Calcium-Dependent Binding of Rabbit C-Reactive Protein to Supported Lipid Monolayers Containing Exposed Phosphorylcholine Group

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ABSTRACT The interaction of rabbit C-reactive protein (rCRP) with a supported monolayer containing a phosphorylcholine moiety was studied. Three types of phospholipids were synthesized, each containing a insertion spacer of eight, six, or three atoms between the phosphorylcholine group and hydrophobic tail. By varying the length of the insertion spacer, we can vary the extension of the phosphorylcholine group from the membrane surface. By varying the monolayer composition, we can control the lateral distance between the exposed phosphorylcholine groups. Using the surface plasmon resonance technique (SPR), we demonstrated that the calcium-dependent binding of rCRP to the model membrane is governed not only by the ability of the ligand to access the binding pocket fully (spacer length), but also by lateral hindrance within the two-dimensional plane of the membrane. The value of the apparent binding constant was estimated by theoretical analysis, which is obviously dependent on the composition of the lipid mixture, and a maximum of $(9.9 \pm 1.5) \times 10^6\ M^{-1}$ was obtained.

INTRODUCTION

C-reactive protein (CRP) is one of the most characteristic acute-phase proteins. It was first discovered in 1930 by Tillett and Francis (1930) and has been found in most vertebrates (Steel and Whitehead, 1994). In response to cell damage, tissue injury, and inflammation, there is a rapid and dramatic increase in the biosynthesis of CRP. In the first 24 h after stimulus the level of CRP in serum can increase by up to 1000-fold over normal trace amounts (~100 ng/ml). CRP was originally identified by its capacity to bind the phosphorylcholine group on C-polysaccharide, which leads to the precipitation of C-polysaccharide on the pneumococcal cell wall in a calcium-dependent manner. In addition, CRP binds specifically to other organic phosphate esters, which include monophosphate and phosphorylethanolamine. Various binding specificities of CRP were characterized. It has been discovered that CRP binds to chromatin, histones, small nuclear ribonucleoproteins, and fibronectin (Robey et al., 1984; Du Clos et al., 1987; Du Clos, 1988; Potempa et al., 1987). In each case, the binding of CRP is calcium dependent and is inhibited by free phosphocholine (PC). This does not seem to be the common feature of CRP. There has been evidence that CRP binds to polycationic molecules (Potempa et al., 1981), which is calcium inhibitable. This binding may take place at a site that is distinct from that proposed for PC binding (Agrawal et al., 1992).

CRP is composed of five identical noncovalently linked monomers arranged in a planar ring with pentameric symmetry (Osmand et al., 1977; Sui et al., 1996). Each monomer has 206 amino acids and a molecular mass of ~21 kDa. It has one binding site for phosphocholine and at least two binding sites for calcium ions. The sequence of the protein was first determined by amino acid sequencing and later confirmed by cDNA sequencing (Woo et al., 1985; Lei et al., 1985). The amino acids within the proposed phosphocholine-binding site are conserved among all of the known CRP sequences, from *Limulus* through mammals. The structure of human CRP at 3.0-Å resolution was reported recently (Shrive et al., 1996). It provided evidence that the calcium-dependent binding of phosphocholine takes place through a hydrophobic pocket and the calcium ions themselves.

The physiological effects of CRP are not yet well understood. Some studies have demonstrated that CRP activates the complement system via the classical pathway. It has also been indicated that CRP promotes phagocytosis, as well as the activation of plateletes. To reveal the biological function of CRP, attention has been focused on the binding properties of CRP. The in vivo experimental data (Kushner and Kaokab, 1961) show that CRP binds to the damaged or necrotic membrane structures but not to intact cell membranes. Other studies suggest that such binding could lead to activation of the classical complement pathway (Kaplan and Volanakis, 1974; Siegel et al., 1974).

As a part of the functional characterization of CRP, extensive studies have been carried out on the interactions between CRP and liposomes (Volanakis and Wirtz, 1979; Richards et al., 1977, 1979; Volanakis and Narkates, 1981; Mold and Gewurz, 1981; Mold et al., 1981). Richards et al. (1977, 1979) reported for liposomal model membranes that the interaction of CRP with PC-containing liposomes can activate the complement system. They found that the complement-dependent membrane damage was strongly dependent upon the liposomal membrane composition. Volanakis

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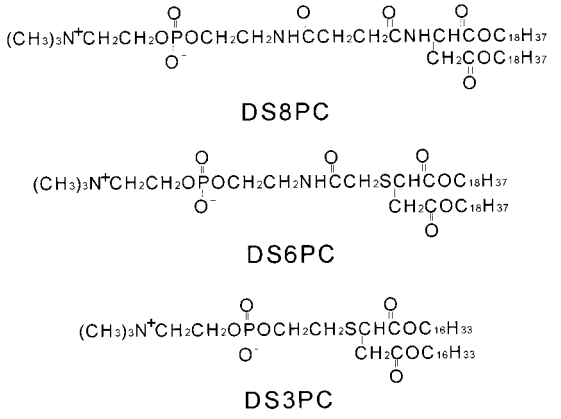
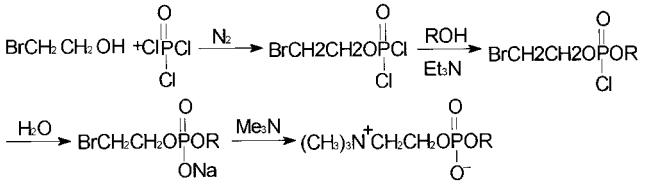
and Wirtz (Volanakis and Wirtz, 1979; Volanakis and Narkates, 1981) reported that the calcium-dependent binding of CRP to model membranes of lecithin required incorporation into the bilayer of lysophosphatidylcholine (lyso-PC). Thus they suggested that a certain disturbance of the molecular organization of the lipid bilayer is necessary for the selective binding of CRP. The molecular mechanism by which CRP binds to the model membrane is still unclear.

