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Study of the Spontaneous Dissociation of Rabbit C-Reactive Protein

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Abstract—C-Reactive protein (CRP) is composed of five identical noncovalently linked monomers and characterized as an important acute-phase protein. The CRP subunit obtained by denaturing treatments, which is termed modified CRP, has also been widely studied. In the current work, we found that there exists some degree of natural dissociation of CRP in stock solution. This dissociation is critically dependent on the absence of Ca^{2+}. Low pH could enhance the dissociation of CRP, while ionic strength has little effect. Anilinonaphthalenesulfonate (ANS) fluorescence detections indicate that the exposure of hydrophobic surface increases during the dissociation. Acidic pH conditions also induce an increase in ANS fluorescence. This suggests that hydrophobic interactions between CRP subunits may contribute to the study of its pentameric structure. Surface plasmon resonance experiments indicate that monomeric CRP does not specifically bind to phosphatidylcholine-containing membrane as native CRP does. Electron microscopy shows that monomeric CRP binds to negatively charged lipid through electrostatic forces, and such lipid may induce the dissociation of CRP due to the acidic pH in the diffuse double layer near the membrane.

Key words: C-reactive protein, modified C-reactive protein, subunits, electron microscopy, surface plasmon resonance, lipid/protein interaction

C-Reactive protein (CRP) was first discovered in 1930 by Tillett and Francis [1], and it was characterized as an important acute-phase protein [2]. CRP is composed of five identical noncovalently linked subunits [3]. Each subunit of CRP has 205 amino acids with a molecular mass of ~22 kD. The primary structures of human CRP and rabbit CRP present more than 70% homology [4, 5]. In response to cell damage, tissue injury, or inflammation, CRP concentration in serum can increase to 300 μg/ml, about 1000-fold over its normal concentration, within the first 24 h. CRP can specifically bind to the phosphatidylcholine (PC) group on C-polysaccharide in the presence of calcium [6], this fact being used in the purification of CRP from serum.

Although CRP is a soluble protein, most of its physiological functions are closely related to the biological membrane. Extensive studies have been carried out to reveal the interaction between CRP and membranes, such as CRP and liposomes [7, 8] and CRP and lipid monolayers [9]. However, a key issue concerning the structure and function of CRP on membranes still remains unclear.

Modified CRP can be obtained by a non-proteolytic conformational change in the CRP subunit structure [10-13] and has biochemical properties and biological functions distinct from those of native CRP [14-18]. For instance, modified CRP does not bind to the classic CRP ligand, PC; modified CRP migrates electrophoretically in the α zone, while CRP migrates in the γ zone; modified CRP can activate and/or potentiate the reactions of leukocytes, monocytes, and platelets. Shields [19] proposed a hypothesis to resolve the apparently disparate activities of native, modified (altered), and peptide forms of CRP. He suggested that native CRP gave rise to the modified and peptide forms of CRP as a consequence of local conditions (such as low pH, oxygen radicals, enzymatic action, etc.).

The traditional preparation of modified CRP involves denaturing treatments such as high temperature (over 60°C), severe acidic condition (pH 2.0), and high level of urea (~8 M). However, it is also observed in solutions of native CRP under Ca^{2+} free condition after long-term storage [20]. Modified CRP has been proven to be a naturally occurring protein in various tissues throughout the body [21-23]. For example, antigens associated with the modified CRP have been found in both normal and

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inflamed human muscle fiber cytoplasm, in certain connective tissues and arterial walls, and in inflamed rabbit liver and muscle [24]. Modified CRP also enhances platelet aggregation and secretion. More recently, it has been shown that modified CRP has profound inhibitory effects on tumor growth and the metastatic ability of an adenocarcinoma in mice [25]. In addition, several authors suggest that modified CRP is bound through a specific receptor on monocytes and macrophages [26, 27].

Although monomeric CRP has been extensively studied, the preparation of this protein form involves rather severe conditions which may not occur *in vivo*. In the present work, we studied the dissociation of CRP in physiological conditions to provide insight into the natural occurrence of CRP subunits.

