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Binding of the monomeric form of C-reactive protein to enzymatically-modified low-density lipoprotein: effects of phosphoethanolamine

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Abstract

Background—The 5 subunits of native pentameric C-reactive protein (CRP) are dissociated to generate monomeric form of CRP (mCRP) in some *in vitro* conditions, both physiological and non-physiological, and also *in vivo*. Many bioactivities of mCRP generated by urea-treatment of CRP and of mCRP generated by mutating the primary structure of CRP have been reported. The bioactivities of mCRP generated by spontaneous dissociation of CRP are largely unexplored.

Methods—We purified mCRP generated by spontaneous dissociation of CRP and investigated the binding of mCRP to enzymatically-modified low-density lipoprotein (E-LDL).

Results—mCRP was approximately 60 times more potent than CRP in binding to E-LDL. In the presence of the small-molecule compound phosphoethanolamine (PEt), at 37°C, the binding of mCRP to E-LDL was enhanced <2-fold, while the binding of CRP to E-LDL was enhanced >10-fold. In contrast, PEt inhibited the binding of both CRP and mCRP to pneumococcal C-polysaccharide, another phosphocholine-containing ligand to which CRP and mCRP were found to bind. We have not investigated yet whether PEt alters the structure of CRP at 37°C.

Conclusions—Combined data suggest that the targeting of CRP with the aim to monomerize CRP *in vivo* may be an effective approach to capture modified forms of LDL.

Keywords

C-reactive protein; Monomeric C-reactive protein; Phosphoethanolamine; Pneumococcal C-polysaccharide; Enzymatically-modified low-density lipoprotein

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1. Introduction

Throughout this paper, the abbreviation CRP represents “native pentameric C-reactive protein”. CRP is composed of 5 identical, non-covalently associated, 23 kDa-subunits. Some of the biologically significant binding specificities of CRP include substances containing phosphocholine (PCh) and phosphoethanolamine (PEt), such as pneumococcal C-polysaccharide (PnC) and damaged cells of the myocardial infarcts. There are 5 PCh-binding sites in CRP, one located on each subunit. Calcium ions are required for the binding of CRP to PCh and PEt [1–7].

Although CRP is secreted by hepatocytes in the form of pentameric molecules, the 5 subunits of CRP are dissociated to generate monomeric form of CRP (mCRP) in some *in vitro* conditions, both physiological and non-physiological, and also *in vivo* [1,8,9]. Four *in vitro* conditions for the generation of mCRP have been described. 1. mCRP can be generated by exposing CRP to protein denaturants such as urea and heat [10]. 2. Recombinant mCRP can be generated by mutating CRP amino acids Cys36 and Cys97 to alanine so that the expressed CRP does not pentamerize [11,12]. 3. mCRP can also be generated by spontaneous dissociation of CRP when CRP is stored in the absence of calcium [13–16]. Also, in the absence of calcium, both CRP and mCRP are susceptible to proteolytic degradation [17,18]. 4. Under physiological conditions, mCRP is generated when CRP is associated with a cell membrane or liposome [19–21]. This mechanism of mCRP generation has been considered to explain the production of mCRP *in vivo* [20,21]. The presence of mCRP has been demonstrated in normal vascular tissue, atherosclerotic lesions, damaged brain tissue, diseased kidney, urine, and in other inflammatory conditions including inflamed tissues in animal studies [22–29]. These demonstrations suggest that mCRP is a major tissue form of CRP, and therefore, it is important to investigate the functions of mCRP.

CRP has been shown to bind to enzymatically-modified low-density lipoprotein (E-LDL) which is an atherogenic form of LDL and a constituent of atherosclerotic lesions [30–34]. The PCh-binding site of CRP participates in the binding of CRP to E-LDL because PCh has been shown to inhibit binding of CRP to E-LDL. We have shown recently that PEt, which, like PCh, presumably also blocks the PCh-binding site of CRP, does not inhibit binding of CRP to E-LDL; instead, PEt enhances the binding of CRP to E-LDL [34,35]. The capacity of CRP to bind to E-LDL is a beneficial function of CRP because, by doing so, CRP can prevent formation of macrophage foam cells [34,35]. mCRP exhibits bioactivities different from the bioactivities of CRP [15,36–39]. However, like CRP, recombinant mCRP has been shown to bind to E-LDL [14]. The PCh and cholesterol moieties of E-LDL molecules serve as the ligands for the binding of CRP and mCRP to E-LDL [21,32,34].