Compared with liposomes, the lipid monolayer is easier to use for mimicking biological membranes. The composition, temperature, and ionic conditions of the monolayer can be easily controlled. In the present work, we designed and synthesized artificial lipids with phosphorylcholine as the polar headgroup. Compared with normal PC-lipids, our synthesized lipids are characterized by the long-arm spacers between the polar headgroups and the hydrophobic tails. By varying the length of the insertion spacer, we can vary the extent to which the phosphorylcholine group protrudes from the lipid/water interface. By varying the monolayer composition, we can control the lateral distance between the exposed phosphorylcholine groups. In this way we investigated the features of CRP binding. Our experimental results provide direct evidence that the calcium-dependent binding of CRP to PC-lipid-containing membrane is indeed restricted by such steric hindrance.



(a)



(b)

Scheme 1.

MATERIALS AND METHODS

Materials

Distearoylphosphatidylcholine (DSPC), dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphoglycerol (DPPG), sheep anti-human C-reactive protein antibody, and PE-Sepharose 4B were purchased from Sigma Chemical Co. and were used without further purification. Other chemicals were purchased locally.

Preparation of the synthetic lipids

Three kinds of home-synthesized lipids are all characterized by long-arm spacers between polar headgroups and hydrophobic tails, which are dioctadecanyl *N*-(*N*′-(trimethylaminoethylphosphatoethyl)succinamido-*N*-yl)-asparate inner salt, dioctadecanyl 2-((trimethylaminoethylphosphatoethyl)aactylmethylthio)succinate inner salt, and dihexadecanyl 2-(aminoethylphosphato-ethythio)succinate inner salt. Their structures are shown in Fig. 1 *a*. The chemical reactions used to synthesize them are schematically displayed in Fig. 1, *b* and *c*; these partly follow the work of Eibl and his co-workers (Diembeck and Eibl, 1979). We have abbreviated the three kinds of synthesized lipids as DS8PC, DS6PC, and DS3PC, respectively, where DS stands for the hydrophobic tail containing 18 carbon atoms, PC for the polar headgroup, and 8′, 6′, and 3′ mean the insertion of eight-, six-, and three-atom chains as spacers. The different lengths of the inserted spacers may make the polar headgroups protrude from the membrane surface to varying extents.

Purification of rabbit C-reactive protein

Rabbit C-reactive protein (rCRP) was purified from the acute phase serum of white rabbits by affinity chromatography according to the published procedure (Bach et al., 1977). Rabbit was injected intramuscularly with turpentine (0.5 ml/kg), 36 h before the withdrawal of blood. The serum was passed through an affinity column of phosphorylethanolamine-Sepharose 4B, which had been equilibrated with TBS-Ca^{2+} buffer (0.02 M Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, pH 7.8) in the presence of Ca^{2+} (10 mM). The fractions containing other proteins were pooled. After the column was washed with TBS-Ca^{2+} buffer, the rCRP was eluted with 0.1 M phosphorylcholine chloride in TBS-Ca^{2+} buffer. The fraction was then passed rapidly through a Sepharose 4B column (equilibrated with TBS-Ca^{2+} buffer) to remove serum amyloid P-component. The eluted protein was dialyzed against TBS-Ca^{2+} buffer and then against TBS-EDTA (0.02 M Tris-HCl, 0.015 M NaCl, 10 mM EDTA, pH 7.8) to dissociate any bound phosphorylcholine and Ca^{2+}. The solution was then dialyzed against pure water to remove the other ions. The rCRP concentration was measured by absorbance at 280 nm ($E_{1\%} = 20.3\ cm^{-1}$). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of rCRP prepared in this way showed a single protein band with a molecular mass of 20 kDa (corresponding to one rCRP monomer). The reactivity of the purified rCRP was examined by immunoprecipitation with C-polysaccharide and with sheep anti-human rCRP antibodies.

Preparation of supported monolayer

Supported membrane is one kind of model membrane system used to study lipid/protein interactions (Sackmann, 1996; Sui et al., 1988). It is particularly useful in the characterization of ligand/receptor binding in combination with surface-sensitive techniques (Sui et al., 1988). In the present work, to prepare supported monolayers on gold-coated cover slides, the slides were treated as follows. The cover slides were cleaned in a mixture of 20 ml water, 20 g $K_2Cr_2O_7$, and 180 ml H_2SO_4 (98%) for 24 h, extensively rinsed with water (distilled and deionized), and dried in air.

