Biochemical and Biophysical Research Communications **288,** 75–79 (2001)
doi:10.1006/bbrc.2001.5733, available online at http://www.idealibrary.com on IDEAL®

Dissociation and Subunit Rearrangement of Membrane-Bound Human C-Reactive Proteins

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Received September 18, 2001

As one of the most important acute-phase reactants in human serum, C-reactive protein plays its physiological roles mainly on membranes. Here we show that the human C-reactive protein is two-dimensionally crystallized upon specific adsorption on the phosphorylcholine ligand containing membranes by monolayer approach. The 2.0-nm resolution projection structure of the two-dimensional crystals analyzed by electron microscopy and image reconstruction reveals open-ring-like pentamers in the crystals. The electron microscope graphs also show that the dissociated pentamers with open-ring-like structure occur in a closed packing region (not two-dimensionally crystallized). These results indicate a membrane-induced dissociation and rearrangement of hCRP, which may relate to the variety of hCRP's physiological functions.

Key Words: **human C-reactive protein; two-dimensional crystallization; electron microscopy; pentamer; dissociation.**

As one of the classical acute-phase reactants in the serum of humans and many other animals (1), C-reactive protein (CRP) has been found either *in vivo* or *in vitro* to be involved in various physiological functions, such as activation of the complement system (2), enhancement of the phagocytosis (3), regulation of the lymphocytes (4), and conjugation with the pathogen to kill them (5, 6). CRP is characterized by its calcium-dependent precipitation of the pneumococcal C-polysaccharide (CPS) (7). CRP has multiple binding activities with different biological molecules and components, including phosphorylcholine (PC) (8), phosphorylethylnoamine (PE) (9), C1q of the complement system (10), polycations (11, 12), chromatin, histones (13), fibronectin (14), and other reactants. The calcium-dependent specific binding of CRP with PC is of great significance since PC is one of the main compositions of phospholipid head groups in natural cell membranes.

CRP can be detected and purified from the serum of human and many other animals. In human serum, the normal level of CRP is around 0.1–3 μg/mL. In acute phase when the body suffers acute inflammation or damage, however, the concentration of CRP in serum can increase to 1000-fold over normal level in 24–48 h. For the patients with diseases such as rheumatoid arthritis and systemic lupus erythematosus, CRP keeps higher level than normal. Since CRP binds with PC in a specific manner, people can purify CRP by affinity methods from the serum of human or animals in acute phase.

Native human CRP (hCRP) in solution is composed of five identical 23-kDa subunits in a planar pentameric arrangement and classified as a pentraxin (15). Each subunit has a calcium-dependent specific binding site for PC. Both monoclonal marking technique (16) and structural analysis (17) revealed that all the PC binding sites are on the same face of the pentameric disk. The other face of the disk was determined to be involved in the binding of other ligands such as C1q (10, 18). The apparent binding constant of hCRP with PC was determined to be 0.62×10^6 M^{-1} with equilibrium gel filtration (19). CRP exits in plasma as a kind of soluble protein; however, most of its physiological roles are related with its interaction with membranes. Studies on the interaction of hCRP with membranes *in vitro* indicated that in the presence of calcium, hCRP binds specifically to liposomes made up of egg-PC and lyso-PC (20, 21).

Although the atomic resolution structure of hCRP in solution has been resolved, the exact structure and function of membrane-bound CRP are still unclear. Concerning the fact that the interaction of CRP with membrane is a basic mechanism related to CRP's physiological function, to reveal the structural state of membrane-bound CRP is of extreme importance. In our previous work, different types of two-dimensional (2D) crystals of rabbit CRP (rCRP) on lipid monolayers

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0006-291X/01 $35.00

were obtained in different conditions (22–24). Two-dimensional crystals composed either of pentameric rCRP and monomeric rCRP were obtained. In the present work, 2D crystal of hCRP induced by specific adsorption on lipid monolayer is obtained. The projection structure in 2.0-nm resolution reveals the pentameric hCRPs dissociated and rearranged during specific adsorption, indicating a variation in quarternary or ultrastructure of membrane-bound hCRP. These findings may be greatly related to the variety of hCRP's physiological roles.