The reported bioactivities of mCRP, including the E-LDL-binding activity, are mostly based on the investigations using either recombinant mCRP or mCRP generated by urea-treatment of CRP. Little is known about the bioactivities of purified mCRP generated by spontaneous dissociation of CRP [38,39]. While investigating CRP-E-LDL interactions [34], we routinely observed that the stored CRP was more efficient than freshly-purified CRP in binding to E-LDL. Gel filtration analysis of the stored preparations of purified CRP revealed the presence of mCRP. In this study, we purified mCRP from the stored CRP preparations and investigated the binding of purified mCRP to E-LDL.

2. Materials and methods

2.1. Purification of CRP and mCRP

As described previously [40], CRP was purified from pleural fluid by 3 steps: a Ca^{++}-dependent affinity chromatography on a PCh-conjugated Sepharose column (Pierce, Rockford, IL),

followed by anion-exchange chromatography on a MonoQ column (GE Healthcare, Piscataway, NJ), and gel filtration on a Superose12 column (10/300 GL, GE Healthcare) using the Biologic Duo Flow Protein Purification System (BioRad, Hercules, CA). Purified CRP was stored in 10 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.2 (TBS) at −80°C. The duration of storage was at least 8 weeks at −80°C followed by 2 weeks at 4°C. We called this preparation of CRP as “stored CRP”.

Stored CRP was centrifuged and subjected to gel filtration again on the Superose12 column to remove any mCRP which might have formed upon storage of CRP. The column was eluted with TBS and the fractions containing CRP were collected, diluted in appropriate Ca^{2+}-containing buffer as described below, and then used in the assays the same day. After the re-purification procedure, we called this preparation of CRP as “freshly-purified CRP”.

During the re-purification of CRP, the mCRP-containing fractions were also collected, diluted in appropriate Ca^{2+}-containing buffer as described below, and then used in the assays. Thus, mCRP, that we report here, was generated by spontaneous dissociation of stored CRP.

2.2. Preparation of E-LDL

E-LDL was prepared as described previously, with some modifications [31,34,41]. First, native LDL was isolated from normal human plasma according to a published method [42]. Native LDL was dialyzed against 5 mmol/l Veronal containing 150 mmol/l NaCl, pH 7.3. Next, native LDL (5 mg/ml) was treated with plasmin (0.1 U/ml; Roche, Indianapolis, IN) and EDTA (0.5 mmol/l) and incubated for 24 h at 37°C with shaking. Cholesterol esterase (40 μg/ml; Roche) was added next and incubated for 24 h at 37°C with shaking. Then, once again, plasmin and cholesterol esterase were added together and incubated overnight at 37°C with shaking. Finally, the mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was stored at 4°C in aliquots. The concentration of protein in the E-LDL preparations was measured using a protein assay kit (Bio-Rad).

2.3. E-LDL-binding assay

E-LDL-binding assays were performed as described previously [34]. Microtiter wells were coated with 10 μg/ml of E-LDL diluted in TBS overnight at 4°C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. CRP and mCRP diluted in TBS containing 0.1% gelatin, 0.02% Tween-20 and 2 mmol/l $CaCl_2$ (TBS-Ca) were added in duplicate wells. After incubation for 3 h at 37°C, the wells were washed with TBS-Ca. Immunoaffinity-purified polyclonal rabbit anti-CRP antibody (1 μg/ml) was used to detect bound CRP. Immunoaffinity-purified polyclonal rabbit anti-CRP antibody was purified from the rabbit anti-human CRP antiserum (Sigma-Aldrich, St. Louis, MO) by affinity chromatography on a CRP-conjugated agarose column prepared by using the AminoLink Immobilization kit (Pierce) [7]. HRP-conjugated goat anti-rabbit IgG (Pierce) were used as the secondary antibody. Color was developed, and the absorbance was read at 405 nm in the microtiter plate reader (Molecular Devices, Sunnyvale, CA). To determine the effects of PEt (Sigma-Aldrich, P0503) on the binding of CRP and mCRP to E-LDL, CRP and mCRP were added to E-LDL-coated wells in the presence of 10 mmol/l PEt diluted in TBS-Ca buffer.

