Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis


From the Departments of Pharmacology and Department of Biochemistry and Molecular Biology, Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614, the Department of Biochemistry, Center for Biomedical Research, University of Texas Health Science Center, Tyler, Texas, 75708, and Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

Running Title: Site-directed mutagenesis of CRP

To whom correspondence should be addressed: Alok Agrawal, Department of Pharmacology, Quillen College of Medicine, P.O. Box 70577, East Tennessee State University, Johnson City, TN 37614, USA. Tel.: 423-439-6336; Fax: 423-439-8773; E-mail: agrawal@etsu.edu

Background: The functions of C-reactive protein (CRP) in the extracellular matrix at the sites of inflammation are unknown.

Results: Mutation of just one amino acid in CRP (E42Q) expands its ligand-binding capacity.

Conclusion: Some functions of CRP depend upon its inflammation-induced structural configuration.

Significance: Investigations using E42Q-CRP in animal models of inflammatory diseases may establish its therapeutic potential, CRP’s functions and a CRP-targeting strategy.

SUMMARY

C-reactive protein (CRP) is a cyclic pentameric protein whose major binding specificity, at physiological pH, is for substances bearing exposed phosphocholine moieties. Another pentameric form of CRP, which exists at acidic pH, displays binding activity for oxidized LDL (ox-LDL). The ox-LDL-binding site in CRP, which is hidden at physiological pH, is exposed by acidic pH-induced structural changes in pentameric CRP. The aim of this study was to expose the hidden ox-LDL-binding site of CRP by site-directed mutagenesis and to generate a CRP mutant that can bind to ox-LDL without the requirement of acidic pH. Mutation of Glu42, an amino acid which participates in intersubunit interactions in the CRP pentamer and is buried, to Gln resulted in a CRP mutant (E42Q) which showed significant binding activity for ox-LDL at physiological pH. For maximal binding to ox-LDL, E42Q CRP required a pH much less acidic than that required by wild-type CRP. At any given pH, E42Q CRP was more efficient than wild-type CRP in binding to ox-LDL. Like wild-type CRP, E42Q CRP remained pentameric at acidic pH. Also, E42Q CRP was more efficient than wild-type CRP in binding to several other deposited, conformationally-altered proteins. The E42Q CRP mutant provides a tool to investigate the functions of CRP in defined animal models of inflammatory diseases including atherosclerosis because wild-type CRP requires acidic pH to bind to deposited, conformationally-altered proteins including ox-LDL, and available animal models may not have sufficient acidosis or other possible modifiers of CRP’s pentameric structure at the sites of inflammation.
CRP is a plasma protein which is also found in the extracellular matrix at the sites of inflammation, such as atherosclerotic lesions (7-10, and reviewed in Ref. 11). Atherosclerosis is an inflammatory disease (12), and generation of an acidic extracellular milieu is a hallmark of inflammatory processes; it has been suggested that at the sites of inflammation, the pH may become acidic due to activated macrophages, hypoxia, lactate generation and proton generation (13-20, reviewed in Ref. 21). Naghavi and co-workers measured the pH of both human and rabbit atherosclerotic plaques, and found the plaques to contain areas in which the pH was decreased to 5.5 (20). The functions of CRP in the development of atherosclerosis are not known.

The binding of CRP to two atherogenic forms of LDL, oxidized LDL (ox-LDL) and enzymatically-modified LDL (E-LDL), has been investigated previously (8, 9, 22-26). We found that CRP did not bind to ox-LDL at physiological pH, but gained the ability to bind to ox-LDL at acidic pH (6). Similarly, CRP bound to E-LDL at physiological pH, but the binding was dramatically enhanced at acidic pH (5). In addition, using E-LDL, we showed that CRP-bound E-LDL did not cause formation of macrophage foam cells (27). These in vitro data suggested that CRP should be able to bind to atherogenic LDL in vivo due to acidosis at the sites of inflammation and prevent foam cell formation.

However, CRP is neither proatherogenic nor atheroprotective in murine and rabbit models of atherosclerosis (28-35). The reason for the unresponsiveness of human CRP in animal models of atherosclerosis is not clear. Because atherogenic LDL can be efficiently bound by CRP only when CRP is present in its acidic pH-modified structural form, and because the animal models of atherosclerosis may lack an inflammatory environment (12, 36), a CRP mutant which can bind to atherogenic LDL at physiological pH is needed to investigate the possible atheroprotective role of CRP using available animal models.

