogens.**

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### 1. Introduction

C-reactive protein (CRP) is the canonical acute phase protein of humans and the founding member of the pentraxin family of proteins [1]. In the American horseshoe crab, *Limulus polyphemus*, pentraxin homologues (LPx) include C-reactive protein (L-CRP) [2,3], *Limulus* serum amyloid P component [4,5] and limulin, a minor hemolytic pentraxin [6]. The LPx are major hemolymph proteins, present at 2.9–20  $\mu\text{M}$  [7]. It is believed that L-CRP participates in plasma-based innate immunity through agglutination and cytolytic destruction of potential pathogens [8–10]. Structurally, L-CRP exists in solution as an oligomer of approximately 350 kDa formed by doubly stacked rings of 7–8 monomers [4,11]. The circular organization of monomers results in a large central pore approximately 2 nm in diameter that appears to be retained during transmembrane insertion in target lipid bilayers [8].

The agglutination of foreign cells that have entered the fluid-filled compartments of metazoans is generally recognized as an important mode of immune defense. Agglutinins are typically polyvalent proteins with multiple binding sites for one or more moieties that are found on the surfaces of microbes and other parasites [12]. Previously we reported that L-CRP agglutinates model liposomes in both a  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent fashion [8].

The present study examines the nature of L-CRP–lipid bilayer interactions with respect to membrane-induced oligomerization of L-CRP and the behavior of bilayer lipids interacting with L-CRP. In particular, we suggest that hyper-oligomerization of L-CRP in the presence of susceptible bilayers is a novel mechanism of agglutination that increases avidity and capture efficiency and that transmembrane insertion occurs concomitantly to contribute to the killing of entrapped potential pathogens.

### 2. Materials and methods

#### 2.1. Protein purification

Adult *Limulus* were purchased from the Marine Resource Center at the Marine Biological Laboratory in Woods Hole, MA. Methods for collection, processing and treatment and storage of *Limulus*

**Abbreviations:** CRP, C-reactive protein; LPx, *Limulus* pentraxins; L-CRP, *Limulus* C-reactive protein; FTIR, Fourier transform infrared spectroscopy; AFM, atomic force microscopy.

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hemolymph have been described [6,13,14]. L-CRP was purified by affinity chromatography on phosphorylethanolamine-agarose as previously described [8].

## 2.2. Bacterial agglutination assay

Two bacterial strains were used: *Escherichia coli* D31 and *Vibrio alginolyticus* (ATCC 14582). Bacteria were grown overnight in either Luria-Bertani (LB) broth (Difco 244620) (*E. coli*) at 37 °C or Marine Broth (Difco 279110) (*V. alginolyticus*) at 25 °C with shaking and subsequently inoculated into the same broth to reach mid-logarithmic phase. Aliquots of L-CRP in 50 mM Tris, 50 mM Na<sub>2</sub>SO<sub>4</sub>, pH 7.8 or 50 mM Tris-maleate, 50 mM Na<sub>2</sub>SO<sub>4</sub>, pH 5.2, both, with or without 10 mM CaCl<sub>2</sub>, were serially diluted in 90 µl of identical buffer. Bacterial broth containing 100 colony forming units (10 µl) was added to each well. The plates were incubated overnight at 37 °C for *E. coli* or at 25 °C for *V. alginolyticus*. Agglutinated bacteria formed a diffuse mat covering the surface of the round-bottom wells; non-agglutinated bacteria formed a compact pellet at the center of the wells. The minimum bacterial agglutinating concentration is defined as the highest dilution of L-CRP that results in visible agglutination.

## 2.3. Electron microscopy

Unilamellar liposomes were constructed of total *E. coli* lipids (Avanti Polar Lipids, Alabaster, AL, cat. 100500) as described previously [8]. Liposomes (100 µg/ml lipid) were incubated with indicated concentrations of L-CRP in 50 mM Tris-maleate, 50 mM Na<sub>2</sub>SO<sub>4</sub>, pH 5.2 with or without 10 mM CaCl<sub>2</sub> for indicated time periods. Samples were adsorbed to glow-discharged, carbon-coated copper grids, blotted, stained with 2% uranyl acetate, blotted and air-dried, and imaged with a JEOL JEM-1230 transmission electron microscope.