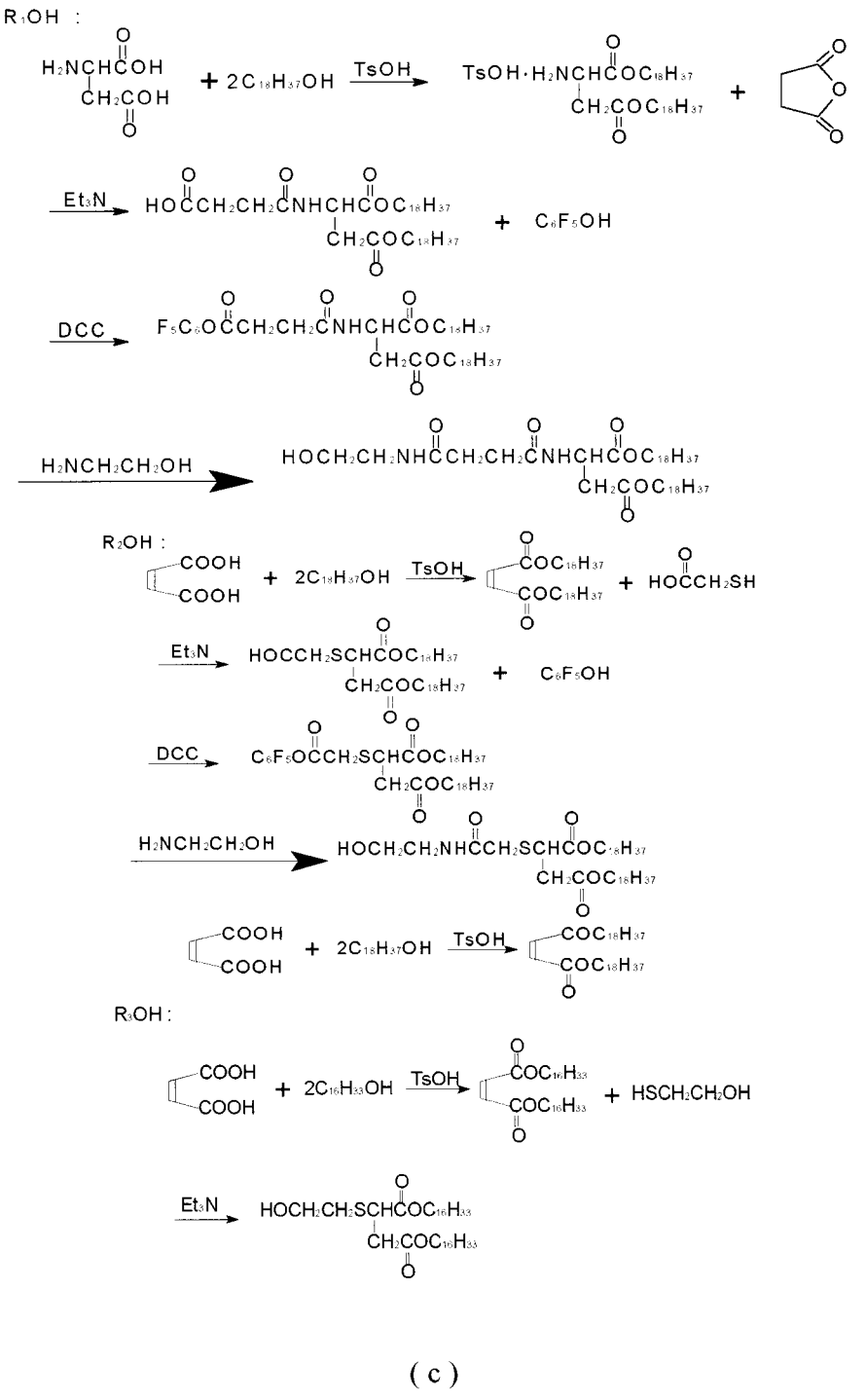


FIGURE 1 The molecular structures of the synthetic lipids and the scheme of the preparation process. (*a*) The structures of the three kinds of synthetic lipids. (*b*) The scheme of the preparation process, where ROH is the tail-connector-spacer alcohol (TSCOH) of the synthetic lipids. (*c*) Synthesis of the tail-connector-spacer alcohol (TSCOH), where R_1OH, R_2OH, and R_3OH represent TSCOH of DS8PC, DS6PC, and DS3PC, respectively.

Then the cleaned slides were hydrophobilized by soaking them in a mixture of dimethyldichlorosilane and chloroform (1:9) for 30 min and rinsed with chloroform and ethanol. After drying gold vapor was used to lay down a 50-nm layer to the slide under vaccuum.

The phospholipid monolayers were transferred to the pretreated slides by the horizontal lifting method. The lipid sample was prepared by mixing DSPC (2 mM, dissolved in chloroform/methanol, 3/1, v/v) and the desired synthetic lipid (2 mM, dissolved in chloroform/methanol, 3/1, v/v) to a suitable molar ratio. To form a lipid monolayer at the air/water interface, a computer-controlled LB film balance was used. The total volume of the trough was 250 ml, and the total area was 370 cm^2. The area covered by the lipid monolayer was changed by a movable barrier, and the surface tension was measured with the Wilhelmy system. The subphase we used was pure water, and the lipid monolayer itself was prepared by deposition of lipid sample in small droplets at different positions on the water surface. To form a supported film, the lipid monolayer was first compressed to a surface pressure of 45 mN/m and then horizontally transferred by hand to a gold-covered slide.

