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# Functional Transformation of C-reactive Protein by Hydrogen Peroxide*

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**Edited by Luke O'Neill**

**C-reactive protein (CRP) is present at sites of inflammation including amyloid plaques, atherosclerotic lesions, and arthritic joints. CRP, in its native pentameric structural conformation, binds to cells and molecules that have exposed phosphocholine (PCh) groups. CRP, in its non-native pentameric structural conformation, binds to a variety of deposited, denatured, and aggregated proteins, in addition to binding to PCh-containing substances. In this study, we investigated the effects of $H_2O_2$, a prototypical reactive oxygen species that is also present at sites of inflammation, on the ligand recognition function of CRP. Controlled $H_2O_2$ treatment of native CRP did not monomerize CRP and did not affect the PCh binding activity of CRP. In solid phase ELISA-based ligand binding assays, purified pentameric $H_2O_2$-treated CRP bound to a number of immobilized proteins including oxidized LDL, IgG, amyloid $\beta$ peptide 1–42, C4b-binding protein, and factor H, in a CRP concentration- and ligand concentration-dependent manner. Using oxidized LDL as a representative protein ligand for $H_2O_2$-treated CRP, we found that the binding occurred in a $Ca^{2+}$-independent manner and did not involve the PCh-binding site of CRP. We conclude that $H_2O_2$ is a biological modifier of the structure and ligand recognition function of CRP. Overall, the data suggest that the ligand recognition function of CRP is dependent on the presence of an inflammatory microenvironment. We hypothesize that one of the functions of CRP at sites of inflammation is to sense the inflammatory microenvironment, change its own structure in response but remain pentameric, and then bind to pathogenic proteins deposited at those sites.**

C-reactive protein (CRP)[3] is a pentameric molecule made up of five non-covalently associated, identical subunits arranged symmetrically around a central pore (1, 2). The ligand recognition function of native pentameric CRP is to bind, in a $Ca^{2+}$-dependent manner, to phosphocholine (PCh)-containing substances, such as pneumococcal cell wall C-polysaccharide (PnC), necrotic cells, platelet-activating factor, PCh-containing molecules on the surface of parasites, enzymatically modified LDL, and oxidized LDL (ox-LDL) if the oxidation was sufficient to expose the PCh groups present in LDL (3–10). Under certain conditions, such as in an acidic pH buffer, CRP adapts a different pentameric configuration that exposes a hidden ligand-binding site for non-PCh ligands and that enables CRP to bind to immobilized, denatured, and aggregated proteins, irrespective of the identity of the native protein (11–13). Mutagenesis of $Glu^{42}$ or $Pro^{115}$, amino acids in the intersubunit contact region in pentameric CRP, to $Gln^{42}$ and $Ala^{115}$, respectively, also converts CRP into molecules that bind to a variety of immobilized, denatured, and aggregated proteins (11, 14).

CRP is a plasma protein that is present at sites of inflammation such as necrotic areas in local inflammatory lesions, synovium of patients with rheumatoid arthritis, inflammatory lesions of experimental allergic encephalomyelitis, inflammatory and arterial atherosclerotic lesions, and neurofibrillary tangles of Alzheimer's disease (15–22). When CRP is present at sites of inflammation, it is exposed to an inflammatory microenvironment. The microenvironment at sites of inflammation including arterial lesions in atherosclerosis, inflammation in the eye and the sites of bacterial infection, is characterized by acidic pH, hypoxia, and increased $O_2$ and energy demand, which result in the production of reactive oxygen species (ROS), including $H_2O_2$, and subsequent dysregulation of the extracellular redox environment, which is known to cause modifications in the proteins present nearby (23–33). The functions of CRP at sites of inflammation have not been defined yet; however, it has been suggested that a structural change in CRP and the resulting shift from the ligand recognition function of CRP in its native conformation to another ligand recognition function in its non-native conformation occur at sites of inflammation (13).

In this study, we used $H_2O_2$ as a prototypical ROS, treated freshly purified native pentameric CRP with $H_2O_2$, and investigated the ligand recognition functions of purified pentameric $H_2O_2$-treated CRP. We found that pentameric $H_2O_2$-treated CRP gained a ligand recognition property not exhibited by native CRP, indicating that $H_2O_2$, like acidic pH reported previously (11), is another modifier of the structure and ligand recognition function of CRP.

