Mapping the Binding Areas of Human C-reactive Protein for Phosphorylcholine and Polycationic Compounds

RELATIONSHIP BETWEEN THE TWO TYPES OF BINDING SITES

We developed a fluorescence-based assay method for determining ligand binding activities of C-reactive protein (CRP) in solution. Using this method, we compared the phosphorylcholine (PC)- and polycation-based binding activities of human CRP. The PC-based binding required calcium, whereas a polycation (e.g. poly-l-lysine) was bound in the presence of either calcium or EDTA, the binding being stronger in the presence of EDTA. The published crystallographic structures of CRP and the CRP-PC complex show it to be a ring-shaped pentamer with a single PC-binding site per subunit facing the same direction. As expected from such a structure, binding affinity of a ligand increased tremendously when multiple PC residues were present on a macromolecular structure. In addition to PC-related structures, certain sugar phosphates (e.g. galactose 6-phosphate) are bound near the PC-binding site, and one of the sugar hydroxyl groups appears to interact with CRP. The best small ligands for the polycationic binding site were Lys-Lys and Lys₄. Because of the presence of multiple Lys-Lys sequences, polylysines have tremendously enhanced affinity. Although PC inhibits both PC- and polycation-based binding, none of the amines that inhibit polylysine binding inhibits PC binding, suggesting that the PC and polycationic binding sites do not overlap.

Human C-reactive protein (CRP) is a prototype acute-phase reactant. At the onset of infection or inflammation, the concentration of CRP may increase as much as 1000-fold. CRP acts as a defense molecule against some pathogens such as pneumococcal bacteria by initially binding to their surface components, e.g. phosphorylcholine (PC), and then activating the complement system or causing opsonization. The other major biological functions of CRP include removal of damaged cells and cell debris, wound healing, and immunomodulatory effect (1–3). Such a diverse range of biological activity of CRP is matched by a wide range of ligand with which it interacts. The known ligands can be classified into three major groups. They are 1) compounds that contain phosphorylcholine or related structures, 2) polycationic compounds such as poly-l-lysine and protamine sulfate, and 3) carbohydrates that contain α-galactose-related structures.

CRP is a member of the pentraxin family and, thus, is a homopentamer whose subunits associate non-covalently side by side to form a wreath-like structure (4). Because all subunits face the same direction, the surface of each side of the wreath has distinct binding characteristics. It is known that the conformation of CRP changes depending on the presence or absence of calcium (5). In addition, the soluble form of CRP and CRP bound to a plastic surface or cell surface (so-called neo-CRP) present different epitopes (6), and therefore, there is a potential for altered binding specificity (7).

We describe here a sensitive assay method for the ligand binding activities of CRP in the solution phase. The assay is based on polyethylene glycol (PEG) precipitation of CRP complexed with Eu-labeled, high affinity ligands. This method allows measurement of the binding activity of all three ligand groups, namely PC-, polycationic-, and galactose-based binding, using a similar assay strategy and the same detection method. We describe in this paper a detailed mapping of the PC-binding site and polycationic binding site of human CRP and the relationship between the two types of ligand.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used in the binding assay and inhibitors listed in the tables with the exception of PC-modified derivatives were from Sigma unless otherwise noted. Organic buffer chemicals MES, HEPES, Tricine, and AMPSO were from Research Organics (Cleveland, OH). Recombinant human CRP was from Oriental Bio-Service Kanto, Inc. (Tsukuba, Japan). Hydrophilic filters (Duranop, 0.45 μm) in 96-well format were from Millipore (Bedford, MA), and 96-well microtiter plates were from Fisher.

The preparation of sugar-containing bovine serum albumin (BSA) derivatives that have a general structure of [sugar-SCH₂CONH(CH₂)ₙNH₂]ₙ protein has been described (8). A BSA derivative containing on the average 40 residues of lactotrioside is designated as Lac₄₄-AD-BSA. Modification of BSA with PC has been described (9). Briefly, PC-glycerol was oxidized by sodium periodate to form choline phosphorylglucitoldehyde (CPGA), which was then conjugated to the amino groups of BSA via reductive amination. These BSA derivatives contain a short linking arm of ethylene group between PC and the BSA amino group and are designated as PCₙ-E₄-BSA, in which n is the average PC content in mol/mol, which was determined by organic phosphate content. The same reaction sequence was used to conjugate PC to poly-l-lysine (PL) of DP 240, and the level of PC incorporation was varied by changing the ratio of CPGA to PL. The efficiency of PC incorporation into PL depended on the degree of substitution attained. At a low occupancy level of 30% (of 240 amino groups), the efficiency of CPGA incorporation was 55%...
of the input, whereas at a high occupancy of 60%, the efficiency was ~33%.