Surface plasmon resonance set-up

The home-made surface plasmon resonance (SPR) apparatus (Liu et al., 1995) is shown schematically in Fig. 2 *a*. A semiconductor laser (wave-

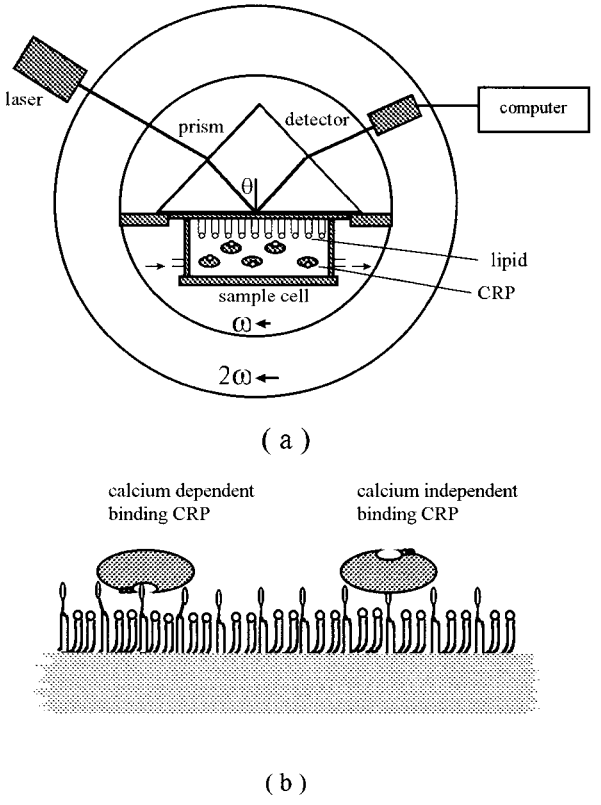


FIGURE 2 (*a*) Schematic illustration of the arrangement of the surface plasmon resonance setup. The triangle prism (refractive index 1.8) is fitted to the small rotating stage of the θ–2θ angle measuring apparatus. The angular accuracy of the θ–2θ angle measuring apparatus is 0.005°. The volume of the sample cell is 200 μl. (*b*) Schematic description of the interaction of rCRP with the supported monolayer containing exposed phosphorylcholine groups.

length 670 nm) was the incident light source. The measurement was performed by varying the incidence angle θ. To characterize the interactions between rCRP and lipid monolayers, the measurements were made as follows. After the lipid monolayer was transferred to the fresh surface of the gold-covered slide, the slide was carefully stuck with index matching fluid to the bottom surface of a triangular prism (as shown in Fig. 2 *a*). Then the sample cell was installed, and the buffer (50 mM Tris-HCl buffer at pH 7.4 containing 2 mM EDTA and 10 mM $CaCl_2$) was slowly pumped into it. The initial peak position of the SPR curve for buffer was recorded. Then the protein solution was pumped into the cell and the second SPR curve was measured after equilibrium. Each measurement was repeated three or four times. The construction shown in Fig. 2 *a* allows us to vary both the surface concentration of the ligands and the bulk concentration of rCRP. Under the condition that the thickness of the film is very thin (less than 10 nm), the displacement of the SPR peak position is linearly related to the layer thickness. Such a condition is sufficient for protein adsorption. The amount of adsorption of rCRP can thus be measured in terms of the shift of the peak position of the SPR curve. In the present work, we follow Stenberg's result (Stenberg et al., 1991) that a 0.1° shift in the SPR peak position is a measure of 1 ng/mm^2 protein absorbed to the membrane surface.

RESULTS

Calcium-independent binding of rCRP to supported monolayers

Our recent work (Mi et al., 1997) reported that rCRP can spontaneously adsorb to phospholipid monolayers at the air/water interface even without Ca^{2+} in the subphase and that the adsorption cannot be inhibited by phosphocholine. Such nonspecific interaction should be taken into account in the study of rCRP binding to supported monolayers. In Fig. 3 we present adsorption and desorption measurements under three different conditions. In the first case, measurement A displays the detachment effect of an excess of EDTA on rCRP binding. We can see that under elution of excess EDTA a large amount of adsorbed rCRP was removed, which is calcium dependent. The remainder is small but not negligible, which is calcium independent. In the second case, as shown in measurement B, a similar phenomenon is observed, but the detachment of adsorbed rCRP is due to competitive inhibition of free phosphocholine. In the third case, measurement C, the sample type of inactive rCRP saturated by PC was used to measure adsorption. In this case, the rCRP-PC complex interacts with ligand-containing membrane in a nonspecific manner. All three measurements shown in Fig. 3 exhibit an obvious nonspecific adsorption of rCRP to PC containing supported monolayer. Such adsorption does not depend on the presence of calcium and is not inhibited by phosphocholine. These results are identical to what we obtained in terms of the phospholipid monolayer at the air/water interface (Mi et al., 1997).

From the results of Fig. 3 we have noticed that the amounts of nonspecific adsorption in all three cases are approximately equal. Further measurements were performed for better understanding. We have measured the surface concentration of rCRP as a function of its bulk concentration in the absence of calcium ions (data not shown). The measurement results showed that when the bulk concentration of rCRP was above 1.0×10^{-8} M, the surface concentration of rCRP in Ca^{2+}-independent adsorp-

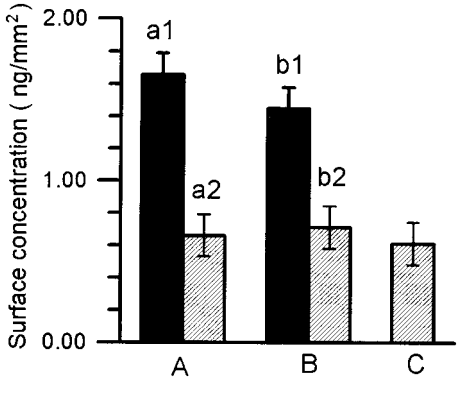


FIGURE 3 Adsorption and desorption measurements of rCRP. Buffer for adsorption: 50 mM Tris-HCl, 2 mM EDTA, 10 mM $CaCl_2$, pH 7.4. Supported monolayer mixture: DS8PC/DSPC (1/12). (*A*) The detachment effect of an excess of EDTA on rCRP binding. a1 and a2 represent the surface concentrations of rCRP before and after elution with 50 mM Tris-HCl buffer containing 2 mM EDTA. (*B*) The effect of a competitive inhibition of phosphocholine on rCRP adsorption. b1 and b2 represent the surface concentrations of rCRP before and after the addition of PC to 500 mM. (*C*) Adsorption of the inactive rCRP, which was saturated by PC before addition. All of the experiments above were performed at room temperature. Bulk concentration of rCRP: 1×10^{-7} M.