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[3] The abbreviations used are: CRP, C-reactive protein; C4BP, C4b-binding protein; $H_2O_2$-CRP, CRP treated with 2% $H_2O_2$; mCRP, monomeric CRP; ox-LDL, oxidized LDL; PCh, phosphocholine; PnC, pneumococcal C-polysaccharide; ROS, reactive oxygen species; TBS-Ca, TBS, pH 7.2, containing 0.1% gelatin, 0.02% Tween 20 and 2 mM $CaCl_2$; TBS-EDTA, TBS, pH 7.2, containing 0.1% gelatin, 0.02% Tween 20 and 5 mM EDTA.

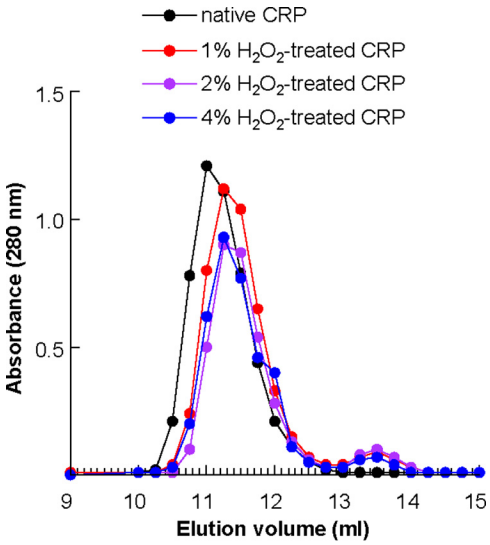


FIGURE 1. **Gel filtration chromatography of native and $H_2O_2$-treated CRP.** Elution profiles of CRP treated with increasing concentrations of $H_2O_2$ for 2 h at 37 °C are shown. Elution profile of untreated native pentameric CRP is shown to locate the elution volume of pentameric CRP. Chromatography was performed in TBS, pH 7.2, containing 2 mM $CaCl_2$. A representative of two chromatograms from the Superose 12 gel filtration column is shown.

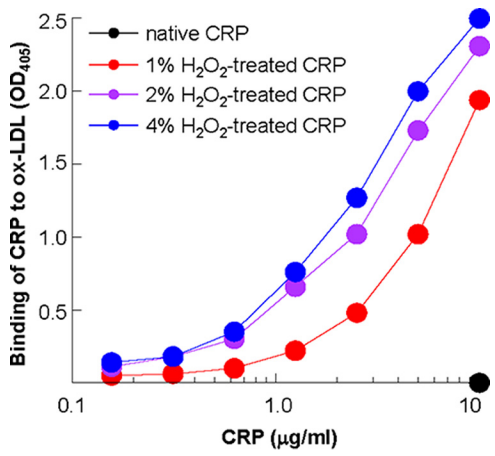


FIGURE 2. **Binding of $H_2O_2$-treated CRP to immobilized ox-LDL.** CRP, in increasing concentrations and diluted in TBS-Ca, was added to ox-LDL-coated wells and incubated overnight at 4 °C. Bound CRP was detected by using rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the *A* value was read at 405 nm. A representative of three experiments is shown.

## Results

All experiments were performed three times, and comparable results were obtained each time. The results of a representative experiment are shown in the figures where the raw data ($A_{405}$) were used to plot the curves.

*Minimal Monomerization of Pentameric CRP upon Treatment with $H_2O_2$*—We tested the effects of 1%, 2 and 4% $H_2O_2$ on the pentameric structure of CRP at a concentration of 100 μg/ml. CRP preparations after treatment with $H_2O_2$ were subjected to gel filtration (Fig. 1). As shown, the elution volumes of native pentameric CRP, $H_2O_2$-treated CRP and monomerized CRP were 11.0, 11.2, and 13.5 ml, respectively. The elution volume of a large proportion of $H_2O_2$-treated CRP was similar to that of native pentameric CRP. Monomerization of CRP was minimal after $H_2O_2$ treatment because only a small proportion of $H_2O_2$-treated CRP was eluted at 13.5 ml as monomeric CRP (mCRP). To isolate pentameric $H_2O_2$-treated CRP, we pooled the fractions falling in the left three-fourth portion of the chromatogram, thus eliminating the possibility of the presence of any mCRP in the final preparations of pentameric $H_2O_2$-treated CRP. The pools of fractions containing pentameric $H_2O_2$-treated CRP were used in all other experiments.