## 2.4. Atomic force microscopy

Log-phase bacteria ( $2 \times 10^8$  colony forming units/ml *E. coli* WBB01 in 0.5 ml buffer: 20 mM HEPES, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, pH 5.2) were incubated alone or with 20 µg/ml L-CRP at 37 °C for 90 min. The bacteria were then placed on freshly cleaved mica, excess liquid was removed and the bacteria were air-dried at room temperature for 24 h. Dried bacteria were rinsed with 3 ml H<sub>2</sub>O and imaged with an atomic force microscope in air using a MFP-3D (Asylum Research, Santa Barbara, CA, USA). Imaging in air was performed with NSG11-B cantilever ( $k = 5.5$  N/m; NT-MDT, Moscow, Russia) in AC (tapping) mode using frequencies of about 150 kHz. The frequency was chosen to result in an amplitude 5% lower than the amplitude at the resonance frequency. The set point was always adjusted to guarantee minimum forces applied to the sample. Further image processing was done with the MFP-3D software under IGOR Pro (Lake Oswego, OR, USA). Images shown are representative for the respective sample.

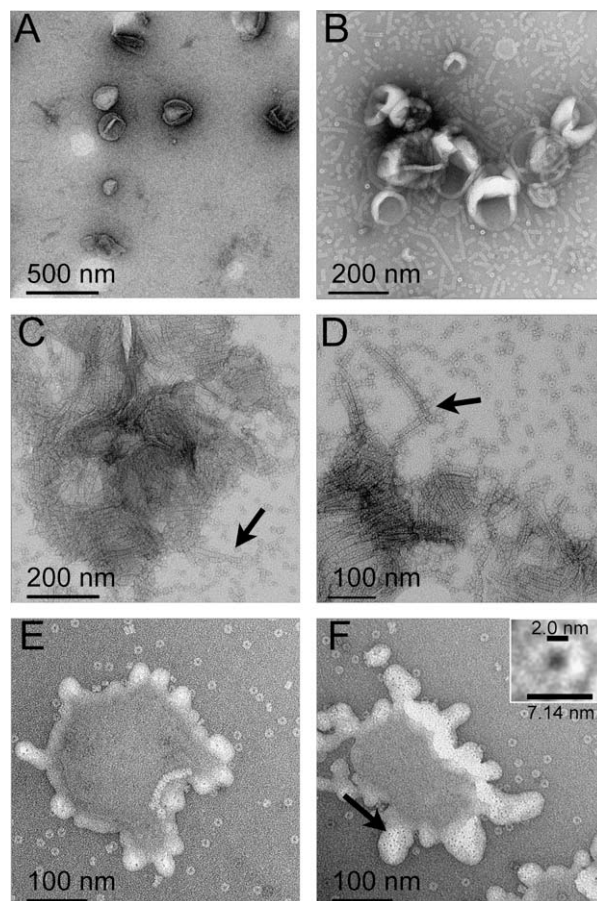
## 2.5. Fourier transform infrared spectroscopy (FTIR)

Liposomes were constructed as described above but to a final concentration of 40 mg/ml lipid. Samples containing 30 mg/ml lipid were incubated with 2.6 µM L-CRP in 50 mM Tris-maleate, 50 mM Na<sub>2</sub>SO<sub>4</sub>, pH 5.2 in the presence or absence of 10 mM CaCl<sub>2</sub> at room temperature for 1 h. Spectra were obtained with a Perkin-Elmer 2000 infrared spectrometer at room temperature. For each spectrum, eight interferograms were collected at 1 cm<sup>-1</sup> resolution and 3600–1000 cm<sup>-1</sup> wavenumber range. Spectral analysis was carried out with Spectrum 2000 software (Perkin-Elmer).