2.4. PnC-binding assay

Binding activity of CRP and mCRP for PCh was evaluated by using PnC (Statens Serum Institut, Copenhagen) as described previously [34]. Briefly, microtiter wells were coated with PnC (10 μg/ml) in TBS. After blocking, serial dilutions of CRP in TBS containing 0.1% BSA, 0.02% Tween-20 and 5 mmol/l $CaCl_2$ were added to the wells and incubated for 3 h at 37°C. Immunoaffinity-purified polyclonal rabbit anti-CRP antibody (1 μg/ml) was used to detect bound CRP. HRP-conjugated goat anti-rabbit IgG were used as the secondary antibody. Color

was developed, and the absorbance was read at 405 nm in the microtiter plate reader. In some assays, an anti-CRP monoclonal antibody (HD2.4) was used to detect PnC-bound CRP [7]. To determine the effects of PEt on the binding of CRP and mCRP to PnC, CRP and mCRP were added to PnC-coated wells in the presence of 10 mmol/l PEt prepared in TBS containing 0.1% BSA, 0.02% Tween-20 and 5 mmol/l $CaCl_2$.

3. Results

3.1. The binding of stored CRP to E-LDL is more efficient than that of freshly-purified CRP

We first compared the binding of stored CRP and freshly-purified CRP to E-LDL using the E-LDL-binding assay (Fig. 1). CRP from both preparations bound to E-LDL in a dose-dependent manner, but with different efficiency. For equivalent binding of CRP to E-LDL, 10 μg/ml of freshly-purified CRP was required compared to 1.73±0.4 μg/ml of stored CRP. Based on the data obtained from 4 assays, we calculated that 80–90% less of stored CRP was required compared to freshly-purified CRP for equivalent binding to E-LDL. Because it has been shown earlier that the storage of CRP under certain conditions causes its dissociation into mCRP and that mCRP also binds to E-LDL [13–16], we hypothesized that the increased binding of stored CRP to E-LDL might be due to the presence of mCRP in stored CRP preparations.

3.2. Purification of mCRP

To test the hypothesis that the increased binding of stored CRP to E-LDL was due to the presence of mCRP, we performed gel filtration chromatography of stored CRP. For comparison, we performed gel filtration chromatography of freshly-purified CRP. The elution profiles of freshly-purified CRP and stored CRP from the gel filtration chromatography column are shown in Figs. 2A and 2B. The elution profile of stored CRP showed two peaks, indicating the presence of mCRP. The first peak corresponded with the elution profile of freshly-purified CRP. The molecular weight of the protein in the second peak was calculated to be approximately 31 kDa. Next, we used reducing SDS-PAGE (Fig. 2C), to determine the molecular weight of the subunits of freshly-purified CRP and the protein present in the second peak (labelled as mCRP in Fig. 2B). The apparent molecular weights for both were found to be approximately 27.5 kDa. The degradation products (Fig. 2C, lane 3) appearing after electrophoresis reflected the unstable nature of mCRP under electrophoresis conditions. We concluded that the protein present in the second peak was mCRP generated from spontaneous dissociation of CRP (further confirmed by the results of the PnC-binding assays; see below), and used it as purified mCRP.

3.3. The binding of purified mCRP to E-LDL is more efficient than that of CRP: Effects of phosphoethanolamine

In the experiments shown in Figures 3 and 4, we used purified mCRP and freshly-purified CRP. In the E-LDL-binding assay (Fig. 3A), mCRP bound to E-LDL more efficiently than did CRP. The amount of mCRP required for 50% of maximal binding to E-LDL was 60.5±27.7 times (98.2%) less than the required amount of CRP. Prompted by our previous finding that PEt enhances the binding of CRP to E-LDL [34], we next determined whether PEt also had an effect on the binding of mCRP to E-LDL. In the presence of 10 mmol/l PEt, the binding of mCRP to E-LDL was slightly enhanced at all concentrations of mCRP (Fig. 3B). In the presence of PEt, the amount of mCRP required for 50% of maximal binding of mCRP to E-LDL was 2.3±1.2 times (56.5%) less compared to that required in the absence of PEt.

Next, we compared the binding of CRP and mCRP to E-LDL, in the presence and absence of PEt, in a single assay (Fig. 3C). PEt again enhanced the binding of both CRP and mCRP to E-LDL. From the data shown in Figure 3C, we determined the concentration of CRP and mCRP, in the presence and absence of PEt, required for equivalent binding to E-LDL (Fig. 3D). CRP

was required at a concentration of 9.4 μg/ml, CRP in the presence of PEt was required at a concentration of 0.92 μg/ml, mCRP was required at a concentration of 0.23 μg/ml, and mCRP in the presence of PEt was required at a concentration of 0.16 μg/ml. Thus, relatively, CRP was least efficient in binding to E-LDL while PEt-bound mCRP was most efficient. We also found that a temperature of 37°C was critical for PEt-dependent enhancement of the binding of CRP to E-LDL; PEt did not enhance the binding of CRP to E-LDL when the E-LDL-binding assays were performed at room temperature (data not shown).