Methods—Amine-containing compounds including polylysines were determined by 2,4,6-trinitrobenzenesulfonic acid method (11). PC-containing compounds were assayed for organic phosphate content (12), and the aldehydo function was determined by neocuproine method (13). Vicinal glycol was determined by combination of periodate oxidation and 2,4,6-tripyridyl-s-triazine method (14) as follows. Sample (20 µl) containing up to 0.1 mmol of vicinal glycol was incubated with sodium metaperiodate (10 mM, 10 l) for 0.5 h at 37 °C. The solution was diluted to 1 ml with water, and 2 ml of 2,4,6-tripyridyl-s-triazine solution (14) was added. Absorbance at 593 nm was linearly proportional to the vicinal glycol concentration up to absorbance of 0.8. 1H NMR spectra were recorded with a Bruker AMX 300 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Preparation of PC-containing BSA Derivatives with a Longer Linking Arm (Scheme 1)—The direct conjugation of CPGA to BSA produces a derivative with only an ethylene group separating PC and the protein amino group. To make a PC-BSA with a longer separation between the peptide and PC, a longer bifunctional linking arm was synthesized. The amino group of 6-aminoheptanoic acid (AHA) was protected with benzoyloxycarbonyl group to yield ZAHAG. The solution was suspended in 1 ml of dry pyridine and shaken for 2 days, during which time pyridine borane was added periodically to maintain saturation. The total amount of pyridine borane added was about 0.6 mmol for 0.5 mmol of CPGA. The excess borane was removed by hydrogenolysis to yield 3-(benzyloxycarbonyl)aminohexanoyl]amino-1,2-dihydroxypropane (ZAHA-AG). The combined yield of two crystalline crops from a mixture was 90%. Elemental analyses for C17H26N2O5 are as follows: C, 60.33; H, 7.75; N, 8.28 (calculated); C, 60.57; H, 7.97; N, 8.24 (experimentally determined). Benzyloxycarbonyl group was then removed by hydrogenolysis to yield 3-N[(6'-benzoxycarbonyl)aminoheptanoyl]amino-1,2-dihydroxypropane (ZAHAG). The combined yield of two crystalline crops from ethyl acetate was ~90%. Elemental analyses for C17H26N2O5 are as follows: C, 60.33; H, 7.75; N, 8.28 (calculated); C, 60.57; H, 7.97; N, 8.24 (experimentally determined). Benzyloxycarbonyl group was then removed by hydrogenolysis to yield 3-N[(6'-aminoheptanoyl]amino-1,2-dihydroxypropane (AHA-AG), which was conjugated to CPGA by reductive amination using a 2-fold excess of AHA-AG over CPGA. The reductive amination was carried out using 10 mg of BSA in 1.2 ml of phosphate buffer with the aldehydo reagent in 5-fold excess over borane. The reductive amination was carried out using 10 mg of BSA in 1.2 ml of 0.2M sodium phosphate buffer, pH 7.5, for 42 h, during which time pyridine borane was added periodically to maintain saturation. The total amount of pyridine borane added was about 0.6 mmol for 0.5 mmol of CPGA. The excess borane was removed from the mixture by extracting twice with ether, and any remaining borane was quenched by acidifying the mixture with glacial acetic acid and stirring with acetaldehyde (50 µl) for several hours. After evaporation, the residue was dissolved in 0.1 M acetic acid and fractionated on a column (2.5 × 140 cm) of Sephadex G-15 in 0.1 M acetic acid, and the fractions that contained both organic phosphate and vicinal glycol were combined and evaporated to yield the product, PC-AHA-AG (see Scheme 1). The conjugation of PC-AHA-AG to BSA is similar to that for PC-glycerol, namely, mild periodate oxidation, Sephadex G-15 separation, and the conjugation of the product to BSA by reductive amination. The reductive amination was carried out using 10 mg of BSA in 1.2 ml of 90%-ethanol buffer with the aldehydo reagent in 5-90-fold excess over the BSA amino group. The derivatized protein was purified on a column (1 × 46 cm) of Sephadex G-50 in 25 mM ammonium acetate, pH 7. The fractions containing protein were combined and lyophilized. The second lyophilization after dissolving the residue in water produced the salt-free BSA derivative. The content of PC in this long-arm PC-BSA (designated as PC-AhAe-BSA) ranged from 20 to 48 mol/mol. N-Acetylation of PC-PLs—A portion from each of the PC-PL preparations was N-acetylated to mask the polycationic determinants. PC-PL (6 to 10 mg) was dissolved in cold, saturated NaHCO3 (0.5 ml), acetic anhydride (20 µl) was added, and the mixture was left overnight in the cold. The mixture was diluted with 0.3 ml of water and fractionated on a Sephadex G-25 column (1.5 × 92 cm) in 15 mM acetic acid. Fractions containing acetylated PC-PL were determined by microBCA assay, combined, and lyophilized. The content of the amino group on these N-acetylated PC-PLs determined by the 2,4,6-trinitrobenzenesulfonic acid method was ~5 mol/mol (~2% total of the amino group).