tion was not sensitive to the bulk protein concentration. A stable maximum of the surface concentration of rCRP adsorbed in such case was obtained around 0.57 ng/mm^2. As the bulk concentration of rCRP fell below 1.0×10^{-8} M, the surface concentration adsorbed decreased. Similar results were also observed with various molar ratios of the mixture, indicating that the nonspecific binding is not dependent on the ratio of DS8PC/DSPC. In the separated experiments we found that such nonspecific binding is not dependent on the type of phospholipids, cannot be detached by bivalent cations such as Ba^{2+}, and is not inhibited by phosphocholine. So we believe that the nonspecific interaction between rCRP and phospholipid membrane may mainly be dominated by hydrophobic interactions.

Calcium-dependent binding of rCRP to supported monolayers

To characterize the specific binding of rCRP to the supported monolayers, the problem of the interference of information from nonspecific adsorption should be considered. As mentioned above, because calcium is essential to the binding of rCRP to the phosphorylcholine group, we can distinguish rCRP-specific binding from its nonspecific adsorption by determining whether the adsorption process is calcium dependent. Thus the amount of specific binding of rCRP to a ligand-containing membrane can be measured by determining how much rCRP can be detached with EDTA. In all of the following experiments we assume that the bound rCRP molecules that can be detached by EDTA are those attached by calcium-dependent binding.

Fig. 4 shows that the surface concentration of rCRP, attached to supported monolayers by calcium-dependent binding, obviously varies with the molar ratio of the monolayer mixture. An optimum molar ratio of DS8PC/DSPC of 1:12 was found. Around this ratio the amount of rCRP specifically bound to the membrane surface is the highest (1.05 ng/mm^2). Above and below this optimum ratio the calcium-dependent adsorption would decrease. The surface concentrations shown at the two terminals of the abscissa (see Fig. 4), however, look quite different. Binding to the pure DSPC monolayer is lowest (0.16 ng/mm^2), and binding to the pure DS8PC monolayer is remarkably higher (0.61 ng/mm^2). A similar phenomenon was observed in the case of avidin binding to biotin-lipid-containing monolayers (Liu et al., 1995).

Fig. 5 shows the results obtained by measuring the surface concentration of rCRP on three kinds of supported monolayers, which contain different synthetic lipids in the same molar ratio (1/12). According to the molecular structures of the synthetic lipids (as shown in Fig. 1 *a*), each of them is characterized by an insertion arm with a certain length. Thus the membrane-bound ligands protrude from the membrane surface to different heights, whereas the lateral spaces between them are equal. From the results of Fig. 5 we can see that the surface concentrations of calcium-dependent adsorption of rCRP to the three kinds of supported monolayers are remarkably different. In the case of DS8PC-containing monolayer, the amount of protein bound is highest (1.05 ng/mm^2). The surface concentration of rCRP bound to DS6PC-containing monolayer is 0.72 ng/mm^2, and that of rCRP bound to DS3PC-containing monolayer is 0.30 ng/mm^2.

Calculation of the apparent binding constant

The structure of rCRP consists of five identical monomers, arranged as a cyclic pentamer. Each monomer contains one binding site for phosphorylcholine, which is mediated through calcium. All of the binding sites are on only one of

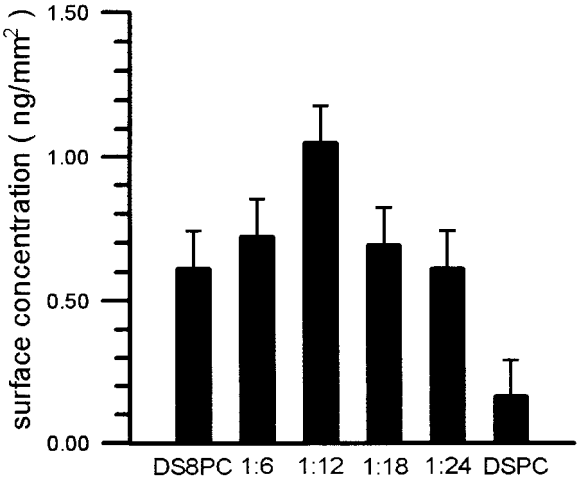


FIGURE 4 The surface concentrations of rCRP Ca^{2+}-dependently adsorbed to the supported monolayers formed by different molar ratios of DS8PC/DSPC, which were obtained by the measurements upon the detachment effect of EDTA. Buffer: 50 mM Tris-HCl containing 2 mM EDTA and 10 mM $CaCl_2$, pH 7.4. Bulk concentration of rCRP: 10^{-7} M.