MATERIALS AND METHODS

Material and reagents. C-reactive protein from human plasma was purchased from Sigma Chemical Co. (St. Louis, MO) with the product number of C-4063. The purity of the protein was confirmed to be over 99% by SDS–PAGE with silver staining. The reactivity of the CRP was examined by immunoprecipitation with C-polysaccharide and with sheep anti-human CRP antiserum. The phospholipid egg-PC and lyso-PC, the phosphorylcholine calcium chloride, and sheep anti-human CRP antiserum were also purchased from Sigma Chemical Co. The distilled water was from the Micro-Electronic Institute of Tsinghua University. All the other reagents were of analytical grade purchased locally.

Two-dimensional crystallization of hCRP on egg-PC/lyso-PC monolayers by specific adsorption. The monolayer two-dimensional crystallization technique has been widely used since its development by Uzgiris and Kornberg (25). We used the same technique as in our previous work (22–24, 26, 27). Briefly, droplets (~15 μl) of 0.1 mg/ml human CRP solution containing 5 mM $CaCl_2$, 100 mM NaCl in 20 mM Tris–HCl buffer with a pH value of 7.4 were placed in a small Teflon well (4 mm in diameter and 0.5 mm in depth). Egg-PC and lyso-PC in 5:1 molar ratio was dissolved in chloroform:methanol (3:1, v/v) with a total concentration of 1 mg/ml. 0.5–1.0 μl of this lipid solution was then spread on the surface of protein solution. Then the whole system was incubated in a humid atmosphere at room temperature or 4°C.

Electron microscopy and image processing. After incubation in the small well for proper period, the lipid monolayers with proteins on them were picked up on hydrophobic carbon-coated grids. Briefly, the grids were placed horizontally on to the film at the air/water interface and picked up after they reached the monolayers. After blotting off the residual solution on the grids, we negatively stained the samples with uranyl acetate (1%, w/v) for 1–2 min.

The grids were examined in a Philips CM120 transmission electron microscope under the accelerating voltage of 100 kV at a magnification from 50,000 to 60,000. Interesting areas of the samples were photographed on Kodak SQ-163 films. The best images selected by optical diffraction were digitized at a step size of 13 μm/pixel for further analysis by an AGFA Duoscan camera system. The numeric images were processed using the I.C.E. (28) and the MRC (29) image processing packages. Briefly, images of crystals with good quality were boxed and Fourier transformed into diffraction spectra. The spectra were analyzed by the SPECTRA program of the I.C.E. package to index the lattice parameters. The crystals' images were then unbent using the CCUNBENDA program and laterally merged together by the ORIGTILTD program of the MRC package. The amplitudes and phases extracted from the Fourier transformed spectra by MMBOX program were analyzed by the ALLSPACE program of the MRC package to determine the symmetry group of the crystal (30). The final average amplitudes and phases were used to calculate the final projection map by CCP4 package (31).