The aim of this study was to generate a CRP mutant that can bind to ox-LDL without the requirement of acidic pH. Because the ox-LDL-binding site in pentameric CRP is formed at acidic pH, our choice of amino acids in CRP for mutagenesis was based on the hypothesis that acidic pH loosens the CRP pentamer to expose amino acids that are otherwise hidden at physiological pH and it may be possible to mimic the effect of acidic pH on CRP by mutating certain amino acids. Accordingly, we focused on the amino acids participating in the intersubunit interactions in the CRP pentamer. The following amino acid pairs have been implicated in intersubunit interactions (37-39): Val^{10-}Ile^{104}, that is, Val^{10} of one subunit interacts with Ile^{104} of adjacent subunit; Pro^{12-}Ser^{118}; Tyr^{40-}Pro^{115}; Tyr^{40-}Val^{117}; Glu^{42-}Glu^{35}; Ser^{46-}Glu^{35}; Glu^{42-}Lys^{119}; Ser^{44-}Lys^{69}; Val^{90-}Pro^{87}; Gly^{101-}Lys^{201}; Ser^{120-}Tyr^{197}; Pro^{115-}Trp^{205}, and Arg^{118-}Asp^{155}. In this study, we mutated two amino acids, Glu^{42} (to Gln) and Pro^{115} (to Ala), from the intersubunit contact region, and one amino acid, Tyr^{175} (to Ala), which is not a part of the intersubunit contact region (Fig. 1), and evaluated the effect of these mutations on the binding of CRP to several immobilized proteins including ox-LDL.

**EXPERIMENTAL PROCEDURES**

*Construction and expression of CRP mutants* - The construction of the CRP mutants E42Q (substitution of Glu^{42} with Gln), P115A (substitution of Pro^{115} with Ala), and Y175A (substitution of Tyr^{175} with Ala) has been reported previously (6). All three mutants were stably expressed in CHO cells, as described previously (41). CHO cell lines expressing each CRP mutant were isolated by a series of subcloning steps.

*Purification of CRP* - Native WT CRP was purified from discarded human pleural fluid, as described previously (6), and stored frozen. Purification of CRP mutants from the CHO cell culture supernatants involved two steps: a Ca^{2+}-dependent affinity chromatography on a PCh-Sepharose column (Pierce) followed by gel filtration on a Superose12 column (GE Healthcare), as described previously (6), and stored frozen. On the day of the experiments, CRP was re-purified by gel filtration on a Superose12 column to remove any form of modified CRP which might have been generated due to storage of CRP. Re-purified CRP was stored in 10 mM TBS, pH 7.2, containing 2 mM CaCl$_2$ at 4 °C and was used within a week.
Preparation of ox-LDL - Native LDL was isolated from human plasma by sequential ultracentrifugation (1.019<d> 1.063 g/ml), as described previously (42). Ox-LDL was prepared by treating LDL with 20 µM CuCl<sub>2</sub> in PBS for 12 h at 37 °C (43). The Cu<sup>2+</sup>-mediated oxidation was terminated by adding EDTA to the reaction at a final concentration of 0.5 mM. Following dialysis against PBS, ox-LDL was passed through chelex-100 resin (Bio-Rad) to remove any traces of Cu<sup>2+</sup>, filter-sterilized, and stored in the dark at 4 °C. The degree of oxidation of LDL was evaluated by agarose gel electrophoresis: ox-LDL had 3.2-3.5-fold higher R<sub>F</sub> values than native LDL. The degree of oxidation was also determined by TBARS assay (Cayman Chemical Company), a colorimetric assay using malondialdehyde as a standard. Ox-LDL used in this study resulted in 30.5 nmoles malondialdehyde/mg of protein. Protein concentrations of the ox-LDL preparations were measured using the microBCA protein assay kit (Pierce).

Ox-LDL-binding assay - Microtiter wells were coated with 10 µg/ml of ox-LDL diluted in TBS (350 µl/well), overnight at 4 °C. Purified CRP (WT and mutants), diluted in TBS, pH 7.2, containing 0.1% gelatin, 0.02% Tween-20 and 2 mM CaCl<sub>2</sub> (TBS-Ca), was added in duplicate wells (100 µl/well), and incubated for 2 h at 37 °C, unless otherwise mentioned in the figure legends. After the CRP incubation step, the wells were washed with TBS-Ca. Rabbit anti-CRP antibody (Sigma), diluted 1/1000 in TBS-Ca, was used (100 µl/well; 1 h at 37 °C) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (GE Healthcare), diluted in TBS-Ca, was used (100 µl/well; 1 h at 37 °C) as the secondary antibody. Color was developed and the absorbance was read at 405 nm in a microtiter plate reader.