MATERIALS AND METHODS

Materials. 2,3-Dimercapto-1-propanesulfonic acid, phosphatidylcholine (PC), lyso-PC, egg-PC, EDTA, ultrapure urea, Sepharose 4B, phosphorylethanolamine-Sepharose 4B, and sheep anti-human CRP antiserum were purchased from Sigma (USA). Deionized water was purchased from the Micro-Electronic Institute of Tsinghua University. All other reagents were of analytical grade purchased locally.

Purification of rabbit CRP. Rabbit C-reactive protein was purified from acute phase serum of rabbits by affinity chromatography on phosphorylethanolamine-Sepharose 4B according to the published procedure [9]. The purity of the protein is confirmed to be over 99% by SDS-PAGE with silver staining. The reactivity of the purified CRP was examined by immunoprecipitation with C-polysaccharide and with sheep anti-human CRP antiserum.

Analysis of CRP solution by ACTA Purifier. For analysis, 200 μl solution with the concentration of 0.2 mg/ml in TBS (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.8, and 0.02% sodium azide) was applied to an ACTA Purifier with a Superdex 200 column (Pharmacia, Sweden). For isolation, we applied 2 ml CRP solution with the concentration of 1 mg/ml in TBS onto the ACTA Purifier with HiLoad 26/60 Superdex 200 column (Pharmacia). The elution buffer was the same as storage buffer. The flow rate for analysis and isolation were 0.5 and 1.0 ml/min, respectively.

The molecular weight of the CRP oligomers was estimated by comparison with the retention times of a set of protein standards of known molecular weight: lysozyme (14.3 kD), trichosanthin (26 kD), ovalbumin (43 kD), albumin (67 kD), alcohol dehydrogenase (150 kD).

Hydrophobic fluorescence detection. Anilinonaphthalenesulfonate (ANS) binding assays were used to detect the nonpolar residues exposed to solvent. Florescence emission spectra were recorded for solutions in quartz cuvettes (1 cm path length) with a Hitachi F-2500 fluorescence spectrophotometer (Japan). For each spectrum, the excitation wavelength was set at 360 nm, and the emission spectrum was recorded every 0.5 nm from 420 to 550 nm with an integration time of 0.08 sec at room temperature. The concentration of ANS in solutions was 20 μM with the CRP concentration of 2 μM. In the experiments a blank made with buffer alone was carefully subtracted from the experimental spectra for correction.

Surface plasmon resonance (SPR) measurements. A homemade SPR system [28, 29] based on the Kretschman configuration was used in the present work. An HL6711G semiconductor laser (wavelength 670 nm) was used as the incident polarized light source, and a photodiode served as a detector to collect the reflected light. The chip was stuck to a prism with a refractive index of 1.8 through index matching oil. A 200 μl sample chamber was made of Teflon, which was rotated by a computer-controlled stepping motor with a minimum step angle of 1/36,000°. The numerical evaluation of each resonance angle has an error of less than 0.001°. Each curve of the resonance unit (the angle change of degrees, unit 0.001°) versus time was measured by varying the incident angle around the resonance point. The supported lipid monolayer for SPR measurements was prepared according to the methods described by Sui et al. [28]. Lipid monolayer was compressed to a surface tension of about 40 mN/m by a computer controlled Langmuir–Blodgett trough, and then horizontally transferred onto a 50 nm gold layer covered glass slide which was previously cleaned and hydrophobized. All experiments were performed at room temperature (25 ± 1°C).

Incubation of CRP with monolayer. To study the structures of CRP on membranes, monolayer technique was used in the current work as previously described [8]. Briefly, a solution of protein was placed in a small Teflon well (4 mm in diameter and 0.5 mm in depth). The lipid sample dissolved in chloroform–methanol (3 : 1 v/v) was then spread on the surface of the protein solution. The amount of lipid sample added to each well was much more than that required to form a monolayer. Due to the aperture effect, the lipid membrane in the middle was a monolayer in the highest pressure that it could reach. The protein molecules in the membrane/solution interface may interact with the membranes by specific, electrostatic, or other types of nonspecific interactions. The structure of proteins on the membranes thus can be studied.

In our experiments, droplets (~15 μl) of protein solution were placed in the Teflon wells, and the surface of the droplets was coated with 0.5-1 μl of lipid mixture of different lipids at different molar ratios as indicated in the text. Then the whole system was incubated in a humid atmosphere at different temperatures and for different periods.