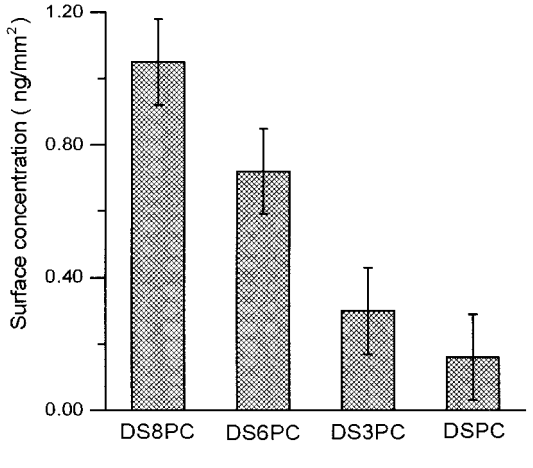


FIGURE 5 The surface concentrations of rCRP Ca^{2+}-dependently adsorbed to the three types of supported monolayers, which were obtained by the measurements upon the detachment effect of EDTA. For the three type monolayers, they have equal molar ratios of DS8PC/DSPC of 1/12. Buffer: 50 mM Tris-HCl containing 2 mM EDTA, 10 mM $CaCl_2$, pH 7.4. Bulk concentration of rCRP: 1×10^{-7} M.

the planar surfaces of the pentameric ring. The calcium-dependent binding of rCRP to membrane-bound phosphorylcholine moieties may be described by the following steps:

$$\text{rCRP} + \text{PC} \leftrightarrow \text{rCRP} \cdot \text{PC} \quad ①$$

$$\text{rCRP} \cdot \text{PC} + \text{PC} \leftrightarrow \text{rCRP} \cdot \text{2PC} \quad ②$$

$$\text{rCRP} \cdot \text{2PC} + \text{PC} \leftrightarrow \text{rCRP} \cdot \text{3PC} \quad ③$$

$$\text{rCRP} \cdot \text{3PC} + \text{PC} \leftrightarrow \text{rCRP} \cdot \text{4PC} \quad ④$$

$$\text{rCRP} \cdot \text{4PC} + \text{PC} \leftrightarrow \text{rCRP} \cdot \text{5PC} \quad ⑤$$

When CRP specifically binds to the lipid monolayers, the binding sites should be facing the monolayer surface. Because the distribution of the membrane-bound ligands normally does not match well with the pentameric geometry of CRP, it is mainly the first step to determine the above reactions when using the SPR technique. This means that as long as one binding site of the pentamer reacts with one phosphorylcholine moiety at the monolayer surface, the whole pentamer will be immobilized on the interface. Once the pentamer is bound to the surface, the SPR responds by a displacement of the resonance angle, regardless of whether the following steps of the ligand/rCRP reaction take place. The binding constant we obtained in terms of SPR thus reflects to a large extent that of the first step of the reactions. In the following we provide a model to describe the reaction of rCRP with ligand containing supported monolayer. In this model we suppose that each molecule (a pentamer) binds specifically to one ligand:

$$\begin{array}{l} \text{CRP} + n\text{Ligand} \leftrightarrow \text{CRP} \cdot \text{Ligand} \otimes (n-1)\text{Ligand} \\ \qquad\quad \downarrow \\ \text{CRP} \otimes n\text{Ligand} \end{array}$$

where CRP is one rCRP molecule; Ligand is one membrane-bound ligand; n is the average number of ligands beneath one bound rCRP molecule; CRP · Ligand indicates that each molecule binds specifically (calcium-dependently) to one ligand; CRP · Ligand $\otimes$ $(n-1)$Ligand indicates that each protein molecule binds a ligand specifically and covers $(n-1)$ ligands nonspecifically; and CRP $\otimes$ nLigand describes the calcium-independent adsorption in which each rCRP molecule can cover n ligands. Because the concentration of calcium is in excess, the influence of calcium is not included when the apparent binding constant is calculated. Here we use the term "apparent binding constant" to emphasize the fact that the reaction is between rCRP and the membrane-bound ligands. Thus the apparent binding constant, K_a (M^{-1}), can be expressed as

$$\begin{aligned} K_a &= [\text{CRP} \cdot \text{ligand}]_{eq}/([\text{CRP}]_{eq} * [\text{ligand}]_{eq}) \\ &= \frac{[\text{CRP} \cdot \text{ligand}]_{eq}}{[\text{CRP}]_{eq} * ([\text{ligand}]_{ini} - [\text{CRP} \cdot \text{ligand}]_{eq} - ((n-1) * [\text{CRP} \cdot \text{ligand}]_{eq} + n[\text{CRP}]_{un})} \end{aligned} \quad (1)$$

In Eq. 1, $[\text{CRP} \cdot \text{ligand}]_{eq}$ is the equilibrium surface concentration of CRP-ligand complex (mol/mm^2); $[\text{CRP}]_{eq}$ is the equilibrium concentration of CRP in bulk solution (mol/liter); $[\text{ligand}]_{ini}$ is the initial surface concentration of ligands (mol/mm^2); $[\text{CRP}]_{un}$ is the surface concentration of CRP nonspecifically adsorbed to the membrane surface (mol/mm^2); and $(n-1) * [\text{CRP} \cdot \text{ligand}]_{eq} + n * [\text{CRP}]_{un}$ expresses the surface concentration of ligands that are nonspecifically covered by CRP (mol/mm^2). Equation 1 can be changed into the following form:

$$\frac{1}{[\text{CRP} \cdot \text{ligand}]_{eq}} = \frac{1}{K_a([\text{ligand}]_{ini} - n[\text{CRP}]_{un})} * \frac{1}{[\text{CRP}]_{eq}} + \frac{n}{[\text{ligand}]_{ini} - n[\text{CRP}]_{un}} \quad (2)$$