PnC-binding assay - Binding activity of CRP for PnC was evaluated by using PnC (Statens Serum Institut) as the ligand. Microtiter wells were coated (350 µl/well) with 10 µg/ml of PnC in TBS, overnight at 4 °C. CRP diluted in TBS-Ca, was used (100 µl/well; 1 h at 37 °C) to detect bound CRP. HRP-conjugated goat anti-mouse IgG, diluted in TBS-Ca, were used (100 µl/well; 1 h at 37 °C) as the secondary antibody. Color was developed and the absorbance was read at 405 nm in a microtiter plate reader.

CD spectroscopy - The CD spectra of CRP (100 µg/ml) were recorded at 25 °C using JASCO J-815 CD spectrometer equipped with a Peltier type temperature control system (JASCO model PTC-423S/15) and interfaced to a personal computer. The instrument was calibrated with (1S)-(+) 10-camphorsulfonic acid. The CD spectra were measured from 200 nm to 250 nm every 0.5 nm with 4 s averaging per point and a 2 nm bandwidth. A 0.1 cm path length cell was used for obtaining the spectra. The CD spectra were signal averaged by adding four scans and baseline corrected.

Gel filtration - Gel filtration analysis of CRP at pH 5.6 was carried out on a Superose12 column. The column was equilibrated and eluted with TBS,
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pH 5.6, containing 2 mM CaCl₂ at a flow rate of 0.3 ml/min. Fractions (60 fractions, 0.25 ml each) were collected and absorbance at 280 nm measured to locate the elution volume of CRP from the column.

1-anilinonaphthalene-8-sulfonic acid (ANS)-binding fluorescence assay - The hydrophobic fluorescent probe ANS was purchased from AnaSpec, Inc. The ANS-binding fluorescence assays were performed as described previously (6) to investigate the structural changes in CRP at pH 5.6. CRP (50 µg/ml) in TBS containing 2 mM CaCl₂, at various pH, was mixed with ANS at a final concentration of 100 µM. The fluorescence intensity of the binding of ANS to CRP was measured by using the excitation and emission wavelengths of 390 nm and 460 nm respectively, in a spectrofluorometer (Fluostar Galaxy, BMG lab technologies).

RESULTS

E42Q CRP binds to ox-LDL more efficiently than WT CRP - The construction, expression, purification and some characterization of E42Q, P115A and Y175A CRP mutants have been reported previously and their overall structure was found not to be different from WT native and WT recombinant CRP (6, 40, 41). The elution profile of each CRP mutant from the gel filtration column was identical to that of WT CRP, suggesting that all CRP mutants were pentameric (Supplemental Fig. 1A). The purity of CRP preparations was confirmed by denaturing SDS-PAGE (Supplemental Fig. 1B). The PCh-binding activity of all CRP mutants was also identical to that of WT CRP (Supplemental Fig. 1C, 1D).

The binding of CRP mutants to ox-LDL was first determined at pH 7.0, a pH at which WT CRP does not bind to ox-LDL (6). To show maximum possible binding of CRP to ox-LDL that can be measured by this assay, pH 5.0 was used, a pH at which WT CRP binds efficiently to ox-LDL (6). At pH 7.0, the binding of E42Q CRP to ox-LDL was significantly higher than the negligible binding of either WT or P115A and Y175A CRP mutants to ox-LDL (Fig. 2A). At pH 7.0, the binding of E42Q CRP to ox-LDL was significantly higher than the negligible binding of either WT or P115A and Y175A CRP mutants to ox-LDL (Fig. 2A). The minimal binding of WT and mutants P115A and Y175A to ox-LDL seen at pH 7.0 may be due to the exposure of a few PCh groups in some ox-LDL molecules (22). As expected, at pH 5.0, the binding of the CRP mutants to ox-LDL was not different from that of WT CRP. These data indicated that E42Q CRP gained the ability to bind to ox-LDL at physiological pH; however, further structural change was required for maximal binding. Therefore, the binding of each CRP species to ox-LDL was evaluated from pH 7.0 down to pH 5.0 where maximal binding for all species occurred (Fig. 2B). The binding of mutants P115A and Y175A to ox-LDL, as a function of pH, was not different from that of WT CRP. However, the binding of E42Q CRP to ox-LDL was enhanced between pH 6.5 and 5.5, as demonstrated by the shift in the E42Q mutant’s binding curve relative to the other forms. Consequently, pH 5.6 was chosen for performing a CRP dose-response assay to compare the efficiency of binding of various CRP species to ox-LDL (Fig. 2C). The resulting curves indicate that E42Q CRP was approximately four-fold more efficient than WT CRP in binding to ox-LDL. These data indicate that E42Q CRP is more potent than WT CRP: E42Q CRP binds to ox-LDL at physiological pH (Fig. 2A), E42Q CRP requires less acidic pH for maximal binding to ox-LDL (Fig. 2B), and more E42Q CRP binds to ox-LDL at any pH (Fig. 2C).