## 3. Results

The relative particle size increase observed when L-CRP interacts with suspensions of liposomes composed of *E. coli* lipids is believed to reflect the protein's role as an agglutinin of potential pathogens [8]. To test this supposition, we incubated *E. coli* or *V. alginolyticus* with serial dilutions of L-CRP. Agglutination of *E. coli* was observed at both pH 5.2 and pH 7.8 and in the presence of 10 mM CaCl<sub>2</sub> for 180 and 714 nM L-CRP, respectively. In the absence of CaCl<sub>2</sub>, the effective agglutinating concentration of L-CRP at pH 7.8 was 1.7 µM. *V. alginolyticus* was agglutinated at pH 7.8, 10 mM CaCl<sub>2</sub> by 357 nM L-CRP.

In order to examine the interaction of L-CRP with membranes, we used transmission electron microscopy to visualize L-CRP bound to liposomes constructed of *E. coli* lipids. At lipid-protein ratios of 1.4:1, by mass, and in conditions that promote rapid, Ca<sup>2+</sup>-dependent liposome agglutination, i.e. 50 mM Tris-maleate, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, pH 5.2 [8], L-CRP formed lengthy fibrils and sheet-like structures that clumped and completely encapsulated liposomes (Fig. 1). Fibrils were oriented perpendicular to the long axis of the oligomerized rings, i.e. perpendicular to the flat faces of the L-CRP doughnuts. Under high ionic strength conditions (1.0 M NaCl) only individual oligomers of L-CRP were observed (data not shown). Longer fibrils and sheets of hyper-



**Fig. 1.** Electron microscopy reveals hyper-oligomerized and membrane associated *Limulus* C-reactive protein (L-CRP). Transmission electron micrographs of liposomes alone (A), liposomes agglutinated by L-CRP in 10 mM EDTA (B), liposomes agglutinated by hyper-oligomerized L-CRP in the presence of 10 mM Ca<sup>2+</sup> (lipid-protein ratio of 1.4:1, arrows indicate protein fibrils extending beyond the boundaries of agglutinated liposomes) (C and D), and liposomes treated with L-CRP and 10 mM Ca<sup>2+</sup> at a higher lipid-protein ratio, 36:1 (E and F). Inset in F is a close up of the ring-structure indicated by arrow.

oligomerized protein were not seen in any preparation that lacked lipid. Protein particles that did not interact with liposomes remained in solution predominantly as single oligomers. Protein fibrils and sheets extended beyond the boundaries of agglutinated liposomes. Liposomes are also agglutinated by L-CRP in the absence of  $\text{CaCl}_2$ . Under these conditions, only small fibrils of protein are seen, both in association with liposomes and in solution. The large sheets of L-CRP were absent. No oligomerization of L-CRP was seen at pH 7.8 in the presence of lipid, with or without 10 mM  $\text{Ca}^{2+}$  (data not shown).

The conditions that facilitate the rapid  $\text{Ca}^{2+}$ -dependent agglutination of liposomes also facilitate transmembrane-pore formation by L-CRP [8], however, the coating of liposomes by protein particles, at the this lipid–protein ratio, precludes any opportunity to visualize membrane-inserted L-CRP. Electron microscopic examination of L-CRP-liposome preparations with higher lipid–protein ratios (36:1 by mass) reveals what appear to be membrane-inserted L-CRP molecules. Areas of closely packed ring-structures appear in the liposomal bilayers. The lipid-associated rings display an