3.4. PEt inhibits the binding of CRP and mCRP to PnC

We performed PnC-binding assay to investigate whether mCRP retained the PCh-binding activity of CRP and, if so, to determine the effects of PEt on the binding of mCRP to PnC. Both CRP and mCRP bound to PnC in a dose-dependent manner (Fig. 4). However, the avidity of the binding of mCRP to PnC was much lower than that of CRP. For comparable binding to PnC, 59.7±7.8 times more mCRP was required than the required amount of CRP. Identical results were obtained when an anti-CRP monoclonal antibody HD2.4 was used to detect mCRP bound to PnC (data not shown). These results showed that the HD2.4 epitope of CRP was present on mCRP generated by spontaneous dissociation of CRP, and also provided information on the specificity of the HD2.4 antibody for future use. Combined data also suggested structural similarities between mCRP and CRP subunits in the pentamer. As expected, PEt inhibited the binding of both CRP and mCRP to PnC, indicating that mCRP retained the PEt-binding activity of CRP and that the function of PEt to enhance the binding of CRP and mCRP to E-LDL was selective for E-LDL.

4. Discussion

In this study, we purified mCRP generated by spontaneous dissociation of CRP, and investigated the binding of mCRP to E-LDL. Our major findings were: 1. mCRP was approximately 60 times more potent than CRP in binding to E-LDL. 2. In the presence of the small-molecule compound PEt, at 37°C, the binding of mCRP to E-LDL was enhanced less than 2-fold, while the binding of CRP to E-LDL was enhanced more than 10-fold. 3. mCRP generated by spontaneous dissociation of CRP retained the PCh-binding and PEt-binding activities of CRP. 4. The PEt-mediated enhancement in the binding of mCRP and CRP to E-LDL was selective for E-LDL because PEt inhibited the binding of both CRP and mCRP to PnC, another PCh-containing ligand to which CRP and mCRP were found to bind.

Investigating the functions of mCRP has implications because mCRP has been found to be generated *in vivo*. Even a mechanism for the processing of CRP into mCRP in physiological conditions has been proposed, that is, when CRP is associated with a cell membrane, it is converted to mCRP [20,21]. In the arterial wall, mCRP has been shown to localize with LDL and macrophages in the atherosclerotic lesions [12]. Not only mCRP, but CRP has also been found deposited and localized with LDL and macrophages in atherosclerotic lesions in humans and experimental animal models [31,43–45]. However, it is important to note that in the studies where CRP had been found deposited in atherosclerotic lesions, polyclonal anti-CRP antibodies were used which did not differentiate between CRP and mCRP. Many polyclonal anti-CRP antibodies, which have been examined for their mCRP specificity to date, have been shown to cross-react with mCRP [46].

It has been reported previously that recombinant mCRP also binds to E-LDL and it has been suggested that the capacity of mCRP to bind to modified forms of LDL is a beneficial function of mCRP [12,14]. Accordingly, mCRP has been found to decrease the uptake of oxidized LDL by macrophages and it has been proposed that the interaction of mCRP with oxidized LDL may contribute to retardation of the foam cell formation by reducing the aggressive macrophage response to oxidized LDL [14]. Using *ApoE*$^{-/-}$ murine model of atherosclerosis, mCRP has

been shown to be atheroprotective [12,47]. The functions of mCRP have not yet been investigated in the *Apob*$^{100/100}$*Ldlr*$^{-/-}$ mouse model of atherosclerosis, a model in which CRP has been shown to be atheroprotective [48].

The mechanism of action of PEt in enhancing the binding of CRP to E-LDL is not known, but it is reasonable to assume that PEt modifies CRP in a way similar to storage-induced modification of CRP. Several effects of PEt, model liposomes, and cell membranes on the integrity of CRP have been described recently [20,21,49,50]. Data obtained from the studies on Limulus CRP suggested that the binding of CRP to liposomes and bacterial membranes causes hyper-oligomerization of CRP [49]. The binding of PEt to Limulus SAP-like pentraxin was shown to alter the structure of the Ca^{2+}-binding site of Limulus SAP-like pentraxin; upon binding PEt, each subunit of the pentraxin bound an additional calcium ion. In the crystal structure of PEt-bound Limulus SAP-like pentraxin, the ethanolamine group was poorly defined, suggesting structural and binding variability of this group [50]. In addition to these effects of PEt and membranes on the structural integrity of CRP, the binding of membranes to CRP has been shown to induce monomerization of CRP, and thereby exposing an otherwise hidden cholesterol-binding site of CRP [20,21]. We have not investigated yet whether PEt induces monomerization of CRP at 37°C.