E42Q CRP is neither monomerized nor aggregated at any pH - Gel filtration, ox-LDL-binding reversibility assays, ANS-binding fluorescence assays and CD spectra were used to investigate the possibility that the mutation and acidic pH might have caused monomerization and/or aggregation of CRP and that the observed binding of CRP to ox-LDL might be due to monomerized and aggregated forms of CRP.

Gel filtration was used to determine whether E42Q CRP remained pentameric at pH 5.6 after incubation for 2 h at 37 ºC, the conditions used in the ox-LDL-binding assays. The elution profiles of E42Q CRP at pH 7.2 and 5.6 were identical (Fig. 3A). These data indicated that E42Q CRP remained pentameric at pH 5.6; it was neither monomerized nor aggregated.

Next, the effect of pH neutralization on the ox-LDL-binding activity of CRP was evaluated (Fig. 3B). Only E42Q CRP showed significant binding to ox-LDL at pH 7.0. At pH 5.6, each CRP species efficiently bound to ox-LDL. When a CRP solution, which had been incubated at pH 5.6 for 2 h at 37 ºC, was neutralized, and the binding assay...
was performed at pH 7.0, no CRP bound to ox-LDL. Because it is known that monomerized CRP binds to ox-LDL at pH 7.0 (25) and monomers of CRP cannot form pentamers in vitro, these data suggested that CRP was not monomerized at acidic pH and that any structural change in CRP at pH 5.6 was reversible at pH 7.0.

ANS-binding fluorescence assays were used to evaluate hydrophobic changes in the structure of CRP at pH 5.6 (Fig. 3C). Incubation of ANS with all forms for CRP at pH 7.0 resulted in negligible increase in fluorescence. In contrast, ANS binding to CRP at pH 5.6 resulted in significantly increased fluorescence as compared to that at pH 7.0. Neutralization of the pH 5.6 CRP solutions, which had been incubated for 2 h at 37°C to pH 7.0, followed by the addition of ANS resulted in no increase in fluorescence consistent with no binding of ANS. These data suggested that the hydrophobicity of CRP was increased at acidic pH, but that the change was reversible. The reversibility of the structural changes in the various forms of CRP indicated that they were neither monomeric nor aggregated at acidic pH.

Finally, we used CD spectroscopy to determine global changes in the secondary structure of CRP due to mutation of Glu42 and acidic pH (Fig. 3D). The comparison of the far UV CD spectra of WT and E42Q CRP at pH 5.0, 5.6 and 7.2 showed minimal changes in the secondary structures of both CRP due to acidic pH, and the changes in the secondary structure of E42Q was not different from the changes in WT CRP. These data indicated that the overall secondary structure of WT CRP was maintained in E42Q CRP at any pH.

Combined data suggested that, at acidic pH, CRP was not monomerized, CRP was not completely denatured, CRP was not aggregated, the pentameric structure of CRP was modified although there were no global changes in the secondary structure, and the modifications were reversible.

Acidic pH causes localized structural changes in CRP - Because E42Q CRP required a buffer less acidic than that required by WT CRP for comparable binding to ox-LDL, and because there were no global changes at acidic pH in any CRP, we hypothesized that the structural changes in CRP in response to acidic pH and mutations were only subtle. Localized conformational changes in CRP were investigated by determining the effects of acidic pH on the Ca2+-binding site of CRP by measuring the Ca2+-requirement of CRP to bind to one of its Ca2+-dependent ligands, PnC. A change in the Ca2+-requirement for comparable PnC-binding activity at different pH would reflect a change in the Ca2+-affinity of CRP. At pH 7.2, WT CRP bound efficiently, even at 0.06 mM Ca2+, to PnC (Fig. 4A). However, at pH 5.0, 2 mM Ca2+ was required for efficient binding of CRP to PnC. The increase in Ca2+-requirement was directly related to the decrease in pH. These results suggested that acidic pH modified the Ca2+-binding site of CRP, reduced the Ca2+-affinity of CRP, and that 2 mM Ca2+ should be used to perform ligand-binding assays at acidic pH. As shown in Fig. 4B, all CRP species were similar in binding to PnC at any pH in 2 mM Ca2+, suggesting that the mutation of CRP did not reduce Ca2+-affinity of CRP.