**Table 1**

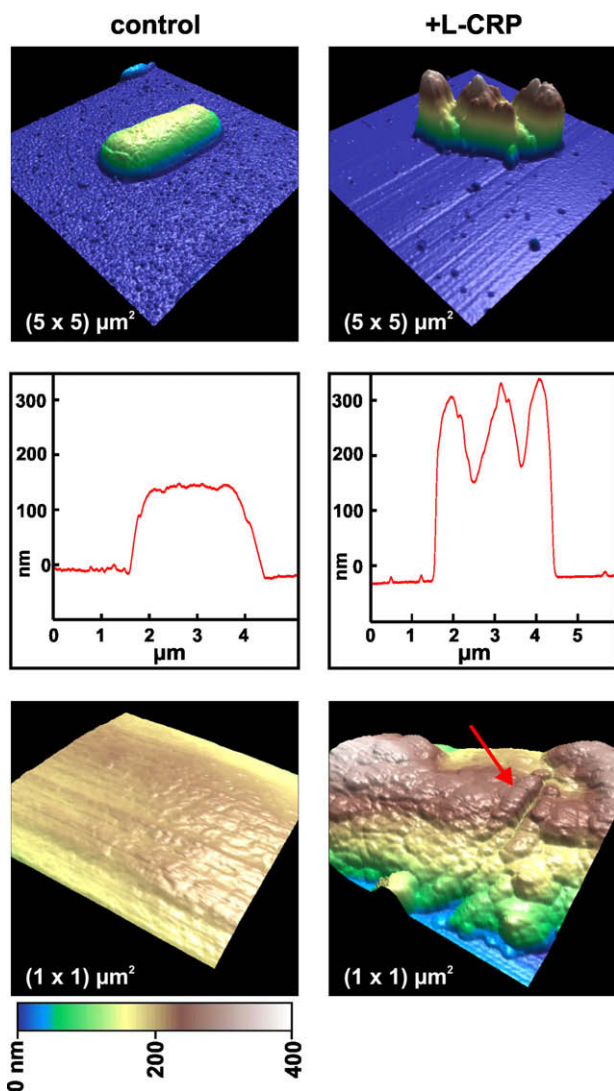
$\text{CH}_2$ -symmetric stretching peak position of *Limulus* C-reactive protein (L-CRP) treated lipid bilayers.

Conditions	Wavenumber ( $\text{cm}^{-1}$ )
Lipid alone	$2853.27 \pm 0.01$
Lipid + L-CRP	$2853.3 \pm 0.03$
Lipid + 10 mM $\text{CaCl}_2$	$2853.22 \pm 0.03$
Lipid + L-CRP + 10 mM $\text{CaCl}_2$	$2852.03 \pm 0.03$

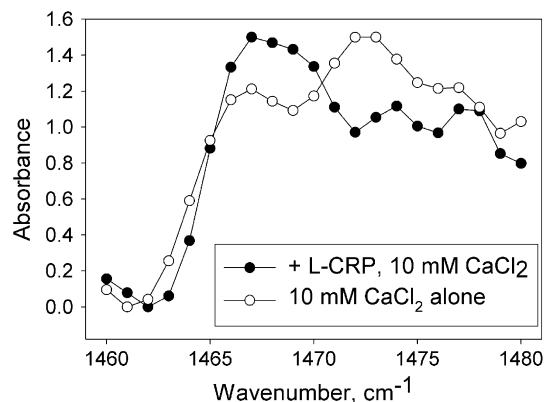
inner diameter of approximately 2.0 nm, corresponding well with that of the free L-CRP molecules. The apparent outer diameter of the lipid-associated rings, 7.14 nm, is significantly smaller than that of free L-CRP molecules, 12.4 nm. Under these conditions L-CRP also induces deformations of the liposomal bilayer. The ring aggregates are associated with outpocketing and tube-like protrusions of the lipid bilayer.

In order to study this phenomenon in a biologically relevant system, we visualized *E. coli* incubated with L-CRP by AFM. Upon drying, L-CRP-treated cells exhibit dramatically different topologies compared to untreated cells (Fig. 2). Untreated cells collapse to a uniform height of approximately 150 nm and display a smooth surface. Cells treated with L-CRP display a rough topology with membrane peaks and valleys of approximately 300–150 nm, respectively. Inspection of the cell surface of L-CRP-treated bacteria at higher resolution reveals the presence of fibrillar structures oriented parallel to the membrane. The cross sectional width of these structures is consistent with that of L-CRP fibrils. Additionally, the fibers seen with AFM on the surface of Gram-negative bacteria are associated with adjacent fibers, also consistent with the sheet-like organization of lipid-associated L-CRP seen with electron microscopy.