*Purified Pentameric $H_2O_2$-treated CRP Binds to ox-LDL in a $H_2O_2$ Concentration-dependent Manner*—To determine the effects of treating CRP with $H_2O_2$ on the binding of CRP to immobilized protein ligands, we first used ox-LDL as the protein ligand. As shown (Fig. 2), native CRP did not bind to ox-LDL. CRP treated with any of the three concentrations of $H_2O_2$ bound to ox-LDL in a CRP concentration-dependent manner. For equivalent binding ($A_{405}$ = 1) of $H_2O_2$-treated CRP to ox-LDL, the required concentrations of CRP treated with 1, 2, and 4% $H_2O_2$ were 6, 3.5, and 2 μg/ml, respectively. Thus, the binding of $H_2O_2$-treated CRP to ox-LDL also occurred in a $H_2O_2$ concentration-dependent manner; the concentration of CRP required for equivalent binding decreased with increasing concentrations of $H_2O_2$. Also, the appearance of the mCRP peak in the elution profile from the gel filtration column (Fig. 1) was critical because it ensured that CRP was indeed modified upon treatment with $H_2O_2$. If we did not see some mCRP in any preparation of $H_2O_2$-treated CRP, then that particular pentameric $H_2O_2$-treated CRP did not bind to ox-LDL (data not shown). These data indicated that $H_2O_2$ changed the structure of CRP, the change occurred gradually in a $H_2O_2$ concentration-dependent manner and that CRP gained the ability to bind to immobilized ox-LDL after treatment with $H_2O_2$. Because $H_2O_2$-treated CRP was purified by gel filtration and did not contain any free $H_2O_2$, the data also indicated that the changes were permanent and not reversible. To show that the $H_2O_2$-induced binding of CRP to ox-LDL was specific to CRP and was not a nonspecific protein·protein interaction, we used BSA as a control. BSA after treatment with $H_2O_2$ did not bind to immobilized ox-LDL (data not shown).

For all subsequent experiments, we used 2% $H_2O_2$-treated CRP and called this preparation $H_2O_2$-CRP. As shown in Fig. 3, the mobilities of both purified pentameric native CRP and purified pentameric $H_2O_2$-CRP in both native and reducing SDS gels were similar, and mCRP was not present in any of the two CRP preparations.

*The PCh-binding Site in $H_2O_2$-CRP Is Intact*—We next determined whether treatment of CRP with $H_2O_2$ perturbed the PCh-binding site of CRP. As shown in Fig. 4*A*, an assay for the binding of native CRP and $H_2O_2$-CRP to PCh-BSA produced essentially overlapping curves. Similarly, assay for the binding of native CRP and $H_2O_2$-CRP to PnC also produced essentially overlapping curves (Fig. 4*B*). The PCh binding activities of native CRP and $H_2O_2$-CRP did not differ from each other. These data indicated that $H_2O_2$-mediated structural change in CRP did not involve the $Ca^{2+}$-binding and the PCh-binding sites of CRP.

*The $Ca^{2+}$-binding and PCh-binding Sites in $H_2O_2$-CRP Do Not Participate in $H_2O_2$-CRP·ox-LDL Interaction*—To determine the role of the PCh-binding site in $H_2O_2$-CRP and the requirement of $Ca^{2+}$ for the binding of $H_2O_2$-CRP to ox-LDL,