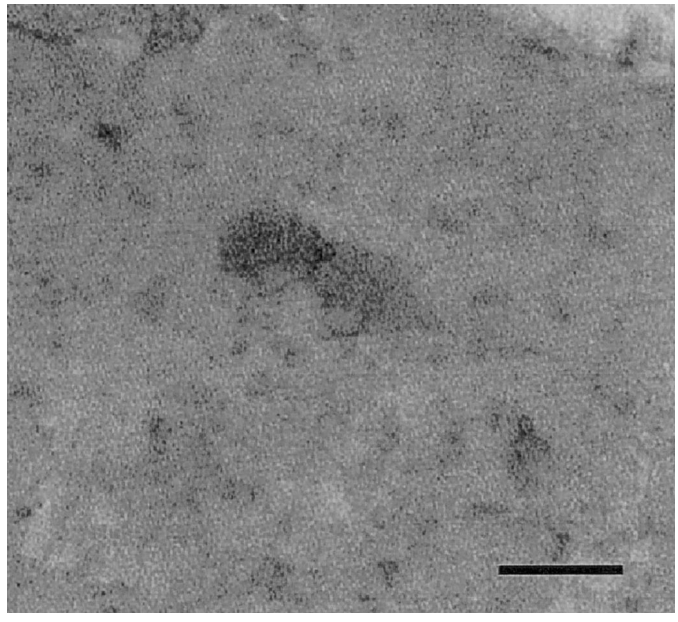

FIG. 1. The 2D crystals of hCRP formed on egg-PC/lyso-PC (in molar ratio of 5:1) monolayers by specific adsorption. The protein was dissolved in 20 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl, 5 mM $CaCl_2$. The concentration of protein is 0.1 mg/ml. Incubation was at room temperature for about 2 h. The scale bar represents 50 nm.

RESULTS AND DISCUSSION

2D Crystals of hCRP on Egg-PC/Lyso-PC Monolayers Are Induced by Specific Adsorption

The specific binding constant of hCRP was measured to be around 7.1×10^{-5} M for egg-PC/lyso-PC liposomes (21), indicating very strong interaction of hCRP with membranes. This is consistent with our results of 2D crystallization of hCRP. At a fairly wide condition (incubation temperature from 4 to 25°C, time from 30 min to 24 h, protein concentration from 20 to 200 μg/ml), two-dimensional crystals of hCRP on egg-PC/lyso-PC monolayers can be obtained (Fig. 1). Incubated less than 30 min, pentameric hCRPs anchored on membranes can be observed. As time goes on, 2D ordered packing of molecules appears and the area of the 2D crystals increases. When incubated for more than 24 h, multilayered 2D crystals with large area form (data not shown). Formation of hCRPs' 2D crystals on membranes was totally inhibited by presence of 1 mM PC or calcium deletion with EDTA in solution (data not shown). This indicated that the 2D crystals were composed of hCRP molecules adsorbed specifically on membranes.

2D Projection Structure of hCRP on Egg-PC/Lyso-PC Monolayers at 2.0-nm Resolution Reveals Open-Ring Pentamers' Assembly

Six images from four films of 2D crystals of hCRP with best quality were analyzed and processed. After