O-Acetylation of 1-t-Decanoyllysophosphatidylcholine—To determine if the free β-OH group of lyssolecithin is needed for binding affinity, a soluble lecithin was prepared by acetylating 1-t-decanoyllysophosphatidylcholine. 1-t-Decanoylphosphatidylcholine (20 mg, 48.6 µmol) was suspended in 1 ml of dry pyridine and shaken for 2 days, during which time pyridine borane was added periodically to maintain saturation. The total amount of pyridine borane added was about 0.6 mmol for 0.5 mmol of CPGA. The excess borane was removed from the mixture by extracting twice with ether, and any remaining borane was quenched by acidifying the mixture with glacial acetic acid and stirring with acetaldehyde (50 µl) for several hours. After evaporation, the residue was dissolved in 0.1 M acetic acid and fractionated on a column (2.5 × 140 cm) of Sephadex G-15 in 0.1 M acetic acid, and the fractions that contained both organic phosphate and vicinal glycol were combined and evaporated to yield the product, PC-AHA-AG (see Scheme 1). The conjugation of PC-AHA-AG to BSA is similar to that for PC-glycerol, namely, mild periodate oxidation, Sephadex G-15 separation, and the conjugation of the product to BSA by reductive amination. The reductive amination was carried out using 10 mg of BSA in 1.2 ml of 90%-ethanol buffer with the aldehydo reagent in 5-90-fold excess over the BSA amino group. The derivatized protein was purified on a column (1 × 46 cm) of Sephadex G-50 in 25 mM ammonium acetate, pH 7. The fractions containing protein were combined and lyophilized. The second lyophilization after dissolving the residue in water produced the salt-free BSA derivative. The content of PC in this long-arm PC-BSA (designated as PC-AhAe-BSA) ranged from 20 to 48 mol/mol.

FIG. 1. Inhibition of Eu-PC40-Et-BSA binding to CRP. F, PC40-Et-BSA; □, PC; △, PC-glycerol.
Binding Sites of Human C-reactive Protein

Solution-phase Ligand-binding Assay for CRP—The principle of our assay involves reacting human CRP with an Eu-labeled ligand in solution: Eu-PC$_{40}$-Et-BSA for the PC-dependent binding, Eu-PL of DP 240 for the polycationic binding, and Eu-Lac$_{40}$-AD-BSA for the galactose-based binding. The complex formed was separated from the unbound Eu-ligand by PEG precipitation, followed by filtration, and Eu fluorescence of the complex trapped on the filter is determined.

Incorporation of Europium into Proteins and Polylysines—For the ligand binding assays to be described below, Eu-labeled, high affinity ligands are needed. PC$_{40}$-Et-BSA, PL of DP 240, and Lac$_{40}$-AD-BSA were chosen as the ligand for PC-dependent, polycation-dependent, and galactose-based binding, respectively, and labeled with a DTPA-labeling reagent. This reagent, which is derived from DTPA-dianhydride, contains four carboxylic acid residues through which Eu is stably chelated and a masked aldehyde as acetal, which is utilized to label proteins/polylysines via reductive amination after unmasking of the acetal. Both the preparation of the reagent and the labeling procedure have been described (31). To minimize the potential alteration of protein characteristics by the Eu-DTPA labeling, we strived for low coupling levels of methyl group signals are alkyl methyl (δ 1.266), O-acetyl methyl (δ 2.111), N-methyl (δ 3.232) (1.1:0.91:3.0).

When CRP at a fixed amount (31.3 pmol) was reacted with varying amounts of Eu-PC$_{40}$-Et-BSA, the fluorescence signal produced was proportional to the amount of Eu-PC$_{40}$-Et-BSA, leveling off at about the molar equivalence of CRP and the Eu-ligand. The reverse experiment of adding different amounts of CRP to a fixed amount of Eu-PC$_{40}$-Et-BSA also produced similar stoichiometry. In contrast, Eu-PL appears to be capable of binding 4 mol/mol of CRP, since the fluorescence signal started to level off only when a 4-fold molar excess of CRP over Eu-PL was present. Although there was a clearly detectable binding of Eu-Lac$_{40}$-AD-BSA by the soluble human CRP, the bound amount was only about 5% of the Eu-PC$_{40}$-Et-BSA bound. This may be because of inherently low affinity of CRP for Eu-Lac$_{40}$-AD-BSA or because of some contaminant in the CRP preparation such as a small fraction of CRP molecules expressing neo-CRP epitope. Indeed when CRP was coated on a plastic surface (neo-CRP), it bound Eu-Lac$_{40}$-AD-BSA in the amount comparable with Eu-PC$_{40}$-Et-BSA. Because of these uncertainties the binding study for Eu-Lac$_{40}$-AD-BSA by the solution-phase CRP was not investigated further.

From the above preliminary experiments, the standard assay was set up as follows. Eu-PC$_{40}$-Et-BSA (8 pmol) or Eu-PL (8 pmol) was incubated with CRP (7 pmol for the Eu-PC$_{40}$-Et-BSA binding assay and 19 pmol for the Eu-PL binding assay) in 25 mM Tris-HCl buffer at pH 7.5. Bound fluorescence value at pH 6.5 was set as 100%.