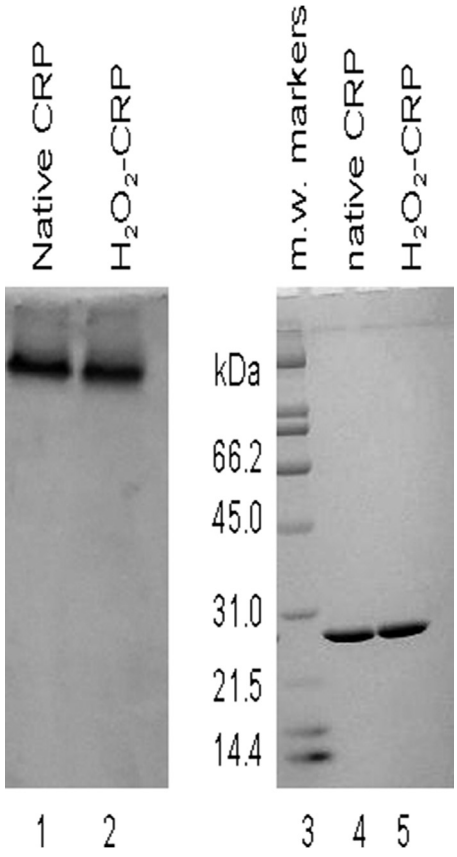


FIGURE 3. **Electrophoresis of native CRP and $H_2O_2$-CRP.** CRP (10 $\mu$g) was subjected to native PAGE in a 7.5% gel (*lanes 1* and *2*). CRP (10 $\mu$g) was subjected to SDS-PAGE under reducing conditions in a 4–20% gradient gel (*lanes 3–5*). A representative of two Coomassie Blue-stained gels is shown.

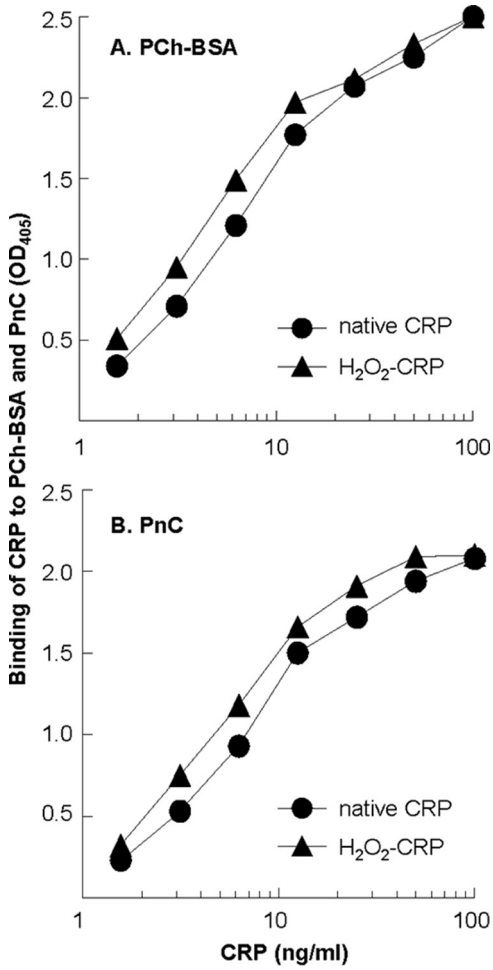


FIGURE 4. **Binding of $H_2O_2$-CRP to PCh.** Microtiter wells were coated with PCh-BSA (*A*) and PnC (*B*). The unreacted sites in the wells were blocked with gelatin. Native CRP and $H_2O_2$-CRP diluted in TBS-Ca were then added to the wells and incubated for overnight at 4 °C. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was developed, and the *A* value was read at 405 nm. A representative of three experiments is shown.

we performed two experiments. In the first experiment, we compared the ox-LDL binding activity of $H_2O_2$-CRP diluted in TBS-EDTA buffer with that of $H_2O_2$-CRP diluted in TBS-Ca buffer. As shown (Fig. 5*A*), the binding curves of $H_2O_2$-CRP·ox-LDL interactions in the presence of either $Ca^{2+}$ or EDTA were similar. Native CRP did not bind to ox-LDL. These results indicated that the binding of $H_2O_2$-CRP to ox-LDL was independent of the $Ca^{2+}$-binding site of CRP. In the second experiment, we determined the effects of PCh on the binding of $H_2O_2$-CRP to ox-LDL. As shown (Fig. 5*B*), in the presence of 10 mM PCh, $H_2O_2$-CRP bound to ox-LDL, as well as in the absence of PCh; PCh did not inhibit the interaction between $H_2O_2$-CRP and ox-LDL, indicating that there was no involvement of the PCh-binding site of CRP in $H_2O_2$-CRP·ox-LDL interaction.