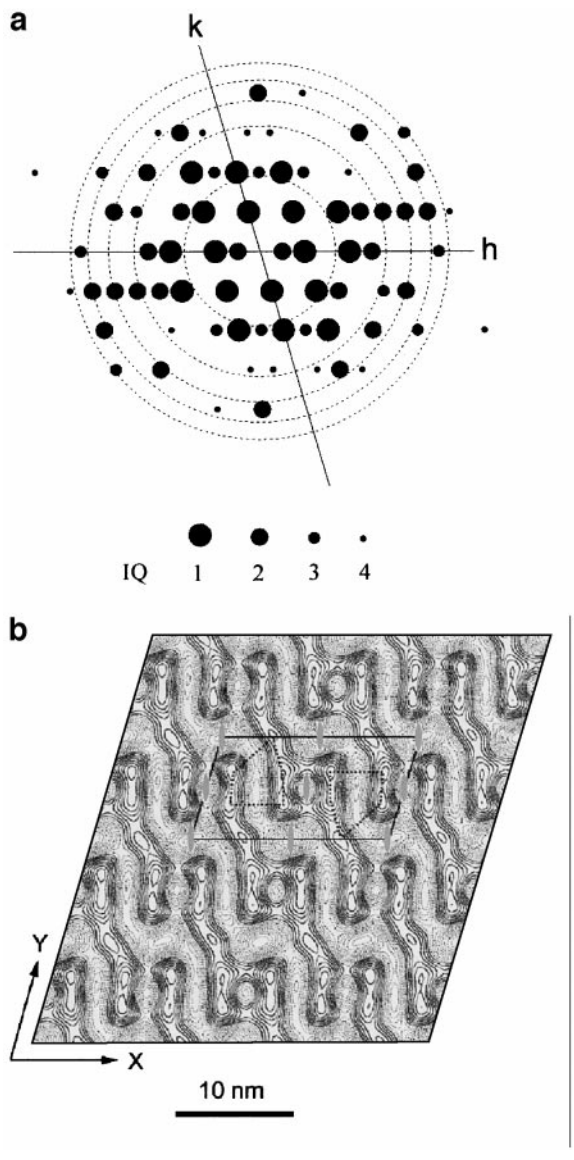

FIG. 2. Image analysis on the 2D crystals of hCRP. (a) Fourier components of the unbent and boxed image No. 21002. The IQ value of each spot is represented by the radius of the spot as indicated in the bottom row. The dash-lined rings from inner to outer represent different resolutions in the Fourier space respectively as 5.0, 3.0, 2.5, 2.2, 2.0 nm. (b) Calculated average projection map at 2.0 nm of hCRPs' crystals from six images of four films. No symmetry is applied to the map, although the pseudo-twofolds are indicated by gray color. In one of the unit cells which is labeled, the probable spatial relationship of the 10 subunits is shown by the dashed pentagons.

unbending, the lattice parameters were determined to be $a = 169.0 \pm 2.2$ Å, $b = 91.4 \pm 2.4$ Å, $\gamma = 74 \pm 1°$, and the resolution may exceed to 2.0 nm (Fig. 2a). The projection map of hCRP's 2D crystals at 2.0-nm resolution was calculated as shown in Fig. 2b. There are altogether 10 motifs with high electron density per unit cell in the crystals. Eight of them are in four pairs parallel to each other in the unit cell. Another two motifs extend to the sides of the unit cell and form lateral links with the paired ones. The dimension of each high electron density motif agrees well with that of a subunit of CRP. This suggests that there are ten subunits in each unit cell of the 2D crystals. The ten subunits in the unit cell may be divided into two groups each of which contains five subunits that arrange in a pentagon shape. Regarding the fashion of compartmentalization, the two pentagons in the same unit cell, however, may have two possible spatial relationships: in the same exposure, or in the opposite exposures. The phase residuals of four images for the $p2$ layer group, 28.2°, 29.1°, 31.0°, and 32.2° separately, are relatively lower than the random value for this symmetry. But for the other two images, the phase residuals were separately 43.1° and 49.2°, larger than the random value. These results indicate the presence of pseudo-twofold axes in the 2D crystals of hCRP. Thus it can be concluded that the two pentagons in the unit cell are in the opposite exposures as suggested by the marked pseudo-twofold axes in the projection map (Fig. 2b). Though hCRP is about 70% homologous to rCRP in sequence, the structures of 2D crystals of the two kind of proteins on eggPC/lysoPC membranes are distinctly different. Different from the previously reported rabbit CRPs' 2D assembly (23, 24), in the present hCRPs' 2D assembly the five subunits in pentagon are arranged in dissociated pentameric structure which has no intra-fivefold symmetry at all.

Dissociation of hCRP Occurs during Assembly on Egg-PC/Lyso-PC Monolayer

Though many ring-like structures can be observed by electron microscopy for the dispersed hCRP molecules in solution and on membranes, no regular pentameric structures can be detected in the 2D crystals. Suggested by the projection map, it is most likely that the pentameric hCRP rings were unclosed to two parts during their 2D assembly on membranes. One part is composed of two subunits and the other part is composed of three subunits. An obvious discrete area between the two parts is present as filled with negative stains. The dissociation of pentamers was not only observed in the crystals, but also in noncrystalline area where dispersed particles aggregated in closed-packing manner (Fig. 3). From Fig. 3 we can see that the fashion of dissociation is the same as that in the 2D crystals; i.e., the pentamers dissociated into two parts.