We found that PEt had minimal effect on the binding of CRP to E-LDL if CRP was already monomerized. The minimal enhancing effect of PEt that was seen on mCRP may be due to possible PEt-mediated separation of self-aggregates of mCRP. Alternatively, mCRP, when alone, may be binding to only a certain population of E-LDL molecules in the E-LDL preparations. PEt, probably by inducing a structural alteration of CRP, may confer mCRP the ability to bind to those E-LDL molecules also to which mCRP cannot bind alone. It is less likely that our mCRP was contaminated with some CRP and the effect of PEt on mCRP was due to the CRP contaminant in purified mCRP preparations. Although mCRP by itself was efficient in binding to E-LDL, the beneficial effect of PEt would be to abolish mCRP's PCh-binding activity while retaining the E-LDL-binding activity.

Using mice, it has been shown that the half-life of mCRP in the circulation is <5 min. mCRP is rapidly cleared from the circulation and migrates to tissues [51]. These findings indicate that the transport of mCRP from circulation to various sites in the body is faster than CRP. We report here that mCRP is much more potent than CRP in binding to E-LDL. Taken together, our data suggest that the targeting of CRP with the aim to monomerize CRP *in vivo* may be an effective approach to capture modified atherogenic forms of LDL.

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Abbreviations

CRP
: pentameric C-reactive protein

mCRP
: monomeric C-reactive protein

LDL
: low-density lipoprotein

E-LDL
: enzymatically-modified LDL

PCh
: phosphocholine

PEt
: phosphoethanolamine

PnC
: pneumococcal C-polysaccharide

TBS
: 10 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.2

TBS-Ca
: TBS containing 0.1% gelatin, 0.02% Tween-20 and 2 mmol/l $CaCl_2$

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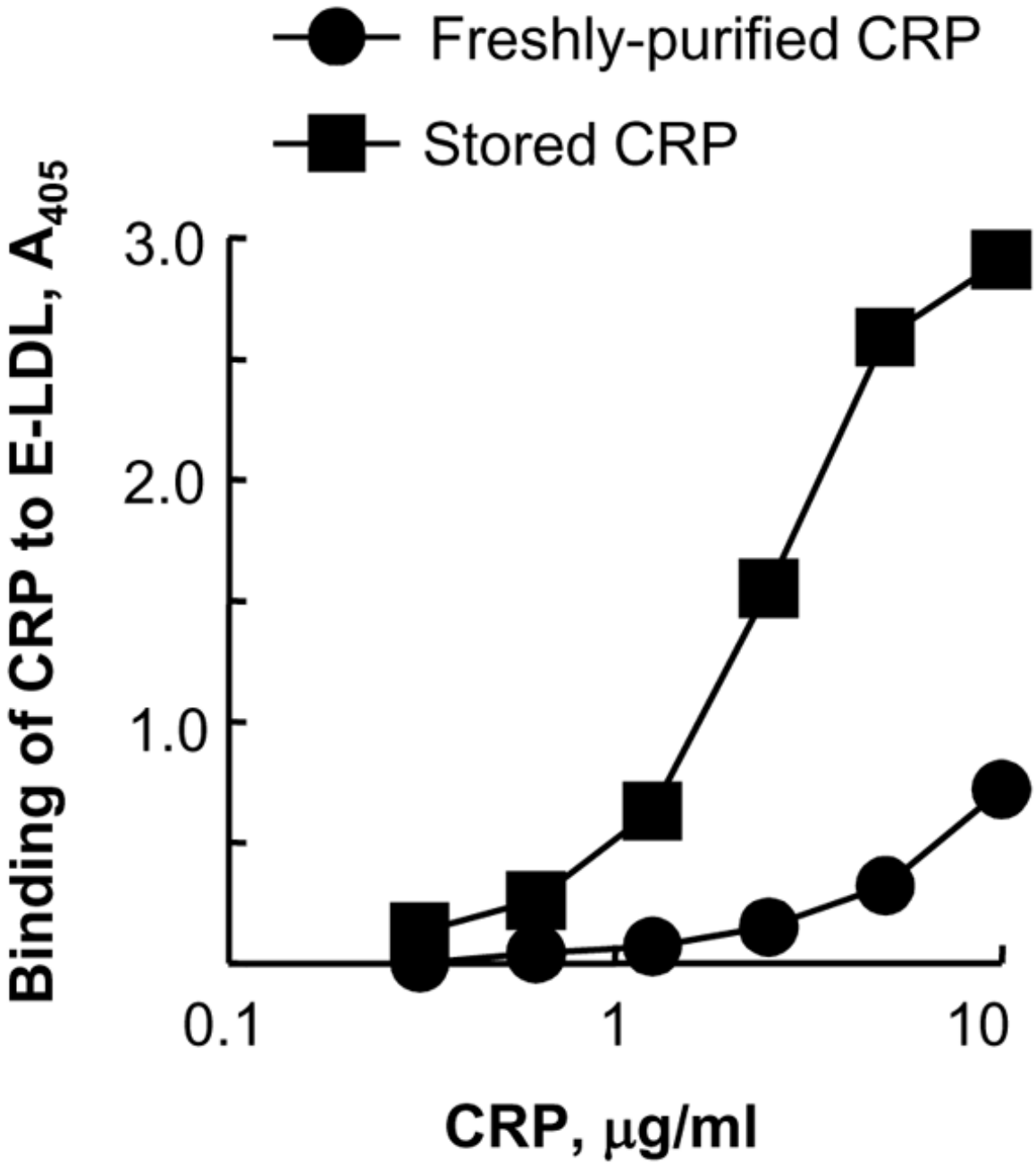