*$H_2O_2$-CRP Binds to a Variety of Immobilized Proteins*—We next investigated the binding of $H_2O_2$-CRP to five more immobilized protein ligands, in addition to ox-LDL. We used IgG, amyloid $\beta$ peptide, C4b-binding protein (C4BP), factor H, and BSA. In the first protein ligand binding assay, we used different concentrations of protein ligands for immobilization on the microtiter wells and a fixed concentration of $H_2O_2$-CRP. As shown (Fig. 6*A*), 0.5 $\mu$g/ml of ox-LDL and IgG was required to coat the wells for maximal binding of $H_2O_2$-CRP. Amyloid $\beta$ peptide was required at 40 $\mu$g/ml to coat the wells for maximal binding of $H_2O_2$-CRP. A concentration of 10 $\mu$g/ml was required for C4BP and factor H to coat the wells for maximal binding of $H_2O_2$-CRP. $H_2O_2$-CRP did not bind to immobilized BSA even when BSA was used at 40 $\mu$g/ml to coat the wells. In the second protein ligand binding assay, we used fixed concentrations of protein ligands for immobilization on the microtiter wells and different concentrations of $H_2O_2$-CRP. As shown (Fig. 6*B*), $H_2O_2$-CRP bound to all ligands in a CRP concentration-dependent manner. These data indicated that $H_2O_2$-CRP had gained the ability to recognize immobilized and hence conformationally altered, denatured, and aggregated proteins.

## Discussion

The aim of this study was to determine whether $H_2O_2$ transforms CRP into a molecule whose ligand recognition functions are different from that of native CRP. Our major findings were: 1) $H_2O_2$-CRP, unlike native CRP, bound to a variety of protein ligands immobilized on microtiter plates in a $H_2O_2$ concentration-dependent and CRP concentration-dependent manner. 2) The PCh-binding site in CRP was not perturbed by treatment of CRP with $H_2O_2$. 3) The absence of calcium or the presence of PCh did not affect the ability of $H_2O_2$-CRP to bind to immobi-

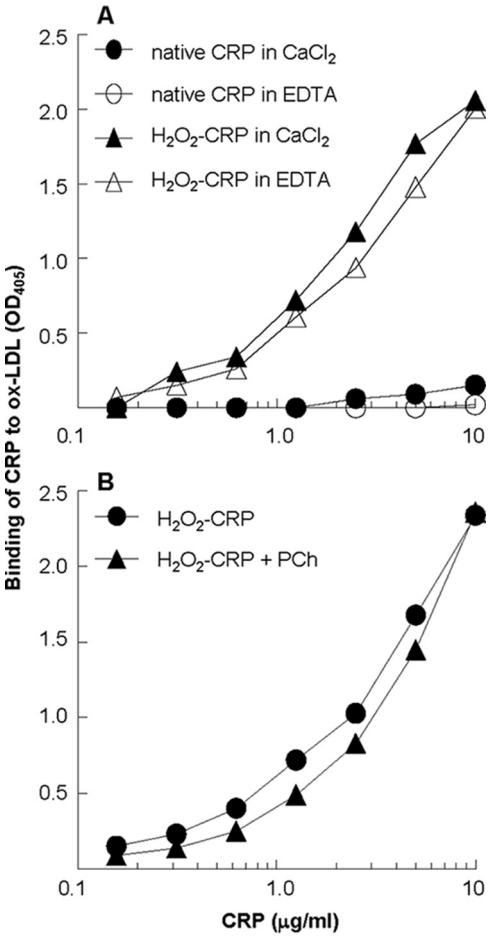


FIGURE 5. **Effects of $Ca^{2+}$ and PCh on the binding of $H_2O_2$-CRP to ox-LDL.** *A*, CRP diluted in TBS-Ca and TBS-EDTA was added to ox-LDL-coated wells and incubated overnight at 4 °C. Bound CRP was detected by using rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the *A* value was read at 405 nm. A representative of three experiments is shown. *B*, $H_2O_2$-CRP at increasing concentrations, diluted in TBS-Ca in the presence and absence of 10 mM PCh was added to ox-LDL-coated wells and incubated overnight at 4 °C. Bound CRP was detected by using rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the *A* value was read at 405 nm. A representative of three experiments is shown.