The principle of our assay involves reacting human CRP with an Eu-labeled ligand in solution: Eu-PC$_{40}$-Et-BSA for the PC-dependent binding, Eu-PL of DP 240 for the polycationic binding, and Eu-Lac$_{40}$-AD-BSA for the galactose-based binding. The complex formed was separated from the unbound Eu-ligand by PEG precipitation, followed by filtration, and Eu fluorescence of the complex trapped on the filter is determined.
**FIG. 3.** Structures of galactose derivatives with negative charge(s) on the C-6 position. GalA, galacturonic; MOβDG, methyl 4,6-(1-carboxyethylidene)-β-D-galactopyranoside.

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**TABLE III**

| Inhibitor | Degree of polymerization | Molecular mass | IC50 | μg/ml | μM |
|-----------|--------------------------|----------------|------|-------|-----|
| Poly-L-Lys | 16                       | 3.4            | 480  | 100   | 480 |
| Poly-L-Arg | 240                      | 50             | 5 ± 1.5 | 0.1 ± 0.03 | 0.2 |
| Poly-L-Orn | 200                      | 38.3           | 12.3 | 0.32  | 0.32 |
| Histonea  |                          |                | NI(300) | NI(21) |
| II-AS     |                          | 14b            | 237 ± 17 | 11 ± 0.8 |
| III-S     |                          | 22c            | 14   | NI(460) | NI(33) |

*a* Approximate molecular mass assuming mostly composed of histones, H2A, H2B, H3, and H4.  
*b* NI, not inhibitory at the concentration shown in parentheses.  
*c* Approximate molecular mass assuming mostly composed of histones, H1.

**Results**

**Effect of pH on Ligand Binding**

The binding activity of CRP for Eu-PC40-Et-BSA was remarkably constant in the pH range of 4.5–8.5 in the presence of CaCl2 (Fig. 2) and showed a slight decrease in the bound amount at pH 9. On the acidic side, however, there was a sharp drop in the binding activity below pH 4.5, reaching essentially no activity at pH 4. The pH dependence of Eu-PL binding was quite similar, with a sharp drop in the binding activity between pH 4.5 and 4.0 (not shown). This sudden loss of activity on the acidic side may be because of dissociation of the pentameric organization of CRP.

**Dependence of Ligand Binding on Calcium**

The conformation of CRP is known to change depending on the presence or absence of calcium ion (5). The binding activity of CRP for Eu-PC40-Et-BSA without added calcium was about 60% of the maximal binding activity, which occurred at around 10 mM calcium, and there was a gradual increase in the bound Eu-PC40-Et-BSA between 0 and 10 mM of added CaCl2. Perhaps the amount of calcium in the CRP sample and buffer is sufficient to mostly maintain the calcium-bound conformation of CRP without any added calcium. The bound amount of Eu-PL in the presence of 0–10 mM calcium did not seem to change much.

To investigate if calcium is truly required for the binding, binding activity was measured in the presence of EDTA. As mentioned under “Experimental Procedures,” EDTA competes Eu off the labeled macromolecules so that they had to be reloaded with Eu after filtration. Using this modified assay method, we found that CRP does not bind Eu-PC40-Et-BSA in the presence of EDTA (0.5 mM), since the fluorescence recovery was essentially the same whether CRP was present or not. This

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**Note:** The text continues with detailed scientific discussion and analysis, including experimental procedures and results, which are not fully transcribed here for brevity. The focus is on the binding sites of human C-reactive protein and the influence of calcium and pH on its binding activity. The document also includes a table that lists the IC50 values for various inhibitors and the molecular mass of different compounds, along with structural diagrams of galactose and related compounds.
observation is in agreement with the prevailing idea that the PC-dependent binding to CRP requires calcium.

As to Eu-PL, DiCamelli et al. (16) report that the binding of PL (measured by nephelometry) was inhibited in the presence of calcium. However, PL apparently can be bound by CRP in the presence of calcium if PC is also present (17). We carried out two parallel assays of CRP binding Eu-PL in the presence of EDTA and in the presence of calcium at the Eu-PL and CRP concentrations of 185 and 380 nM, respectively. The bound amount in the presence of EDTA was 0.274 million counts/s (Mcps) as compared with 0.11 Mcps in the presence of 2 mM calcium, suggesting that Eu-PL binds better to the calcium-free conformation. To find out if this increase in the bound amount is because of an increased number of binding sites or because of increased affinity, IC<sub>50</sub> of PL (DP-240) was determined in the presence of 0.5 mM EDTA and in the presence of 2 mM CaCl<sub>2</sub> using the same washing/reloading protocol. IC<sub>50</sub> in the presence of EDTA was 150 nM as compared with 600 nM in the presence of calcium, indicating that Eu-PL bound tighter to calcium, indicating that Eu-PL bound tighter to the calcium-free conformation. It should be noted, however, that the IC<sub>50</sub> determined in the presence of calcium by certain amino acids.