Fig. 1.
Stored CRP is more potent than freshly-purified CRP in binding to E-LDL. CRP was added to microtiter wells coated with E-LDL. Bound CRP was detected by using anti-CRP antibody. A representative of four experiments using four different preparations of CRP is shown.

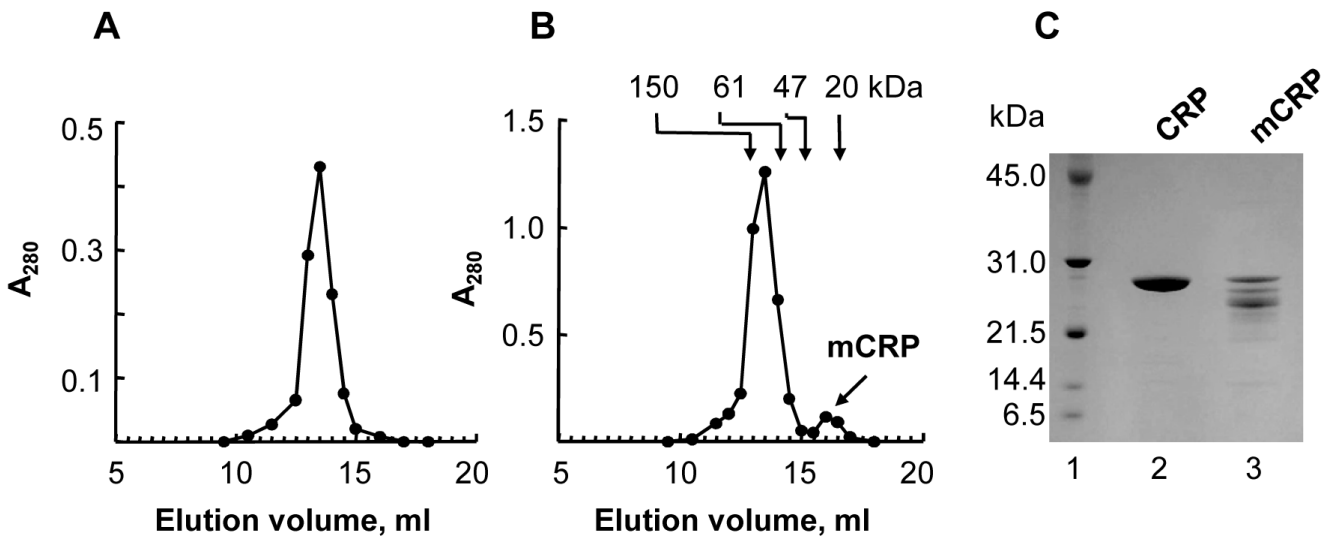

Fig. 2.

Purification of mCRP. (A) Gel filtration chromatography of freshly-purified CRP. A representative of 5 chromatograms from a Superose12 column is shown. (B) Gel filtration chromatography of stored CRP. A representative of 5 chromatograms from a Superose12 column is shown. The arrows point to the elution volumes of molecular weight marker proteins. The first peak contained CRP while the second peak contained mCRP. (C) CRP and mCRP were subjected to 10–20% SDS-PAGE under reducing conditions. A representative Coomassie blue-stained gel is shown. Lane 1, molecular weight marker; Lane 2, freshly-purified CRP (10 μg); Lane 3, purified mCRP (10 μg).