Under the condition that $[\text{CRP}]_{un}$ is not changed with $[\text{CRP}]_{eq}$, we can measure $[\text{CRP} \cdot \text{ligand}]_{eq}$ with different $[\text{CRP}]_{eq}$. Then we obtain the following linear relation by graphing with $1/[\text{CRP} \cdot \text{ligand}]_{eq}$ versus $1/[\text{CRP}]_{eq}$:

$$1/[\text{CRP} \cdot \text{ligand}]_{eq} = A/[\text{CRP}]_{eq} + B$$

Comparing the above equation with Eq. 2, the slope A and the intercept B can be expressed as

$$A = 1/(K_a * ([\text{ligand}]_{ini} - n[\text{CRP}]_{un}))$$

$$B = n/([\text{ligand}]_{ini} - n[\text{CRP}]_{un})$$

Thus the apparent binding constant can be given by

$$K_a = B/(n * A) \quad (3)$$

When deducing Eq. 3 we assume that the surface concentration of nonspecific adsorption, $[\text{CRP}]_{un}$, is a constant value. This condition is satisfied in the present work. The data shown in Fig. 4 were all obtained under the condition that $[\text{CRP}]_{eq}$ was taken to be 1×10^{-7} M. At such a rCRP concentration, as we have already discussed above, the amount of nonspecific adsorption is approximately a constant that is not sensitive to the mixture composition. Thus the calcium-dependent binding constant of rCRP binding to the supported monolayer can be calculated in terms of Eq. 3. The values A and B were obtained by a line-fit procedure. The numeral value of n is determined by the section area of rCRP and that of the lipid molecules. In the present work the section area of one rCRP pentamer is taken as 15,000 Å^2 according to our recent results on two-dimensional crystals of rCRP (Sui et al., 1996). The molecular areas of DS8PC and DSPC are 55 Å^2 and 44 Å^2, respectively, which were obtained from our measurements of the π-A curves. Thereby, as the molar ratios of DS8PC/DSPC are 1:0, 1:6, 1:12, 1:18, and 1:24, the corresponding values of n are 283, 47.3, 28.4, 17.8, and 13.5. From the calculation of Eq. 3, Fig. 6 shows the characteristics of the calcium-dependent apparent binding constant of rCRP, which depends, remarkably, on the composition of the lipid monolayer. The value of the apparent binding constant first increases as the molar

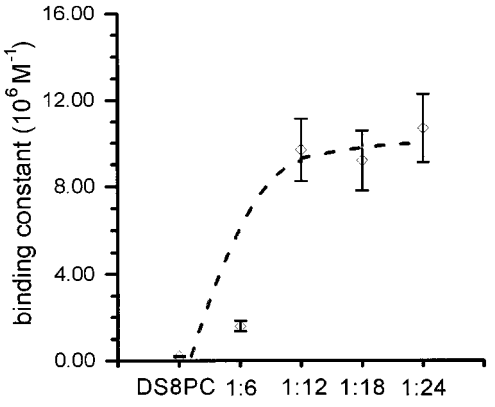


FIGURE 6 The composition dependence of the calcium-dependent apparent binding constant of rCRP to supported monolayer mixtures.

ratio of DS8PC/DSPC decreases and finally reaches to its maximum around a molar ratio of 1:12. A maximum value of $(9.9 \pm 1.5) \times 10^6\ M^{-1}$ was obtained, which is ~50 times of that in the case of binding to pure DC8PC monolayer.

DISCUSSIONS

Although PC is the polar headgroup of major membrane phospholipids, CRP cannot bind to normal cell membranes. Volanakis and Wirtz (1979) reported that CRP bound to, for example, egg PC liposomes in a calcium-dependent manner only if lysophosphatidylcholine (lyso-PC) was present in the liposomes. They suggested that the membrane interface formed by PC and lyso-PC exposed the phosphorylcholine group to the extent that CRP could form a complex perfectly. Using a monolayer technique in the present work, we found (as shown in Fig. 4) that rCRP could hardly bind to a monolayer of pure DSPC, which is identical to the result reported by Volanakis and Wirtz (1979). Experimental results from both monolayer and liposome suggested that the calcium-dependent binding of CRP to phospholipid membrane may be restricted by some kind of steric hindrance. According to a recent report on the three-dimensional structure of human CRP (Shrive et al., 1996), calcium-dependent PC binding takes place through a hydrophobic pocket and the calcium ions themselves. This provides a platform from which the effect of steric hindrance on selective binding can be understood.

Using monolayer technique to study the steric hindrance effect Ringsdorf and co-workers first reported their work on streptavidin (Blankenburg et al., 1989). They chemically synthesized several kinds of biotinyl-lipids with insertion arms of different lengths. Their study showed that a certain length of insertion arm was necessary for streptavidin in combination with the biotinyl-lipid containing monolayers. This steric hindrance effect is a lengthwise one. Liu and Sui studied the characteristics of avidin binding to biotinyl-lipid-containing supported monolayers (Liu et al., 1995). Their work showed that the lateral space between biotin groups is another requirement restricting protein binding. This is the lateral steric hindrance. In this work the results shown in Fig. 4 and Fig. 5 clearly exhibit the steric hindrance effects in these two different types.

From a physical chemistry view, there is a critical surface pressure above which the lipid mixture becomes homogeneous. In the present experiments, all of the supported monolayer samples were prepared by transferring at 45 mN/m from the air/water interface. In a separated measurement we obtained a rough phase diagram of the mixture of DS8PC/DSPC. Based on the phase diagram, the lipid mixture is in a homogeneous state at a surface pressure of 45 mN/m. Thus, by varying the molar ratio of DS8PC/DSPC, the lateral space between PC groups can be precisely controlled. An optimum molar ratio exists (as shown in Fig. 4), indicating that under this condition the ligand-containing monolayer is at its highest efficiency. The value of the molar ratio is higher than the optimum one, 1/12, the effect of the steric hindrance becomes stronger, and therefore the surface concentration of protein bound is smaller. In contrast, when the value of the ratio is lower than the optimum, the surface concentration of the membrane-bound ligands becomes smaller and then the proteins bound specifically become rarer. It should be pointed out that although the surface concentrations of the proteins bound are decreased on both sides around the optimum value of 1:12, the molecular mechanisms causing the decrease in CRP binding are totally different on these two sides.