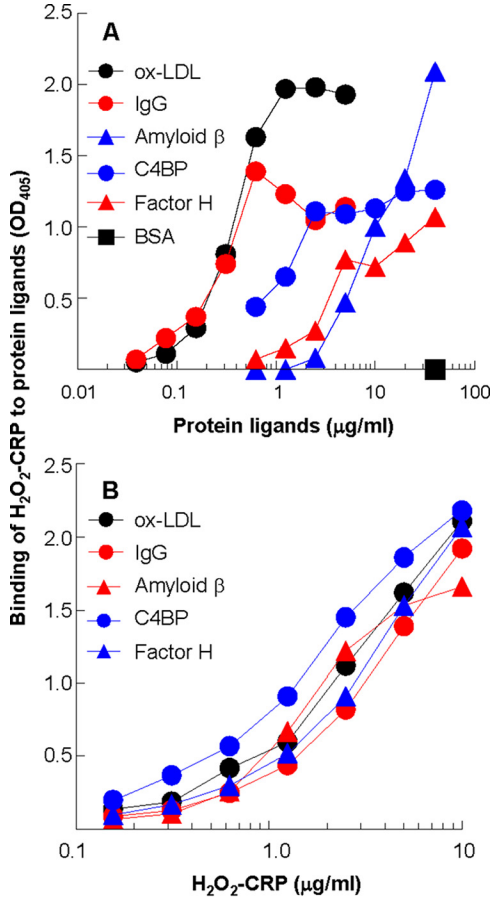


FIGURE 6. **Binding of $H_2O_2$-CRP to six different immobilized proteins.** *A*, microtiter wells were coated with increasing concentrations of ox-LDL, IgG, amyloid β peptide 1–42, C4BP, factor H, and BSA. $H_2O_2$-CRP (10 μg/ml) diluted in TBS-Ca was then added to the wells and incubated overnight at 4 °C. Bound CRP was detected by using rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the *A* value was read at 405 nm. A representative of three experiments is shown. *B*, microtiter wells were coated with 10 μg/ml of ox-LDL, IgG, C4BP, and factor H and with 40 μg/ml of amyloid β peptide 1–42. $H_2O_2$-CRP at increasing concentrations diluted in TBS-Ca was then added to the wells and incubated overnight at 4 °C. The wells were developed as in *A*. A representative of three experiments is shown.

lized protein ligands. Changes in the redox environment have been shown previously to modify the functions of CRP: ROS-mediated modification of CRP has been shown to regulate the stimulus-dependent activation of platelets and reduction of the single intrasubunit disulfide bond in CRP has been shown to induce pro-inflammatory functions of mCRP (34, 35). In our study, for short term treatment of CRP, we used a concentration of $H_2O_2$, which was higher than its physiological concentration. However, in chronically inflamed individuals, it is possible that prolonged exposure of CRP to lower concentration of $H_2O_2$ results in similar changes in CRP. Collectively, these findings provide a proof of concept that ROS are modifiers of the structure and function of CRP.

In addition to $H_2O_2$-CRP, there are other modified forms of CRP including mCRP, all derived from native CRP, whose ligand recognition functions are similar to each other but different from that of native CRP. At acidic pH, CRP binds to any protein that is aggregated and conformationally altered by immobilization, including the extracellular matrix protein fibronectin (11, 12, 36–39). Acidic pH also affects the ability of CRP to activate the complement system (40). Mutagenesis of Glu and Pro in CRP at positions 42 and 115, respectively, also confers additional ligand binding property to CRP (11, 14). When native CRP, or any protein for that matter, is immobilized directly to a surface, the immobilized protein is no longer native and is aggregated on the surface. Aggregated CRP has also been shown to exhibit ligand binding properties overlapping with that of $H_2O_2$-CRP (38, 41–46). Aggregation of CRP on a PCh-coated surface also changes the ligand-binding function of CRP; it binds C1q (41, 47). Trimers of CRP pentamers have also been reported to bind C1q (48). Under certain conditions, CRP forms stacks of two pentamers, and CRP decamers have altered ligand binding properties (49, 50). Possible oligomers of CRP pentamers have also been found *in vivo* in circulation (51). Commercially available CRP has been shown to bind to misfolded proteins (52), which could be due to the presence of mCRP in commercial preparations, as has been pointed out earlier (53, 54). Denatured CRP binds to factor H (55).

mCRP is most highly reactive, binds to a broad range of proteins, and exhibits highest avidity for interacting with other immobilized proteins that have been tested so far (53, 56–61).