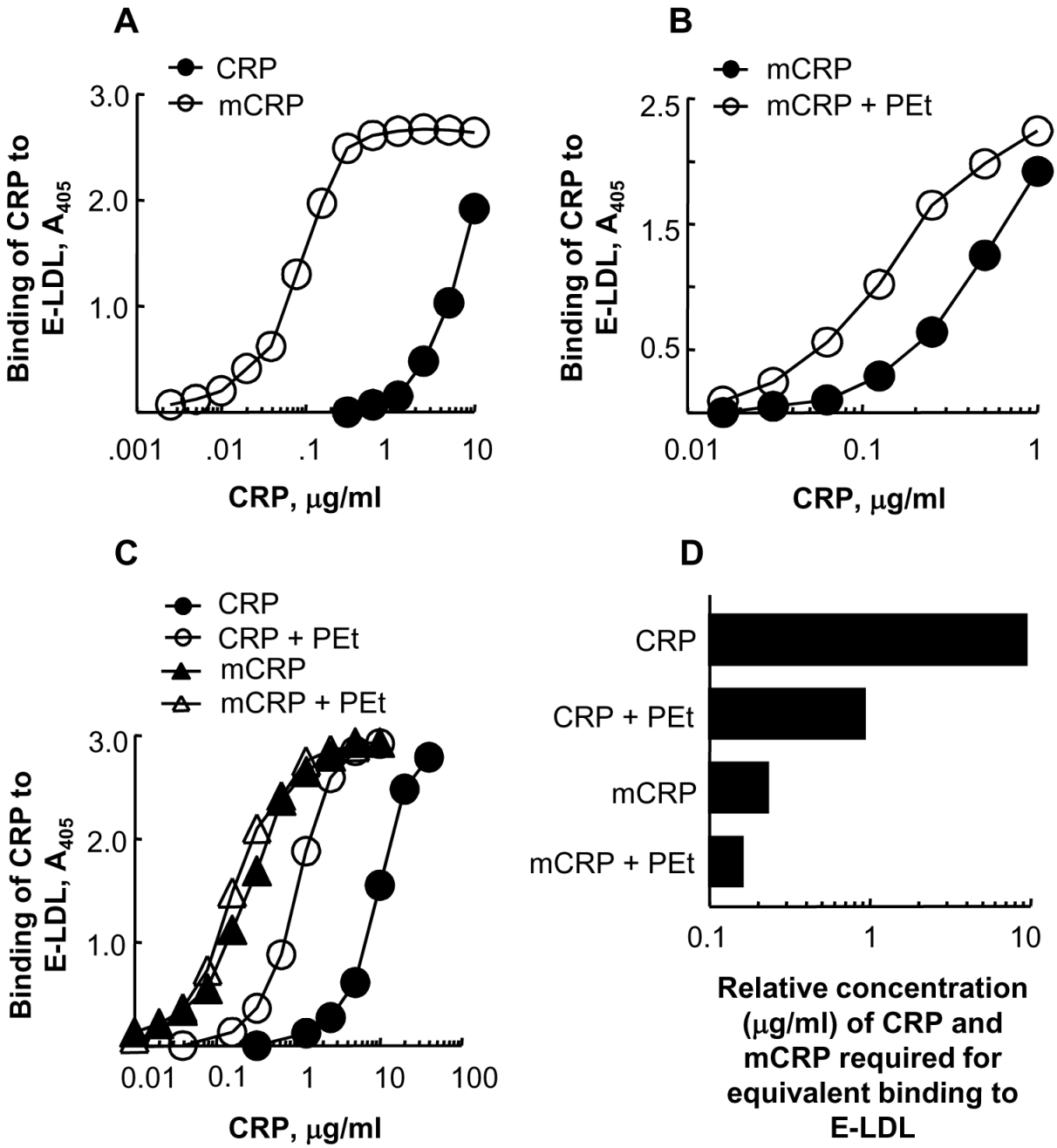

Fig. 3.

Both mCRP and PEt-bound CRP are more potent than CRP in binding to E-LDL. CRP and mCRP were added to E-LDL-coated wells. Bound CRP and mCRP were detected by using anti-CRP antibody. (A) Comparison of the binding of CRP and mCRP to E-LDL. A representative of three experiments is shown. (B) Increasing concentration of mCRP, with and without 10 mmol/l PEt, was added to E-LDL-coated wells. A representative of three experiments is shown. (C) Increasing concentration of CRP and mCRP, with and without 10 mmol/l PEt, was added to E-LDL-coated wells. A representative of two experiments is shown. (D) Relative E-LDL-binding capacity of CRP and mCRP, in the absence and presence of PEt. Values on the x-axis were derived from the experiment shown in Fig. 3C, and represent the

relative concentration of CRP and mCRP, in the absence and presence of PEt, required for their equivalent binding to E-LDL.