In order to investigate the effect of lipid bilayer binding by L-CRP on the lamellar structure of target bilayers we monitored lateral chain interactions with FTIR. Bilayers composed of total *E. coli* lipids were treated with 2.6  $\mu\text{M}$  L-CRP in the presence of 10 mM  $\text{CaCl}_2$  at pH 5.2. These bilayers exhibited a significant rigidification, e.g. increase in lateral interchain interactions, as revealed by the decrease in wavenumber peak position of the  $\text{CH}_2$ -symmetric stretching mode (Table 1). This rigidification was not seen in bilayers treated with L-CRP in the absence of  $\text{CaCl}_2$  or with  $\text{CaCl}_2$  alone. Additionally, the bilayer lipids treated with L-CRP and  $\text{CaCl}_2$  exhibit a change in the relative intensities of the  $\text{CH}_2$ -scissoring peaks (1480–1460). This indicates a rearrangement of small lipid domains (>100 acyl chains) (Fig. 3).



**Fig. 2.** *Limulus* C-reactive protein (L-CRP) dramatically affects the surface properties of Gram-negative bacteria. *Escherichia coli* incubated with or without L-CRP were imaged with atomic force microscopy (AFM). Upon drying the bacteria collapse to a non-uniform height with peaks and valleys of approximately 300 and 150 nm, respectively. Structures consistent with L-CRP fibrils are visible on the bacterial surface (indicated by arrow).



**Fig. 3.** Fourier transform infrared spectroscopy (FTIR) absorbance spectra of the  $\text{CH}_2$ -scissoring region. In the presence of  $\text{Ca}^{2+}$ , L-CRP associated lipid bilayers undergo a reorganization of lipid domains. The relative intensity increase at approximately  $1468 \text{ cm}^{-1}$  in the presence of protein indicates an increase in hexagonal chain packing and is consistent with the rigidification of bilayer lipids seen in the  $\text{CH}_2$ -symmetric stretching region (Table 1).

#### 4. Discussion

The pentraxins from horseshoe crabs exhibit agglutinating activity against a variety of foreign cells, i.e. mammalian erythrocytes and bacteria [9,11,13]. We have recently reported that acidic pH facilitates a  $\text{Ca}^{2+}$ -dependent agglutination by L-CRP of liposomes derived from Gram-negative bacterial membranes [8]. The four-fold increase in L-CRP-dependent agglutination of *E. coli* with a reduction of pH from 7.8 to 5.2 is consistent with the observations of increased L-CRP mediated liposome agglutination at low pH. Conflicting reports exist on the  $\text{Ca}^{2+}$ -dependence of bacterial agglutination [9,11]. Integrating our previously reported observations of liposome agglutination [8] with the data we present here, we suggest that  $\text{Ca}^{2+}$  does indeed potentiate, but is not necessary for bacterial agglutination. It is likely that these data are the result of L-CRP binding a variety of cell surface ligands, predominantly LPS [8,15] and as discussed below, electron microscopy reveals a different membrane binding behavior by L-CRP in the presence and absence of  $\text{Ca}^{2+}$ .

Under conditions that facilitate a rapid agglutination of liposomes, we observed the aggregation of L-CRP into long fibrils and sheets completely encapsulating multiple liposomes. This hyper-oligomerization of L-CRP particles is presumably triggered by interaction with divalent cations and lipid bilayers because only much shorter fibrils were seen in the absence of  $\text{Ca}^{2+}$  or liposomes. These shorter fibrils may serve a nucleating function in the formation of the much larger lipid-associated L-CRP structures. Indeed it appears that fibrils in solution are preferentially utilized as the building blocks for the lipid-associated structures as the small fibrils disappear from solution in the presence of  $\text{Ca}^{2+}$  and liposomes, leaving predominantly single oligomers of L-CRP not associated with lipid. We suggest the formation of these large structures on the surface of liposomes facilitates the efficiency of agglutination by increasing both the valency, which can be expected to increase the avidity of L-CRP for foreign particles, and the hydrodynamic radius of the particles, which can be expected to increase the capture efficiency of the coated liposomes. We are not aware of other reports of immune agglutinins that undergo the sort of hyper-oligomerization found here for L-CRP.