We propose that monomerization of CRP is neither necessary nor a prerequisite for CRP to bind to immobilized, denatured, aggregated, and misfolded proteins. A subtle change in CRP generating a conformation that is an intermediate between native CRP and mCRP is sufficient for CRP to execute its functions. Generation of an intermediate form of CRP has been reported earlier (62). As we have discussed previously (54), mCRP is likely to be a by-product of the functions of pentameric CRP when it is in its non-native conformation and that such mCRP must be deposited, localized, and unable to move around. The presence of mCRP at inflammatory lesions has been reported (62–66); however, it is not known how mCRP is cleared from the sites where it is generated.

The determinant in immobilized protein ligands which is recognized by CRP in its non-native conformation is not known. It is possible that the protein ligands after immobilization form and expose an amyloid-like structure that is recognized by CRP in its non-native conformation. Similarly, the binding site in CRP in its non-native conformation, including in mCRP, that recognizes immobilized protein ligands is not known. It is possible that the cholesterol-binding sequence in CRP, forming an intrinsically disordered motif, is responsible for diverse actions of CRP in its non-native states (67).

CRP is not the only protein in the innate immune system that modifies its own structure and gains novel ligand binding properties in response to the conditions seen at sites of inflammation. At acidic pH, serum amyloid P component and PTX3, the two other proteins of the pentraxin family, also bind to immobilized proteins and exhibit functions different from that under normal physiological conditions (68, 69). IgG also binds to immobilized proteins at acidic pH (70). An inflammatory disease-related, redox-dependent, conformational isoform of macrophage migration inhibitory factor has also been reported (71). L-Ficolin, which binds to PCh groups like CRP does, also changes its own structure in inflammatory conditions (72, 73). Thus, conformational changes are necessary not just for the functions of CRP but also for the functions of a few other molecules involved in immunity and host defense.

*In vivo* experiments using native CRP in animal models of inflammatory diseases suggest that CRP could be a molecule with anti-pneumococcal, anti-atherosclerotic, anti-arthritic, and anti-amyloidogenic functions. CRP has been shown to protect mice against pneumococcal infection via both PCh-dependent and PCh-independent mechanisms (74, 75). CRP slows atherosclerosis development in a mouse model with human-like hypercholesterolemia (76). mCRP has also been found to be atheroprotective *in vivo* (77). For the limited atheroprotective activity of CRP *in vivo*, it has been suggested that the mouse model used to investigate functions of human CRP was inappropriate (54, 78). *Ex vivo* experiments on atherosclerosis also suggest anti-atherosclerotic functions of CRP. It has been shown that CRP binds to enzymatically modified LDL and, as a consequence, prevents the formation of macrophage foam cells and limits complement activation (8, 20). The pro-atherogenic effects of macrophages were found to be reduced upon formation of a complex between CRP and lysophosphatidylcholine (79). CRP also affects the physicochemical properties of LDL and inhibits further oxidation of ox-LDL (80, 81). In addition, mCRP has been found to decrease the uptake of acetylated LDL by endothelial cells (82). CRP has also been shown to inhibit the development of antigen-induced arthritis and collagen-induced arthritis in mice (83, 84). Recently, it has been reported that CRP exhibits antiamyloidogenic functions (85). The significance of the recognition function of CRP in its alternate structural pentameric conformation in host defense is unknown. We propose that, as has been suggested earlier (13), the ligand recognition function of CRP in its native and non-native pentameric structural conformations play a role in its host defense functions, including in ROS-rich inflammatory conditions.

We conclude that $H_2O_2$ is a biological modifier of the structure and ligand recognition functions of CRP. We hypothesize that one of the functions of CRP at sites of inflammation is to first sense the inflammatory microenvironment, change its own structure in response but remain pentameric, and then bind to pathogenic proteins deposited at those sites. The consequences of the binding of modified CRP to pathogenic proteins remain undefined.

## Experimental Procedures

*Preparation and Purification of Pentameric $H_2O_2$-treated CRP*—Native CRP was purified from discarded human pleural fluid by $Ca^{2+}$-dependent affinity chromatography on a PCh-conjugated Sepharose column, followed by anion exchange chromatography on a Mono Q column and gel filtration on a Superose 12 column, as described previously (14). Purified CRP was stored in TBS, pH 7.2, containing 2 mM $CaCl_2$ at 4 °C and was used within a week.