Next, we evaluated the structural change in CRP caused by the substitution of Glu42 to Gln by using molecular modeling (Fig. 4C). In WT CRP, there is an ionic bond between Glu42 and Lys119 at a distance of 3.787 Å. In E42Q CRP, this ionic bond is lost. Instead, there is the possibility of a weak H-bond between Gln42 and Lys119. The shortest distance between the H on the Lys119 ε-amino group and the O on the side chain of Gln42 is 2.821 Å, whereas H-bonds are normally about 2.0 Å. The modeling of WT and E42Q CRP further suggested that there might be a localized structural change in CRP caused by the substitution of Glu42 to Gln.

Re-examination of our previously reported findings (6) - Recently we reported that, at pH 4.6 and in 0.1 mM Ca2+, CRP did not bind to PnC, a temperature of 37°C was required for the binding of CRP to ox-LDL, and the P115A CRP mutant was more efficient than WT CRP in binding to ox-LDL (6). Our current finding (Fig. 4A) that the affinity of CRP for Ca2+ was drastically reduced at pH 5.0 prompted us to re-examine our previously reported findings.

As shown in Fig. 5, at pH 4.6, CRP did not bind to PnC even in 2 mM Ca2+. Just for this reason we did not include pH 4.6 in the current investigation. As shown in Supplemental Fig. 2, at acidic pH and in 2 mM Ca2+, 37°C was not necessary for the binding of E42Q CRP to ox-LDL, suggesting that the efficiency of binding of
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CRP to ox-LDL depends upon all three factors: pH, temperature and the extent of the structural change in CRP. As shown in Fig. 6, P115A CRP bound to ox-LDL more efficiently than WT CRP in 0.1 mM Ca^{2+} but not in 2 mM Ca^{2+}, while E42Q CRP bound to ox-LDL more efficiently than WT CRP at 2 mM Ca^{2+} also. Thus, a decrease in the concentration of Ca^{2+} is not necessary for E42Q CRP to bind to ox-LDL.

E42Q CRP has higher avidity than WT CRP for binding to a variety of other immobilized proteins - Previously, we reported that the binding of CRP to ox-LDL at acidic pH was not due to its specificity for binding to ox-LDL but due to its specificity for binding to immobilized, modified and conformationally altered proteins, irrespective of the identity of the protein (5, 6). Therefore, we investigated the binding activity of E42Q CRP at acidic pH for a few immobilized proteins other than ox-LDL. Binding curves of WT CRP and E42Q CRP to the immobilized proteins, factor H, amyloid β, BSA and gelatin, over a pH range from 7.0 down to 5.0, are shown in Fig. 7. In every case more E42Q CRP was bound to the tested proteins and this binding required less acidic pH compared to that required by WT CRP. Interestingly, E42Q CRP also bound to gelatin at acidic pH; however, the presence of 0.1% gelatin in the binding buffer used in the assays did not inhibit the binding of E42Q CRP to immobilized gelatin (data not shown). These results indicated that the substitution of Glu^{42} to Gln exposed a hidden ligand-binding site in CRP for deposited and conformationally altered proteins present in an acidic pH environment. These findings also suggested that the protein component of ox-LDL was the CRP ligand.

DISCUSSION

The goal of this investigation was to use site-directed mutagenesis to generate a CRP mutant capable of binding to ox-LDL without the requirement of acidic pH. Our major findings were: 1. Mutation of Glu^{42}, an amino acid which participates in intersubunit interactions in the CRP pentamer, to Gln resulted in a CRP mutant (E42Q) which showed significant binding to ox-LDL at physiological pH. For maximal binding to ox-LDL, the E42Q CRP required a pH less acidic than that required by WT CRP. Also, at any given pH, the binding of E42Q CRP to ox-LDL was more efficient than the binding of WT CRP to ox-LDL. 2. The acidic pH did not measurably change the secondary structures of WT or E42Q CRP and and did not monomerize or aggregate CRP. However, the acidic pH changed the hydrophobicity of CRP and reduced affinity for Ca^{2+}. These acidic pH induced changes were reversible at physiological pH. 3. E42Q CRP had higher avidity than WT CRP for binding to not only ox-LDL but also to other immobilized, and therefore conformationally-altered, proteins when both CRP and immobilized proteins were exposed to acidic pH.