Electron microscopy. After incubation in the small well for an appropriate period, the protein bound lipid monolayers were picked up on carbon-coated grids. Briefly, the grids were placed horizontally onto the film at

the air/water interface and picked up after they reached the monolayers. After blotting off the residual solution on the grids, they were negatively stained with uranyl acetate (1% w/v) for 1 min. The grids were then examined in a Philips CM120 transmission electron microscope under the accelerating voltage of 100 kV at a magnification from 50,000 to 60,000. Areas with ordered sample were photographed on Kodak SQ-163 films.

RESULTS

CRP stored in buffers lacking Ca^{2+} shows a time course of dissociation. Previous studies have revealed that native CRP composed of five subunits could undergo dissociation in solution lacking Ca^{2+} [10, 30, 31]. In present work, we use size-exclusion chromatography to examine the oligomeric state of CRP in solution.

It was obvious that in the absence of calcium, freshly purified native CRP continuously dissociated over time during storage (Fig. 1). Figure 1 shows that there is only one sharp peak (~120 kD) that represents a pentamer in the freshly purified CRP solution. After three days of storage, the pentamer peak was somewhat widened indicating the quaternary structure of CRP is loosed to some extent; while the peak representing pentameric CRP in Fig. 1 is very sharp reflecting the tight quaternary structure of the fresh CRP. When the storage time was over one week, the elution volume of the pentamer peak showed a delay in the chromatographic retention time suggesting the presence of forms with small molecular weights in the storage solution. After storage for three weeks, the chromatographs revealed two apparent peaks at weights appropriate for pentamer and monomer forms (120 and 22 kD, respectively). In addition, SDS-PAGE analysis was applied for both fresh CRP and CRP after three-week storage, and the results showed only one band at the molecular weight of monomeric CRP (data now shown), which implied that this conversion is not a result of contaminating protease activity.

On the other hand, the protein solution stored in TBS, pH 7.4, buffers with Ca^{2+} rarely suffered dissociation (data not shown). Consistent with previous reports [10, 11], our results indicate that Ca^{2+} is important for the stabilization of pentameric CRP.

Acidic pH can promote the dissociation of pentameric CRP. Although monomeric CRP has been extensively studied, the preparation of this protein form requires rather severe conditions. For example, acid modified CRP is obtained by the following procedure: CRP containing solution is adjusted to pH 2.0 with HCl and incubated at ambient temperature for 1 min prior to neutralization with NaOH [13]. Such severe conditions do not occur *in vivo*. However, local mild acidic micro-environment [32, 33] may be induced by cell damage or tissue injury, and this can induce an increase in native CRP.

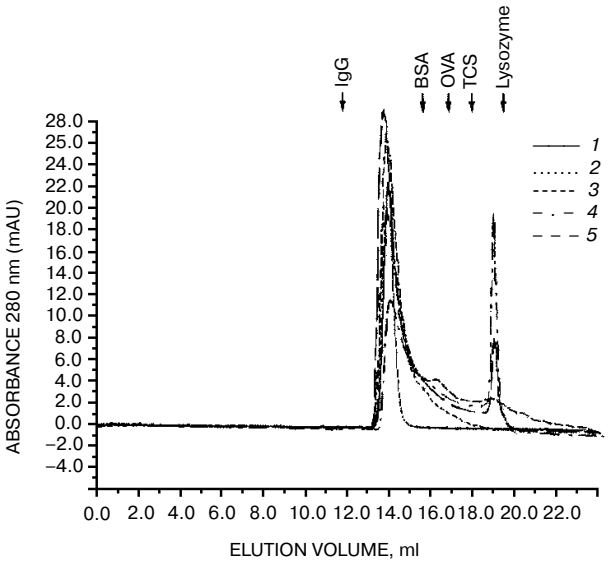

Fig. 1. Chromatography of fresh CRP (*1*), CRP stored for three days (*2*), one week (*3*), and over three weeks (*4*) in neutral buffer. The Superdex 200 column was equilibrated with 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.8, and 0.02% sodium azide at room temperature. The flow rate was 0.5 ml/min. Control protein used: IgG (150,000 kD), bovine serum albumin (68,000 kD), ovalbumin (45,000 kD), trichosanthin (TCS) (26,000 kD), lysozyme (14,000 kD). In addition, fresh CRP incubated in acidic buffer for 48 h (*5*) was eluted with 0.02 M Tris-HCl, 0.15 M NaCl, pH 6.0, and 0.02% sodium azide at room temperature at 0.5 ml/min flow rate after equilibration with the same buffer.