Inhibition Assays

At least two separate series of inhibition assay were carried out for each inhibitor. Inhibition curves generally had an appearance similar to those of PC<sub>5</sub>-Et-BSA and PC-glycerol shown in Fig. 1. In each case, 10–90% inhibition was observed within 2 decades of inhibitor concentration. The only exception to this was PC, which showed a very steep inhibition curve (Fig. 1). This may mean that there is cooperative inhibitory effect among five subunits of CRP, i.e. binding of the first PC molecule somehow facilitates the subsequent binding events.

**Inhibition of the Eu-PC<sub>40</sub>-Et-BSA Binding**

**Polyvalent Inhibitors**—As shown in Table I, binding affinity of the short-armed, PC-substituted BSA derivatives (PC-Et-BSA) was highly dependent on the PC content, showing a 42-fold increase in affinity when the PC content was increased by 6-fold. In contrast, there was only a small increase in affinity of the long-armed derivatives (PC-AhAe-BSA) beyond the PC content of 27 mol/mol. Affinity even higher than the PC-BSA derivatives could be attained when PC residues were on the flexible backbone of polylysine (DP 240). However, we found that the high inhibitory potency of PC-PLs is dependent not only on the PC content but also on its polycationic nature because when the three PC-PL preparations were PC-dependent binding to CRP requires calcium.

**TABLE IV**

| Inhibitor                  | IC<sub>50</sub> (µM) |
|----------------------------|----------------------|
| Lys-Lys                    | 370 ± 50             |
| Lys                        | 230 ± 30             |
| Lys, Arg, His              | Enh                  |
| N<sub>4</sub>-Acetyl-Lys   | Enh                  |
| Lys methyl ester           | 15,000 ± 3,000       |
| N<sub>4</sub>-Acetyl-Lys   | 13,000 ± 1,000       |
| 1,2-Diaminoethane          | 62,000               |
| 1,6-Diaminohexane          | 17,000               |
| 1,8-Diaminooctane          | 3,500 ± 500          |
| 1,12-Diaminododecane       | 27,000 ± 0           |
| Octylamine                 | 20,000 ± 4,000       |
| Choline chloride           | 20,000 ± 4,000       |
| PC                         | 4,700 ± 700          |

* A transient increase in the bound amount of ligand does not allow accurate IC<sub>50</sub> determination.

Small Inhibitors—The IC<sub>50</sub> value for PC of 40 µM obtained by us (Table II) is quite comparable with what was reported by others, e.g. 42.5 µM (18) and 75 µM (19). These values are slightly higher than K<sub>p</sub> of 18 µM determined by capillary electrophoresis (20). A K<sub>p</sub> value as low as 3 µM was obtained using a less direct enzyme-linked immunosorbent assay method (21). The relative IC<sub>50</sub> values were also comparable; the values for PC, PC-glycerol, and phosphoryl ethanolamine were 1:18:32 in our assay and 1:22:55 by Volanakis and Kaplan (19). Shown also in Table II are IC<sub>50</sub> values of a number of other phosphate-containing compounds and related structures. These non-choline-containing phosphates can be divided into three groups: 1) Polyvalent Inhibitors—As shown in Table I, binding affinity of the short-armed, PC-substituted BSA derivatives (PC-Et-BSA) was highly dependent on the PC content, showing a 42-fold increase in affinity when the PC content was increased by 6-fold. In contrast, there was only a small increase in affinity of the long-armed derivatives (PC-AhAe-BSA) beyond the PC content of 27 mol/mol. Affinity even higher than the PC-BSA derivatives could be attained when PC residues were on the flexible backbone of polylysine (DP 240). However, we found that the high inhibitory potency of PC-PLs is dependent not only on the PC content but also on its polycationic nature because when the three PC-PL preparations were PC-dependent binding to CRP requires calcium.

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| Lys-Lys                    | 370 ± 50             |
| Lys                        | 230 ± 30             |
| Lys, Arg, His              | Enh                  |
| N<sub>4</sub>-Acetyl-Lys   | Enh                  |
| Lys methyl ester           | 15,000 ± 3,000       |
| N<sub>4</sub>-Acetyl-Lys   | 13,000 ± 1,000       |
| 1,2-Diaminoethane          | 62,000               |
| 1,6-Diaminohexane          | 17,000               |
| 1,8-Diaminooctane          | 3,500 ± 500          |
| 1,12-Diaminododecane       | 27,000 ± 0           |
| Octylamine                 | 20,000 ± 4,000       |
| Choline chloride           | 20,000 ± 4,000       |
| PC                         | 4,700 ± 700          |