Our data suggest that CRP undergoes a pH-dependent reversible transition between two conformational forms without a significant disruption of its secondary structure, providing new insight into the functions of CRP in inflammatory diseases. The data also suggest that the binding of CRP to ox-LDL at acidic pH is due to the acidic pH-dependent changes in CRP, perhaps due to the loosening of the CRP pentamer. In CRP, the side chain of Glu^{42} in one subunit ionically interacts with the main chain of Glu^{85} (the distance between the Glu^{42} and the α amino of Glu^{85} is 6.242 Å away, which is beyond a salt bridge distance of usually only 4 Å) and with the side chain of Lys^{119} of adjacent subunit. The increased binding of E42Q CRP at pH values closer to physiological conditions are consistent with the loss of an ionic interaction between Glu^{42} and Lys^{119}, and suggestive of a loosening of the pentameric structure. Partial exposure of the hidden ox-LDL-binding site of CRP by mutagenesis raises the possibility that conditions other than acidic pH may also be able to switch the structure of CRP to a more active configuration.

Acidic pH is not the only characteristic of inflammatory sites. Free radicals and other oxidants may also be present at the sites of inflammation in arteries and may oxidize CRP in addition to oxidizing LDL to facilitate the binding of CRP to ox-LDL. Although acidic pH modifies the structure of CRP (6, 48), and is just one possible in vivo modifier of CRP’s structure, other modifiers of the structure of CRP have also been reported (49, 50). CRP modified with active oxygen species has been shown to modulate stimulus-dependent activation of platelets (49). A redox switch in CRP, involving the reduction of its
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single disulfide bond, has been shown to modulate activation of endothelial cells by CRP (50). Also, CRP is not the only host defense protein which is activated by a conformational change; other host defense proteins of the immune system, for example, β-defensin 1 and NPR1, have been recently shown to be activated by conformational changes in response to redox conditions (51, 52) and nitrosylation (52). We are currently investigating the effects of several other inflammation-related protein modifiers, including redox conditions and nitrosylation, on the binding of CRP to deposited, conformationally-altered and amyloidogenic proteins.

In animal models of atherosclerosis, WT CRP is neither atheroprotective nor proatherosclerotic (28-35). We hypothesize that the lack of an effect of CRP in animal models is due to the absence of an inflammatory environment that is needed for appropriately altering the structure of CRP so it can capture atherogenic LDL. Indeed, atherosclerosis is not naturally developed in these animal models and in humans it is developed over a period of several years or decades (12, 36). The E42Q CRP might be useful in investigating the functions of CRP in defined animal models of atherosclerosis. E42Q CRP may provide a better molecule for testing the hypothesis that CRP requires a structural change to bind to ox-LDL in vivo. Our finding that the binding of E42Q CRP to ox-LDL was dramatically increased at physiological pH when the concentration of Ca^{2+} was 20-fold lower than normal (0.1 mM compared to 2 mM) is also significant because it suggests that E42Q CRP will remain active even at low Ca^{2+} concentrations at the sites of inflammation. Therefore, we propose that E42Q CRP will bind to atherogenic LDL in vivo even if the acidic pH component of inflammation is missing or mild in the arterial walls of the animal models of atherosclerosis to prevent foam cell formation, and thus reduce the development of atherosclerosis.

The reversibility of the structural changes in the CRP pentamer, and of its ligand-binding activities, at physiological pH indicates that the functions of CRP in circulation are different than that at the localized sites of inflammation where both CRP and the ligands of CRP are exposed to an inflammatory environment. Interestingly, E42Q CRP efficiently bound not only to ox-LDL but to a variety of immobilized proteins, which include proteins that might be deposited or bound to structures in the body. For example, deposition of factor H has been implicated in age-related macular degeneration and pneumococcal infection (53-58). It is of interest that chaperone proteins, the family of proteins recognizing misfolded proteins, are also, like CRP, ancient proteins (59). Overall, our findings suggest that E42Q CRP may serve as a tool to investigate the functions of CRP in each and every inflammatory disease involving deposition of proteins, such as autoimmune diseases, and in which CRP has been implicated (60-63). Investigations using E42Q CRP in animal models of inflammatory diseases may also establish the therapeutic potential of E42Q CRP. It may also be possible to design small-molecule compounds which can target and change the structure of endogenous native CRP to mimic the structures of acidic pH-treated CRP or E42Q CRP.