Therefore, the molecular weight distributions of fresh CRP in mild acidic solution (i.e., pH 6.0) lacking Ca^{2+} were examined. The results given in Fig. 1 show that the apparently monomeric CRP peak occurs only after 48-h incubation. This means acid pH rapidly promotes the dissociation rate of pentameric CRP, which may also partly account for the contingent occurrence of monomers observed on negatively charged membrane. Experiments were also performed to test the effect of ionic strength on the dissociation of native CRP. The results indicate ionic strength has little effect, since no apparent increase in dissociation rate or monomer content ratio was found (data not shown).

Dissociation of CRP is accompanied by exposure of hydrophobic sites. ANS is a hydrophobic fluorescent probe used in studies of protein tertiary conformation. ANS has very low fluorescence intensity in aqueous solution and an emission maximum at 515 nm; however, in a nonpolar environment, it fluoresces strongly and exhibits an emission maximum at 454 nm [34]. This increase in the fluorescence intensity and the blue shift in the emission maximum are due to the transfer of the ANS molecule from a polar environment to a nonpolar region of the protein molecule.

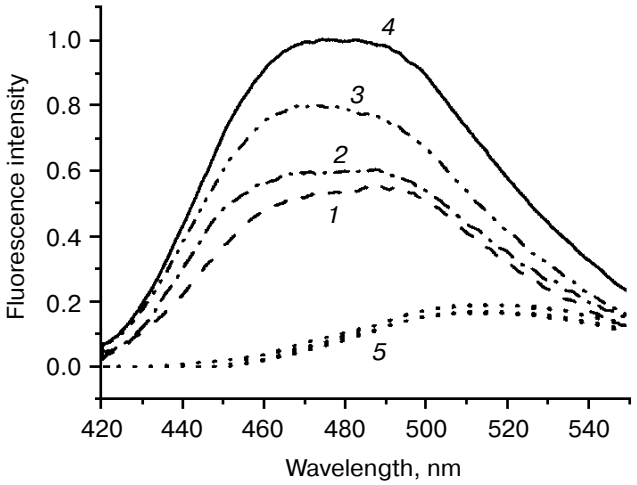

Fig. 2. ANS fluorescence of hydrophobic sites of fresh CRP (*1*) and CRP stored for three days (*2*), one week (*3*), and over three weeks (*4*). Lines of group (*5*) (they virtually do not differ) correspond to buffer without protein.

In TBS buffer at pH 6.0 and 7.0, ANS has very low fluorescence intensity and an emission maximum at 523 nm. In the presence of CRP (Fig. 2), the fluorescence intensity increases with the increase in storage time. The emission maximum shifts to about 480 nm under these conditions. This increase in the fluorescence intensity and the blue shift in the emission maximum indicate the binding of ANS to CRP (pentamer or monomer). This suggests that CRP has some of its nonpolar residues and polypeptide backbone exposed to solvent, and the degree of exposure increases as the dissociation progresses. Further experiments indicate that acidic pH could also induce the increase in hydrophobic exposure (data not shown).