* A transient increase in the bound amount of ligand does not allow accurate IC<sub>50</sub> determination.
compounds with the binding affinity comparable with Pi (IC$_{50}$ 20 mM); Glec-6-P and Man-6-P; 2) compounds with ~10-fold higher affinity than Pi; glycerol phosphate, Gal-6-P, Rib-5-P, 2-deoxy-d-ribose-5-P as well as AMP and dAMP; 3) compounds that have very poor inhibitory potency: α-Glc-1-P and α-Gal-1-P. Interestingly, Gal-6-sulfate, although quite similar in structure to Gal-6-P (Fig. 3), was totally inactive at 50 mM. As will be described under “Discussion,” the phosphate group of PC is held in the binding site by direct coordination to a pair of calcium ions. The failure of Gal-6-sulfate to bind suggests that somehow sulfate cannot make strong coordination bonds to calcium ions. Galacturonic acid (at 47 mM) was also totally ineffective as an inhibitor. However, serum amyloid P-component, another pentraxin structurally homologous to CRP, binds methyl 4,6-(1-carboxyethylidene)-β-D-galactopyranoside (Fig. 3) via its carboxylate interacting with calcium atoms much the same way as phosphate does in CRP (22). Perhaps in the case of galacturonic acid the carboxylate group is unfavorably oriented for the calcium chelation, probably because of close proximity of carboxylate to the sugar ring.

Lyssolecithin in the lipid vesicle or on the cell surface is known to be bound by CRP, whereas totally acylated lecithin is not (23). To assess the inhibitory potency of the individual lyssolecithin molecule in solution as well as to assess the importance of having free β-OH for binding, two lyssolechitins, each with one short fatty acid chain (C6 and C10) and 1-decanoyl-2-acetyl-phosphatidylcholine, were tested. All three phosphatidylcholine derivatives were soluble in water and expected to remain dispersed as monomers because of short acyl chains and the presence of a zwiterionic PC residue. The IC$_{50}$ values of these acylated phosphatidylcholine derivatives were all in 1–2 mM range, only slightly higher than that of PC-glycerol (0.5 mM), suggesting that the presence of free β-OH is not a requirement for binding. Perhaps, as suggested by others, physical disposition of lyssolecithin on the membrane and/or disruption of membrane structure caused by the presence of lyssolecithin are the reasons for preferential binding of lyssolecithin-containing vesicles. The compounds with amino groups, Lys (50 mM), Arg (11 mM), Lys-Lys (8 mM), and Lys$_4$ (4 mM), choline chloride (30 mM) as well as PL of DP 240 (5.6 μM) and histones (400 μM) did not inhibit the PC-dependent binding at the highest concentration tested, which is indicated in parenthesis.

**Inhibition of the Eu-PL Binding**

**Polyvalent Inhibitors**—Table III lists the inhibitory potency of all the polyvalent inhibitors tested. As observed for Eu-PC$_{40}$-Et-BSA binding, the inhibitory potency of PL is highly dependent on the number of cationic groups present, which is proportional to the molecular weight. A comparison of PL of DP 16 and DP 240 shows that an increase in DP of 15-fold resulted in the affinity increase of 1000-fold. Poly-t-Arg and poly-t-ornithine, although tested at only one molecular size, seem to inhibit at the concentration comparable to polylysines. Among the basic proteins tested, only a lysine-rich histone preparation (enriched in H-1) had a measurable IC$_{50}$ (220 μg/ml), although its affinity was much weaker than polyllysine of the comparable size, i.e. DP-60 PL (13 kDa) had IC$_{50}$ of 23 μg/ml. PC$_{27}$-Et-BSA could inhibit the Eu-PL binding but much less effectively (IC$_{50}$ = 2.7 μM) than it inhibited the Eu-PC$_{40}$-Et-BSA binding (IC$_{50}$ = 0.1 μM).

**Small Inhibitors**—Basic amino acids, Lys, Arg, and His, all showed a large affinity enhancement effect in the intermediate concentration range as shown in Fig. 4, the maximum bound amount being as high as 200% that bound in the absence of inhibitor. The reason for this interesting phenomenon is unclear, but to find out which groups are responsible for this effect, we tested various substituted Lys derivatives. As shown in Table IV, the presence of both unmodified α-carboxylic acid and α-amino group may be responsible, since N$_2$-acyethyl-Lys (slight activation) and Lys methyl ester had normal inhibition curves, whereas N$_2$-acyethyl Lys gave the activation (190%). A neutral amino acid, Ala, gave activation, although only up to 140%. Of all the amino-containing compounds tested, only Lys-Lys and Lys$_4$ had sub-mM IC$_{50}$ values, whereas all other amines were much poorer inhibitors.