$H_2O_2$ (30% stock) was purchased from Fisher Scientific. To prepare $H_2O_2$-treated pentameric CRP, equal volumes of CRP (200 $\mu$g/ml) and $H_2O_2$ (2%, 4% or 8%) were mixed to prepare CRP (100 $\mu$g/ml) treated with 1, 2, and 4% $H_2O_2$. The mixtures were incubated at 37 °C for 2 h and then subjected to gel filtration to isolate pentameric $H_2O_2$-treated CRP. Gel filtration chromatography of $H_2O_2$-treated CRP mixtures was carried out on a Superose 12 column (GE Healthcare) using the Biologic Duo Flow protein purification system (Bio-Rad). The column was equilibrated and eluted with TBS containing 2 mM $CaCl_2$. Fractions were collected, and absorbance at 280 nm was measured to locate the elution volume of pentameric CRP from the column. SDS-PAGE under reducing conditions and native PAGE of purified CRP preparations were performed according to standard procedures.

*PCh Binding Assay*—Binding activity of native CRP and $H_2O_2$-treated CRP for PCh was measured by using PCh-conjugated BSA and PnC (Statens Serum Institut) as ligands, as described previously (14). Briefly, microtiter wells were coated with 10 $\mu$g/ml of PCh-BSA or PnC. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin (Sigma-Aldrich). After blocking, CRP diluted in TBS containing 2 mM $CaCl_2$, 0.1% gelatin, and 0.02% Tween 20 (TBS-Ca) was added to the wells and incubated overnight at 4 °C. After washing the wells with TBS-Ca, the anti-CRP mAb HD2.4, diluted in TBS-Ca, was used to detect bound CRP. HRP-conjugated goat anti-

mouse IgG, diluted in TBS-Ca, was used as the secondary antibody. Color was developed, and the *A* value was read at 405 nm.

*Protein Ligand Binding Assay*—The protein ligand binding assay was used to determine the binding of pentameric $H_2O_2$-treated CRP to a variety of immobilized proteins. Factor H (Complement Technology), C4BP (Athens Research and Technology), IgG (Sigma-Aldrich), BSA (Sigma-Aldrich), amyloid $\beta$ peptide 1–42 (Bachem), and ox-LDL prepared as described previously (14) were used as protein ligands. Microtiter wells were coated with protein ligands diluted in TBS (100 $\mu$l/well) and incubated overnight at 4 °C. The concentrations of protein ligands used for coating are mentioned in the figure legends. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. Freshly purified native and $H_2O_2$-treated CRP were diluted in TBS-Ca, added to the wells, and incubated overnight at 4 °C. In some assays, CRP was diluted in TBS containing 5 mM EDTA, 0.1% gelatin, and 0.02% Tween 20 (TBS-EDTA), added to the wells, and incubated overnight at 4 °C. The wells were then washed with the respective buffers, TBS-Ca or TBS-EDTA. Immunoaffinity-purified polyclonal rabbit anti-human CRP antibody (1 $\mu$g/ml), diluted in TBS-Ca, was used (1 h at 37 °C) to detect bound CRP, as described previously (14). HRP-conjugated donkey anti-rabbit IgG (GE Healthcare) diluted in TBS-Ca was used (1 h at 37 °C) as secondary antibody. Color was developed, and the *A* value was read at 405 nm. To determine the effects of PCh (Sigma-Aldrich) on $H_2O_2$-CRP·protein ligand interaction, ox-LDL was used as a representative protein ligand. The assay was performed as described above, except that $H_2O_2$-treated CRP diluted in TBS-Ca was added to ox-LDL-coated wells in the presence and absence of 10 mM PCh and incubated overnight at 4 °C. Bound CRP was detected as described above.

*Author Contributions*—S. K. S., A. T., A. P., and D. N. N. conducted the experiments. S. K. S. conducted most of the experiments. A. A. conceived the idea of the project. A. T. and A. A. wrote the paper. A. A., A. T., and S. K. S. analyzed and interpreted all of the data. All authors reviewed the results and approved the final version of the manuscript.

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