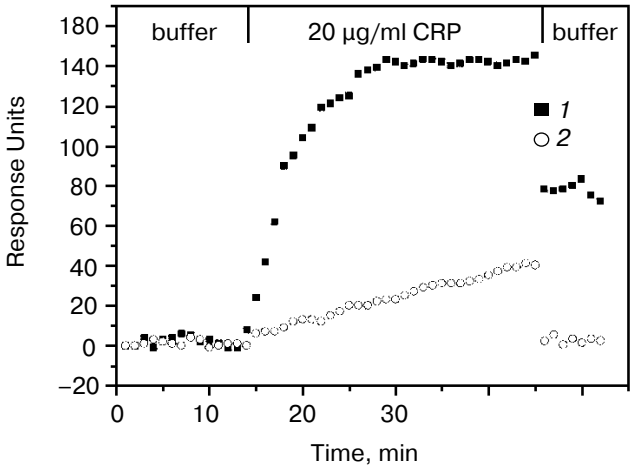

Fig. 3. Surface plasmon resonance curves of RU versus time for native and monomeric CRP binding to PC monolayer. Experiments were carried out on a homemade surface plasmon resonance device at room temperature (25 ± 1°C). The supported lipid layer used here was egg-PC/lyso-PC (5 : 1, molar ratio) monolayer and in experiment was first blocked with 0.1 mg/ml bovine serum albumin, and washed with running buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.8, and 0.02% sodium azide). Then either native (*1*) or monomeric CRP (*2*) was injected into the sample chamber to perform the binding process. When the binding came to a balance, running buffer was used to wash out excess protein. The bulk concentration of CRP is 20 μg/ml.

Although the atomic model of CRP indicates that the interaction between protomers in the pentamer is mainly through salt bridges, hydrophobic interaction may also contribute to the stabilization of its ring-like structure. The dissociation of pentameric CRP is probably due to the disruption of these salt bridges in the depolarized or acid environment. When a normally folded protein is destabilized so that some of its hydrophobic residues and main-chain hydrogen bonds become exposed to solvent, a partially folded or molten globule state of the protein arises. Thus the weakening of hydrophobic interactions may be an early stage in dissociation of native CRP.

Monomeric CRP does not specifically bind to PC ligand. CRP could specifically bind to PC in the presence of calcium [6]. In our previous work, we observed that ring structures were commonly formed on monolayers composed of egg-PC and lyso-PC in molar ratio of 5 : 1 when calcium was present in buffer, and that these structures could further assemble into 2D crystals under proper conditions [35, 36]. In our current work, SPR combined with a supported lipid monolayer system was used to examine whether monomeric CRP has similar PC specific binding.

According to the above size-exclusion chromatographic analysis, we know that CRP stock contains pentameric CRP and monomeric CRP (modified CRP produced by natural dissociation). Using an ACTA Purifier with HiLoad 26/60 Superdex 200 column, the monomeric component was isolated (data not shown). The adsorption of native CRP and monomeric CRP to supported egg-PC/lyso-PC monolayer were then measured by SPR at a protein concentration of 20 μg/ml as shown in Fig. 3. From the SPR curves of response units (RU) versus time, we could see that RU value increased significantly when native CRP with calcium was injected, indicating a strong protein–lipid interaction; while monomeric CRP only led to a fairly small increase in RU value. When eluted by buffer to disrupt non-specific adsorption, the RU value of native CRP remained about 60 units, reflecting its specific binding nature. However the RU value of monomeric CRP decreased almost to zero, which meant the previous increase was mainly caused by nonspecific adsorption. Modified CRP obtained by traditional acidic denaturation was also tried in SPR experiments, and no specific binding was found (data not shown). The above data suggest monomeric CRP has no specific calcium-dependent PC binding.

Monomeric CRP binds to negatively charged membrane by electrostatic force. When using the monomeric

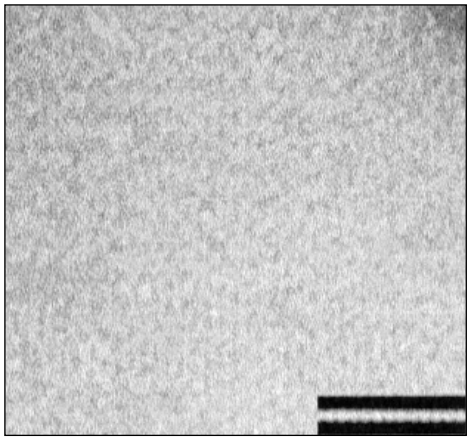

Fig. 4. Monomeric CRP on negatively charged monolayer. The membrane was monolayer composed of 2,3-dimercapto-1-propanesulfonic acid and egg-PC in molar ratio 1 : 5. CRP solution (0.1 mg/ml) in the subphase was incubated underneath the monolayer for more than 24 h at room temperature. The scale bar represents 40 nm.