REFERENCES

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FOOTNOTES

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† These authors contributed equally to this work and should be considered as first authors.

‡ To whom correspondence should be addressed: Department of Pharmacology, Quillen College of Medicine, P.O. Box 70577, East Tennessee State University, Johnson City, TN 37614. Tel.: 423-439-6336; Fax: 423-439-8773; E-mail: agrawal@etsu.edu.

The abbreviations used are: ANS, 1-anilinonaphthalene-8-sulfonic acid; CRP, C-reactive protein; ox-LDL, oxidized LDL; PCh, phosphocholine; PnC, pneumococcal C-polysaccharide; TBS-Ca, TBS, pH 7.2, containing 0.1% gelatin, 0.02% Tween-20 and 2 mM CaCl2.

FIGURE LEGENDS

FIGURE 1. The structure of CRP. A, Discovery Studio Visualizer 3.0 software (Accelrys Software Inc.) was used to generate ribbon diagram of the crystal structure of pentameric CRP obtained from RCSB Protein Data Bank (PDB ID 1B09). B, Two of the five subunits are shown. The side chains of Tyr40, Glu42, Pro115, Lys119 and Tyr175, relevant for this work, are highlighted. Calcium ions are shown as green balls.

FIGURE 2. Binding of CRP to ox-LDL. A, CRP (10 µg/ml), diluted in TBS-Ca, pH 7.0 and 5.0, containing 2 mM CaCl2, was added to ox-LDL-coated wells. Bound CRP was detected by using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm. Results are shown as mean ± S.D. of five assays. * indicates p < 0.005 between WT and E42Q CRP. B, CRP (10 µg/ml), diluted in TBS-Ca, pH 7.0 to 5.0, containing 2 mM CaCl2, was added to ox-LDL-coated wells. Bound CRP was detected as in A. C, Increasing concentrations of CRP, diluted in TBS-Ca, pH 5.6, containing 2 mM CaCl2, was added to ox-LDL-coated wells. Bound CRP was detected as in A. A representative of four experiments is shown for B and C.

FIGURE 3. CRP is neither monomerized nor aggregated at acidic pH. A, Elution profiles of E42Q CRP from the Superose12 gel filtration column. CRP (250 µl of 5.6 mg/ml) in TBS, pH 7.2, containing 2 mM CaCl2, was applied to the column equilibrated with the same buffer (black). CRP (250 µl of 2.2 mg/ml), after incubation for 2 h at 37 ºC in TBS, pH 5.6, containing 2 mM CaCl2), was applied to the column equilibrated with the same buffer (red). CRP was eluted with the respective buffers. A representative of two experiments is shown. B, Reversibility of the ox-LDL-binding activity of CRP. CRP at pH 5.6 was first incubated for 2 h at 37 ºC. After removing an aliquot, the pH was neutralized. These CRP (10 µg/ml) samples were then added to ox-LDL-coated microtiter wells. Bound CRP was detected as in Fig. 2. Red bars, CRP in TBS-Ca, pH 7.2. Black bars, CRP in TBS-Ca, pH 5.6, and incubated at 37 ºC for 2 h before adding to the wells. Blue bars, As in black, except that the pH was neutralized before adding CRP to the wells. Results are shown as mean ± S.D. of five independent assays. C, ANS-binding fluorescence of CRP samples used in B. Red bars, CRP at pH 7.2; Black bars, CRP at pH 5.6 preincubated at 37 ºC for 2 h; Blue bars, After incubating CRP at pH 5.6 at 37 ºC for 2 h, the pH was neutralized. Results are shown as mean ± S.D. of five experiments. D, Far UV CD spectra of WT and E42Q CRP in TBS-Ca at various pH are shown.

FIGURE 4. Determination of local structural changes in CRP. A, PnC-binding activity of WT CRP as a function of pH and Ca2+ concentration. WT CRP (50 ng/ml), diluted in TBS-Ca (various pH, various concentrations of Ca2+) was added to PnC-coated wells. A representative of three experiments is shown. B, PnC-binding activity of CRP mutants as a function of pH and Ca2+ concentration. Increasing
concentrations of CRP, diluted in TBS-Ca (various pH) containing 2 mM CaCl$_2$ was added to PnC-coated wells. In both $A$ and $B$, bound CRP was detected by using a mouse anti-CRP antibody and HRP-conjugated goat anti-mouse IgG. The absorbance of the color was read at 405 nm. A representative of three experiments is shown. $C$, Molecular modeling of CRP based on the X-ray crystal structure of WT CRP-PCh complex (1B09.pdb) is shown.