The conditions that produce the hyper-oligomerized encapsulation of liposomes by L-CRP also result in permeabilization of the liposomes [8]. We observed ring-structures on the surface of L-CRP-treated liposomes that display a central pore consistent in diameter with soluble L-CRP (2.0 nm). This pore size is in excellent agreement with the functional pore size of  $1.9 \pm 0.2$  nm, observed with electrical measurements in planar bilayers treated with L-CRP [8]. We have shown previously that the  $\text{Ca}^{2+}$ -independent mode of membrane binding is sufficient for membrane permeabilization by L-CRP. It may be the case that the fibrillar form of L-CRP is inhibited from binding in a productive manner with respect to transmembrane-pore formation and indeed it appears that binding in a  $\text{Ca}^{2+}$ -dependent manner to liposomes effectively sequesters the pool of L-CRP available to permeabilize liposomes as the specific pore-forming activity of L-CRP is greater at high ionic strength (a chemical condition that inhibits  $\text{Ca}^{2+}$ -dependent binding) [8].

Following the introduction of protein to liposomes in the presence of  $\text{Ca}^{2+}$ , the bilayer lipids show evidence of an increase in conformational order ( $-1.25 \text{ cm}^{-1}$ ), certainly smaller than but comparable to a full scale phase transition (approximately  $2-3 \text{ cm}^{-1}$ ) [16]. The rigidification might result from an increase in lateral pressure as multiple L-CRP molecules insert into the membrane. Alternatively it is possible that the hyper-oligomerized L-CRP fibrils that associate with liposomes act as a protein-scaffold, constricting the overall motion of the bilayer lipids. This interpretation is consistent with the membrane topology observed

for L-CRP-treated bacteria. These cells do not collapse to a uniform height as do control, untreated cells, but rather they retain a higher profile, as though an additional structural component is present, i.e. an exoskeleton of L-CRP rods. Also, apparent in the  $\text{CH}_2$ -scissoring mode is a change in small domain organization, a phenomenon associated with surface-active antimicrobial compounds [17]. This may arise from the peripheral association of L-CRP or from a reorientation of lipid in the immediate vicinity of transmembrane protein domains due to a mismatch in the transverse length of the hydrophobic domain of the protein and the width of the lipid bilayer.

The membrane interacting behavior of L-CRP is believed to reflect its role as an immune effector protein involved in pathogen recognition. The divalent cation-dependent membrane activity is consistent with the well known  $\text{Ca}^{2+}$ -binding activity of vertebrate pentraxins [18] and the hemolytic activity of limulin [6] and CRP from the Southeast Asian species of horseshoe crab, *Tachypleus* [11]. However, the dependence on low pH for  $\text{Ca}^{2+}$ -dependent membrane binding and insertion by L-CRP is not understood. A number of membrane-active proteins and peptides are activated by low pH, e.g. the amoebapores [19] and annexins [18,20]. We suggest that, as is the case for the aforementioned examples, the requirement for low pH represents a biochemical mechanism of regulating the membrane-activity of L-CRP in the serum of the horseshoe crab. It may be the case that L-CRP-bound cells are phagocytosed and permeabilized in the acidified lysosomes of the animal's hemocytes. The low pH environment certainly results in the protonation of anionic groups of both protein and lipid origin. The increased surface area available for van der Waals interaction may facilitate hyper-oligomerization of L-CRP molecules seen in the presence of  $\text{Ca}^{2+}$ , as well as stable interaction with the acyl-chain region of the lipid bilayers.

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