**DISCUSSION**

**Affinity Enhancement by Polyvalent Ligands**—Because CRP is a doughnut-shaped pentamer with a PC-binding site on each subunit, a polyvalent ligand that can bind at multiple PC-binding sites should have much stronger affinity than the monovalent ligand. Indeed, as shown in Table I, the presence of 9 residues of PC on PC-ET-BSA resulted in a 370-fold higher affinity (IC$_{50}$ = 1.5 μM) than the monovalent parent structure, PC-glycerol (IC$_{50}$ = 550 μM). Moreover, the affinities of these short-armed PC-Et-BSAs continued to increase with a further increase in the PC content, which presumably means an improved chance of multiple PC-binding sites being occupied when a larger number of PC residues are present on the BSA molecule. A longer span of PC residues from the BSA surface appears to improve the chance of occupancy of a PC-binding site, since PC$_{27}$-AhAe-BSA had an affinity equivalent to PC$_{40}$-Et-BSA of the shorter arm length. However, with these long-armed PC-AhAe-BSAs, there was only a small increase in affinity with PC content beyond 27 mol/mol, suggesting that larger numbers of PC did not improve the chance of the binding-site occupancy. As mentioned under “Results,” PC-PLs showed affinity enhancement that was dependent on both PC and cationic contents. This fact strongly suggests that PC- and polycationic-binding sites are present on the same side of the CRP molecule, and binding at the two types of site has a synergistic effect on affinity.

As to the unmodified PL binding to CRP via polycationic binding sites, they too exhibited affinity enhancement because of multiple site occupancy. The smallest PL of DP-16 had a 3.7-fold higher affinity than Lys-Lys. A larger increase in the affinity of 1000-fold, going from a DP of 16 to 240, probably resulted from both the increased content of polycationic determinant and the longer span of the molecule.

**PC-binding Site**—The crystallographic structure of liganded human CRP showed that PC is bound in a shallow groove near the Ca$^{2+}$-binding site. At one end of the groove, two oxygen atoms of PO$_3^-$ make a direct coordination bond to calcium ions (Fig. 5), and at the opposite end a glutamate anion is in a proper distance to form an ion-pair with the positively charged nitrogen of choline (24). The sole unliganded oxygen of the phosphate group points away from the groove toward the solvent, so that a second ester group can be accommodated. As observed by others, we also found that the conversion of a phosphate monoester (PC) to a diester (PC-glycerol) lowers affinity substantially. This suggests that either the presence of a glycerol group physically interferes with binding or the formation of a diester somehow weakens chelation of calcium. We favor the latter explanation, since the affinity of glycerol phosphate is higher than inorganic phosphate by 5.5-fold, suggesting that the glycerol group actually has a small positive effect on the binding. It may be that the formation of diester changes the charge distribution and/or relative orientation of oxygen atoms sufficiently so that the calcium is ligated less efficiently.

As noted under “Results,” sugar phosphates can be divided into three groups depending on their inhibitory potency. Glec-6-P and Man-6-P, which have affinities comparable with inorganic phosphate, probably neither contribute nor interfere with
the binding, whereas Galα1-P and Glcα1-P were considerably less inhibitory than inorganic phosphate, suggesting that α-orientation and/or a shorter distance between the sugar ring and phosphate interfere with the binding (Fig. 6). The third group, which includes Gal-6-P, Rib-5-P, and 2-deoxy-β-ribose-5-P as well as adenylated derivatives of the latter two (AMP and dAMP) had a 10-fold higher affinity than P⁰₈, suggesting that these sugar residues contribute to the binding. The fact that the purine residue does not decrease the affinity means that the area is spacious enough to accommodate it easily. Thus, it is likely that the carbohydrate portion of the sugar phosphates are not bound in the choline binding area but in the adjacent space, where the glycerol moiety of PC-glycerol is bound, which is also the same space occupied by the acylated glycerol moiety of lecithins. Examination of the structures (Fig. 6) shows that, in all the sugar phosphates of higher binding affinity, the OH group closest to the phosphate is oriented axial to the sugar ring, i.e. vertical to the average plane of the ring. Moreover, although there are three additional OH groups present in Gal-6-P, only this proximal, vertical OH is present in 2-deoxy-β-ribose-5-P and dAMP. We speculate, therefore, that this axial OH group participates in the binding, perhaps via a hydrogen bond, whereas the presence of the rest of the sugar moiety does not matter much. Similarly, since the IC₅₀ of glycerol phosphate is lower than that of inorganic phosphate, one or both OH groups of glycerol phosphate may contribute energetically to the binding.

The enhanced affinity of Gal-6-P as compared with other hexose-6-Ps may have a direct bearing on the following biochemical observations. CRP binds and precipitates pneumococcal group-specific C-substance (whence the name C-reactive protein originates) via its PC residues (25). However, a capsular polysaccharide of type IV pneumococcus known as S IV, after removal of ketal-linked pyruvic acid, can precipitate CRP as effectively as C-substance despite the fact that S IV has a very low PC content, and Gal-6-P, its major component, is considered to be the likely target of CRP binding (26). More recently, CRP was found to bind with high affinity to the promastigote form of _Leishmania donovani_ via the (6Galβ1,4Manα1-1-P) repeating units of its lipophosphoglycan, which resulted in complement activation as well as parasite opsonization and subsequent entry into macrophage (27). However, CRP does not bind to another species (_Leishmania major_), which has a heavily substituted form of the disaccharide repeating unit. These results suggest that high density of Gal-6-P alone is sufficient to generate a high binding affinity in the absence of PC.