The electron microscope pictures show that almost all the dissociated pentamers were observed in 2D crystal and in closed packing region. So, it is probably that the pentameric hCRP binds specifically on the monolayers and undergoes dissociation and rearrangement during laterally aggregation. The adhesive force between subunits in the homo-pentamer of hCRP, as revealed by the atomic structural model, is mainly due to the salt bridges (17). On the monolayer plain, the

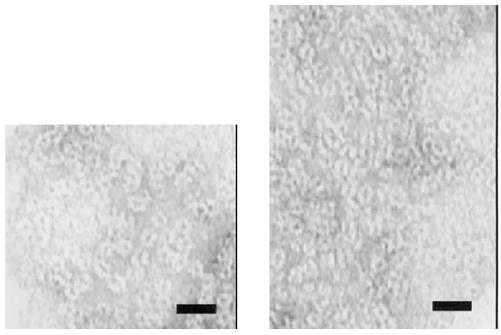

FIG. 3. Dissociation of hCRP rings. The two images show the dissociation of hCRP rings specifically bound on eggPC/lysoPC monolayers. The left image shows some open-ring-like proteins along with pentamers, while the right image shows more dissociated hCRP rings in aggregation closed-packing manner. The scale bar represents 20 nm.

interaction between the homopentamers is mainly due to the lateral electrostatic force (24). As the value of the dielectric parameter near membrane surface of membrane is much lower than that in solution, the stability energy for intramolecular subunits' interaction may be weakened. Thus, when the surface density of proteins increases on the membrane, the comparatively stronger intermolecular subunits' interaction may lead to the dissociation and rearrangement of the pentamers.

The Physiological Significance of the Dissociation of hCRP Specifically Bound on Membranes

Since its discovery, many different physiological roles of CRP have been found and studied. Among them only the mechanism of activation of complement pathway by pentameric CRP ("native CRP") may be studied most clearly. In 1983, Potempa *et al.* found that under certain conditions, CRP may dissociate into monomers (32). The monomeric CRP was found to contribute *in vivo* (33, 34) and to have extensive physiological roles either *in vitro* (35–37) or *in vivo* (38), indicating the factuality and stability of such kind of structural form of CRP. It is believed that the exposure of the new epitope of each monomer (neo-antigen) leads to some of the new physiological roles of monomeric CRP (39). In the present study, hCRP anchors on PC ligand containing membranes specifically, which means that all the subunits have their PC binding site buried on the membrane and their C1q binding site exposed toward solution. The dissociation of the pentamers does not change the orientation of the subunits thus has no influence on the subunits' C1q binding property. But it exposes the intersubunit domain, which is probably part of the neoantigen epitopes. So without losing its complement activation function, hCRP may emerge many other functions at the same time during its dissociation when specifically binding on membranes. Such a high efficiency of physiological roles of biological macromolecules is very common in evolution.

C-reactive protein is of essential significance in animals for the lethality of CRP's absence. Researches in the past decade suggest that CRP probably plays significant roles not only in acute phase but also in normal conditions. CRPs' different forms of structures may be related to various functions of the protein. The current work revealed the dissociation and subunit rearrangement of human C-reactive protein specifically bound on membranes. In our experiments, for two-dimensional crystals' formation, the concentration of hCRP in solution may be as low as 20 μg/mL. In fact, at only 5 μg/mL concentration, aggregated open-ring-like proteins can be observed on membranes. This is very close to the physiological concentration of hCRP in serum. This hints that hCRP may emerge its various structures and thus play its multifunction generally in healthy bodies. Future studies of the proteins' physiological roles on membranes may give more insight to its function/structure relationship.

ACKNOWLEDGMENTS

We thank Dr. Z. H. Zhou for help in installing the ICE Image Processing Package and Dr. R. Henderson providing the MRC programs to Tsinghua University. This work was supported by a grant from the China National Natural Science Foundation.

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