**FIGURE 5.** Acidic pH reduces the affinity of CRP for Ca$^{2+}$. As in Fig. 4$A$, PnC-binding activity of WT CRP as a function of pH and Ca$^{2+}$ concentration is shown. CRP, diluted in TBS-Ca (pH 5.0 and 4.6) containing 0.1 mM or 2 mM CaCl$_2$ was added to PnC-coated wells. After 2 h at 37 ºC, the wells were washed once with respective TBS-Ca and then with TBS-Ca, pH 7.2, containing 2 mM CaCl$_2$. Bound CRP was detected by using a mouse anti-CRP antibody and HRP-conjugated goat anti-mouse IgG. The absorbance of the color was read at 405 nm. A representative of three experiments is shown.

**FIGURE 6.** Effect of Ca$^{2+}$ on the binding of WT and mutant CRP to ox-LDL. **Left panel,** CRP (10 µg/ml), diluted in TBS-Ca (2 mM or 0.1 mM CaCl$_2$), pH 7.0 to 5.0, was added to ox-LDL-coated wells. **Right panel,** Increasing concentrations of CRP, diluted in TBS-Ca (2 mM or 0.1 mM CaCl$_2$), pH 5.6, was added to ox-LDL-coated wells. Bound CRP was detected by using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the color was read at 405 nm. $A$, Comparison of WT and E42Q CRP. $B$, Comparison of WT and P115A CRP. $C$, Comparison of WT and Y175A CRP. $D$, Comparison of E42Q and P115A CRP. A representative of three experiments is shown.

**FIGURE 7.** Binding of CRP to immobilized proteins at acidic pH. Results of a protein ligand-binding assay are shown. Microtiter wells were coated with complement factor H, amyloid β, BSA and gelatin. CRP (10 µg/ml), diluted in TBS-Ca, pH 7.0 to 5.0, was then added to the wells. Bound CRP was detected by using a rabbit anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. The absorbance of the developed color was read at 405 nm. A representative of three experiments is shown.
Fig. 2

**A**

Binding of CRP to ox-LDL (A₄₀₅)

<table>
<thead>
<tr>
<th>pH</th>
<th>WT</th>
<th>E42Q</th>
<th>P115A</th>
<th>Y175A</th>
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<td>6.0</td>
</tr>
</tbody>
</table>

* Binding increased significantly at pH 5.0

**B**

Binding of CRP to ox-LDL (A₄₀₅)

- WT
- E42Q
- P115A
- Y175A

**C**

Binding of CRP to ox-LDL (A₄₀₅)

- WT
- E42Q
- P115A
- Y175A

CRP (µg/ml)

0.1, 1, 10
Fig. 3
Fig. 4

A

Binding of CRP to PnC (A405)

CaCl₂ (mM)

0.01 0.1 1 10

pH 7.2

pH 5.8

pH 5.6

pH 5.4

pH 5.2

pH 5.0

B

Binding of CRP to PnC (A405)

WT

E42Q

P115A

Y175A

CRP (ng/ml)

1 10 100

1.0

2.0

3.0

1 10 100

1.0

2.0

3.0

C

Ionic Bond Distance

Native CRP

Subunit A

Subunit B

H-Bond Distance

E42Q CRP

O2

X113

3.787

K113

Q2

X113

3.821

Subunit A

Subunit B
Fig. 5

Binding of CRP to PnC (A$_{405}$)

- pH 5.0, 2.0 mM CaCl$_2$
- pH 5.0, 0.1 mM CaCl$_2$
- pH 4.6, 2.0 mM CaCl$_2$
- pH 4.6, 0.1 mM CaCl$_2$
Fig. 6

(A) WT, 2.0 mM CaCl₂
(B) WT, 0.1 mM CaCl₂
(C) E42Q, 2.0 mM CaCl₂
(D) E42Q, 0.1 mM CaCl₂

Binding of CRP to ox-LDL (A₄₀₅)

CRP (µg/ml)

pH

(CRP, 10 µg/ml)

(pH 5.6)
Fig. 7

[Graph showing binding of CRP (A405) to Factor H, Amyloid β, BSA, and Gelatin at different pH levels (7.0 to 5.0) for WT and E42Q variants.]
Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis

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