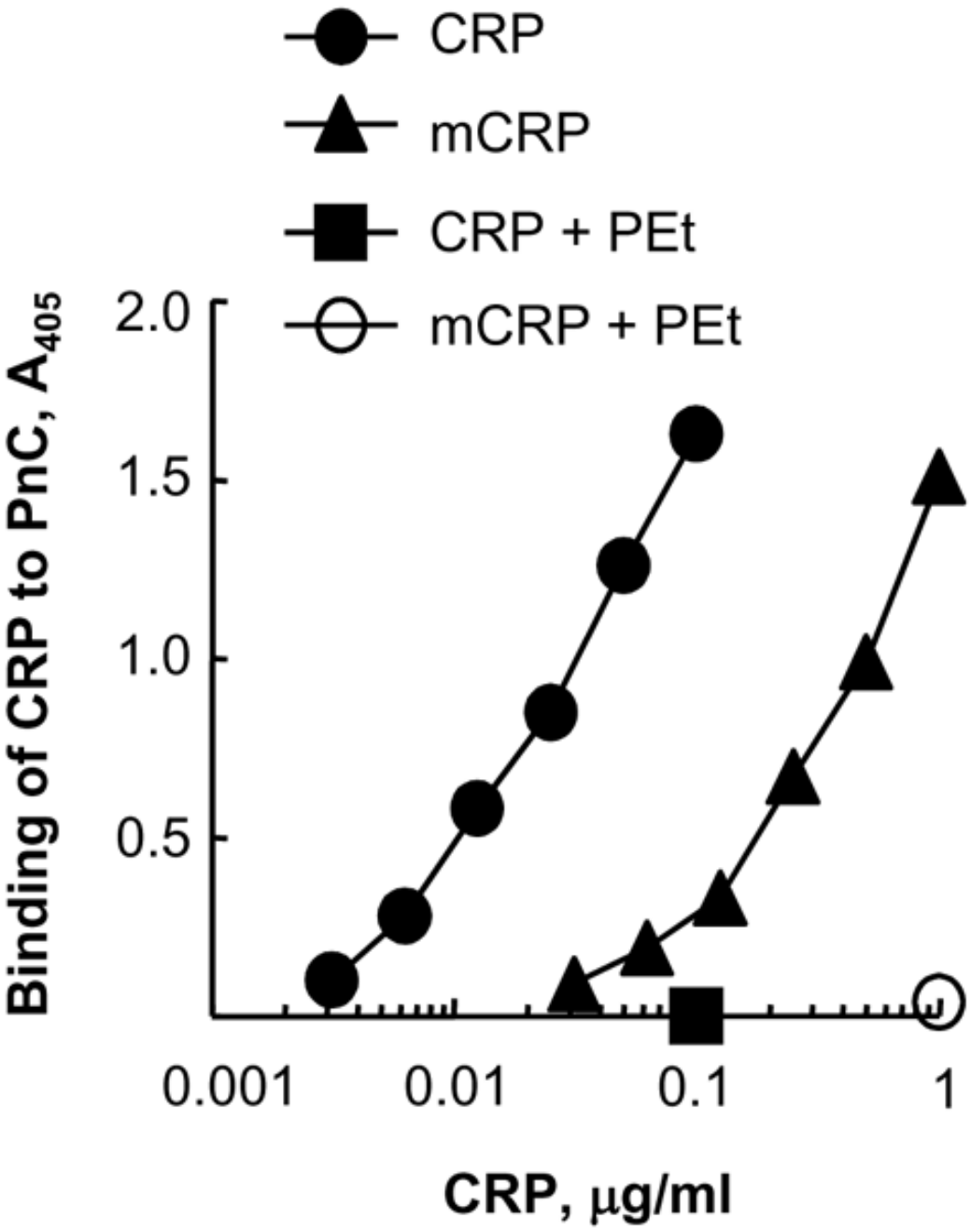

Fig. 4.

PEt inhibits the binding of CRP and mCRP to PnC. Increasing concentration of CRP and mCRP, with and without 10 mmol/l PEt, was added to PnC-coated wells. Bound CRP and mCRP were detected by using polyclonal anti-CRP antibody. A representative of 5 experiments is shown.