**Binding Site for Polycationic Compounds**—Because Lys-Lys and Lys₄ have a comparable but much higher inhibitory potency against Eu-PL than all other amines tested, two cation-binding sites that easily accommodate two ε-amino groups of Lys-Lys seem to be the basic binding unit on each subunit. PLs manifest much higher affinity than Lys-Lys, since PLs can simultaneously occupy similar sites on other subunits. The preferred orientation of two ε-amino groups of Lys-Lys should put them on the opposite side of the peptide backbone, which means that the secondary amine-binding sites may be as far away as 15 Å. We tested several diaminoalkanes of various lengths (from C₂ to C₁₂) to see if affinity close to Lys-Lys can be attained. Most of the diamines had IC₅₀ values in the range of 22 ± 5 mM, the value comparable with that of a monoamine (e.g. octylamine), meaning that there is no affinity enhancement by the presence of the second amino group. Diaminoctane was the best inhibitor among them with its IC₅₀ lower by 6-fold, but even this IC₅₀ value is still considerably higher than that of Lys-Lys. It may be that there is another structural element in Lys-Lys that is augmenting the binding affinity or that structural rigidity imposed by the presence of peptide

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**Fig. 5. PC-binding area of CRP.** The filled circles indicate two calcium ions.

**Fig. 6. Structure-IC₅₀ relationship among phosphate-containing inhibitors.**
bond improves the binding.

CRP binds a number of nuclear components such as nucleosome, ribonucleoprotein, chromatin, and histones (28, 29). Binding of chromatin and nucleosome by CRP and subsequent dissolution are thought to be an important mechanism of removing potential self-antigens from the site of tissue injury (30). Histones, which are integral components of chromatin and nucleosome, have been shown to bind to CRP (29). Of the two histone preparations we tested, only type IIIS, which is a Lys-rich preparation containing mostly H1 histone, was inhibitory against Eu-PL with an IC50 in the 10 μM level. Neither preparation was inhibitory against Eu-PC40-Et-BSA. Therefore, it appears that histone is binding at the polycationic site and not at the PC-binding site. The binding of H1 is presumably via Lys-Lys and possibly Lys-Arg (or Arg-Lys) sequences, since human histone H1 has 14 such sequences out of 193 amino acid residues. A partial sequence of bovine H1 also shows a few such sequences. The fact that H1 has a 10-fold lower affinity as compared with PL of similar size (DP-60, 13 kDa) can be easily explained by the difference in the Lys-Lys content.

**Relationship between PC-combining Area and Polycationic Binding Area**—The PC-combining area contains both phosphate and cation (trimethylammonium) binding areas (Fig. 5). Interestingly, none of the amines, including choline chloride at 30 mM, showed any discernible inhibition of Eu-PC40-Et-BSA binding to CRP. From the IC50 values of PC (0.05 mM) and of phosphate (25 mM), one would expect the KD of choline chloride to be ~2 mM if the binding energies involved at the two areas are additive and IC50 values are proportional to KD. The failure of choline chloride to show any inhibition, therefore, suggests to us that the binding area for the quaternary amino group of PC is inaccessible to amines unless the phosphate site is occupied first. This means that the PC-binding site is not one of the polycationic binding sites, and thus, there must be at least three cation-binding sites on each CRP subunit. PC apparently can bind at all three cationic sites, since it inhibits the Eu-PL binding as effectively as amines. This hypothesis can also explain the result that PC40-Et-BSA inhibits Eu-PL binding, albeit less effectively (IC50 1.0 μM) than its inhibition of Eu-PC40-Et-BSA (IC50 0.1 μM), whereas PL cannot inhibit Eu-PC40-Et-BSA at all.

The ability of PC to bind to both the PC and polycationic sites means that inhibitory activity of PC by does not necessarily mean that the binding in question is via the PC-binding site. For instance, it has been proposed that the binding of histones to CRP is via the PC-binding site, since PC competed effectively against the histone binding (29). As discussed earlier, we believe that histone H1 is bound at the polycationic site, and the effective inhibition of histone binding by PC can easily be explained on the basis of PC competing effectively at the polycationic site.

**Summary**—The x-ray crystallography of PC-CRP complex shows that the phosphate group of PC coordinates directly to the bound calcium ions. A sole unliganded oxygen of phosphate can form a second ester bond, which would point toward the solvent space away from the protein surface.

We observed that CRP interacts with hydroxylated ligands such as Gal-6-P in the area where the second ester is located, most likely via hydrogen bonding to a hydroxyl group located near the phosphate group. This is based on the observation that the inhibitory potency of various sugar phosphates differs by more than 10-fold and that this area can accommodate large molecules such as dsAMP and acylated phosphatidylinolines.

The basic binding unit of polycationic compounds such as PL is likely to be a pair of amine-binding sites on each subunit of CRP. So far as we have tested, the two ε-amino groups of Lys-Lys represent the best pair of amino groups for this site. Although the PC-binding site has a cation binding element, none of the amines tested can gain access to this site. Therefore it appears that the polycationic binding site does not overlap with the PC-binding site.

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