CRP isolated from CRP stocks, globule-like particles could be found on the monolayer containing acidic phospholipids such as PC (Fig. 4). The sizes of these particles were diameter of about 3-4 nm, which is consistent with the size of a subunit of pentameric CRP. When the ionic strength of the buffer was increased, the amount of monomer decreased. Few monomers could be found on neutral or positively charged monolayers (data not shown). These results suggest that the CRP monomers are prone to be adsorbed on negatively charged membranes and that the interaction of the monomers with negatively charged monolayers may be due to the nonspecific electrostatic forces.

DISCUSSION

As one of the major acute phase proteins in many vertebrate animals including humans, C-reactive protein performs most of its biological function while sitting on cell membranes. The discovery of modified CRP with different biochemical and physiological properties to native pentameric CRP led scientists to consider the variation and conversion of this protein to different forms *in vivo* [19]. In a study on the distribution of injected ^{125}I-CRP in mice, Potempa et al. [17] suggested that the injected CRP was converted *in situ* to modified CRP. Motie et al. [20] reported that CRP stored in buffers lacking calcium or containing chelator could spontaneous express modified CRP. During 3D crystallization, Myles et al. [31] found that lower oligomeric forms of CRP were present in the solution by nondenaturing polyacrylamide gel electrophoresis analysis, and further assay indicated that these lower oligomers included trimer and monomer forms [10, 11]. By antigenity determination, both forms of structures were discovered to locate to different organs and tissues *in vivo*. This suggests the presence of different forms of the protein *in vivo*, which led us to ask the question of what underlying factors are involved in the conversion from the pentameric to monomeric form.

By size exclusion gel filtration chromatography, we also found both pentamer and monomer existed in stored CRP solution. This indicates that the natural dissociation of native CRP occurs in solution along with the extension of storage time. From the chromatography of stored CRP, some minor components also could be found between monomer and pentamer (Fig. 1), implying the existence of intermediate states of CRP dissociation. Relaxation in quaternary structure of CRP leads to a final obvious sharp peak for the monomeric form indicating that such a form is the final product of the dissociation with homogeneous structure and relative stability.

Under certain circumstance such as in acidic buffer (pH 6.0), the dissociation rate of CRP rapidly increases. Eisenberg et al. [37] proved that the pH value on the surface of negatively charged membranes is more acidic than that in the subphase. These facts may give an explanation as to why the monomeric form of CRP is often found on monolayers containing negatively charged lipid after a long incubation time when using fresh CRP under neutral condition. We suggest that during the long time scale after nonspecific adsorption to the monolayer, pentameric CRP might release free subunits due to the acidic microenvironment, and that these free subunits finally induce the monomeric structure on the membrane.

Potempa et al. [11] found that urea, heat, or acid treatment in the absence of calcium may induce native pentameric CRP conversion to modified CRP, which was determined to be composed of monomers of CRP. Such a conversion is irreversible and spontaneous as indicated by Kresl et al. [38]. Unlike in previous work, we show that monomers of CRP were obtained directly from purified native CRP in the absence of calcium. These monomers were also observed directly on membranes. The fact that dissociation of CRP is accompanied with the exposure of hydrophobic surface patches implies hydrophobic interactions contribute to the stabilization of its ring-like structure, and that hydrophobic exposure may be an early event in CRP dissociation. We also show that dissociated monomers do not interact with PC ligand containing membranes, which agrees with previous reports.

An asymmetric membrane model of cell membranes shows that the negatively charged lipids such as PC are mostly distributed in the inner membranes. When the cells are necrotic or damaged, the inner membranes may be exposed to the matrix [39]. Membranes of necrotic cells and damaged cells are exposed to the serum and share of the lyso-PC and phosphatidylsulfonic ligands in

lipid composition of the membranes increases. Shields et al. [19] suggested that distinct forms of CRP were created in succession from bound native CRP at the inflammatory site due to local conditions, and that these distinct forms of CRP play their roles in a temporal sequential manner. Pentameric CRP may then interact with the cells by specific binding, while monomeric CRP, either formed due to the local environmental changes or directly derived from the dissociation of pentamers on the acid membrane surfaces, would interact with the cells by electrostatic forces. The specific interaction of CRP with cells will cause the complement system to attack the cells, however the nonspecific interaction of monomeric CRP with cells may lead to physiological events other than the resolution of injured cells.

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