The above explanation is also supported by the analyzed result shown in Fig. 6, which shows that the character of the behavior of the binding constant varies with the molar ratio of DS8PC/DSPC. It is reasonable that the value of the binding constant is approximately a constant one if the molar ratio is lower than the optimum, because the effect of steric hindrance is negligible. On the other side, if the molar ratio is in the region higher than the optimum, the apparent binding constant will be rapidly decreased because of the steric hindrance effect.

As mentioned above, the steric hindrance may be divided into lengthwise and lateral components. According to the 3D structure of CRP (Shrive et al., 1996), it is required for the specific binding that the three choline methyl groups of phosphorylcholine insert into a hydrophobic pocket (formed by Phe^{66}, Leu^{64}, and Thr^{76}) of CRP. Therefore, in the case where the phosphorylcholine group behaves like a membrane-bound ligand, some extension of this group beyond the membrane is essential for specific binding. Fig. 5 mainly displays an effect of the lengthwise steric hindrance. From Fig. 5 we found that the amount of adsorption of rCRP to DS3PC-containing monolayer (0.3 ng/mm^2) is much smaller than the adsorption to DS8PC-containing (1.05 ng/mm^2) and DS6PC-containing (0.72 ng/mm^2) monolayers. This result indicates that the influence of steric hindrance is more remarkable in the case of DS3PC-containing monolayer. Considering that the measurement error is ± 0.13 ng/mm^2, the significant binding of rCRP to DS3PC-containing monolayer is too small, and we can hardly believe that

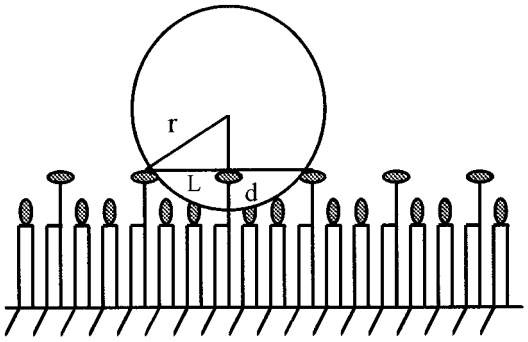


FIGURE 7 The model for calculating the section area of the rCRP subunit. In the model, r is the radius of the rCRP subunit, d is the depth of the binding site of rCRP, and L is the distance between the near exposed headgroups.

the phosphorylcholine group is perfectly accessible to the hydrophobic pocket of the binding site.

On the basis of the above results and discussions, a schematic view of the molecular mechanism of the specific and nonspecific binding of rCRP to ligand-containing supported monolayer is presented in Fig. 2 *b*. According to this model, one can even quantitatively evaluate the section area of the rCRP subunit. Assuming that the rCRP subunit is spherical, the radius of the sphere is r and the depth of the PC-binding site of CRP is d (as shown in Fig. 7). In the case where the molar ratio of DS8PC/DSPC is 1:12, the space L between the near molecules of DS8PC is 24.1 Å. Then taking the radius of DS8PC (or DS6PC) as 4.1 Å, the radius of the rCRP subunit r becomes

$$r = (400 + d^2)/2d$$

If $d = 0.8$ nm, then $r = 29$ Å. Thus the section area of the rCRP subunit is ~2700 Å^2. Comparing with the value of the section area of the rCRP subunit obtained with a two-dimensional crystal on a lipid monolayer, 2400 Å^2 (Sui et al., 1996), it is ~13% larger. Because rCRP molecules binding to a supported monolayer cannot order themselves as perfectly as in a two-dimensional crystal, the section area of the protein obtained with the model of Fig. 7 is reasonable.

An apparent binding constant of rCRP binding to the exposed phosphorylcholine moiety of $(9.9 \pm 1.5) \times 10^6$ M^{-1} is obtained. This is higher than the value of 0.62×10^6 M^{-1} reported by Bach et al. (1977). For the first time they determined the binding affinity of rCRP for phosphocholine with equilibrium gel filtration. There may be two reasons for this inconsistency. First, the methods used are different. The SPR technique is sensitive only to the first binding event, as we have already discussed above, whereas equilibrium gel filtration gives an average binding affinity of the five binding sites. Second, the evaluated value of parameter n in Eq. 3 may also induce a certain error. The numeral value of n was determined according to the section areas of protein and lipids. In fact, the effective section area of the protein molecule should be larger than that obtained for a two-dimensional crystal. Thus, from Eq. 3, the value of the apparent binding constant should decrease as n increases.

The significance of the interaction of CRP with membrane has been accepted in the literature. However, the molecular mechanism of the binding is still unclear. The experimental data in vivo indicated that CRP binds to the damaged or necrotic cell membranes but not intact ones, although there are many PC groups in the outer layer of the membrane. Using an artificial membrane system, we investigated the effects of binding site density and the spacer length on the interaction of CRP with membrane. Our studies provide direct evidence that some extension of binding sites beyond the general membrane surface is required for efficient binding and that a lateral steric exclusion is introduced at high surface density of binding sites. These findings are essential to an understanding of the actual physiological effects of CRP.

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