Glycoconj J (2006) 23:317–327
DOI 10.1007/s10719-006-6173-x

Carbohydrate ligands of human C-reactive protein: Binding of neoglycoproteins containing galactose-6-phosphate and galactose-terminated disaccharide

Reiko T. Lee · Yuan C. Lee

Received: 1 September 2005 / Revised: 31 October 2005 / Accepted: 11 November 2005

Abstract Binding of carbohydrate ligand by human C-reactive protein (CRP), in both native form and structurally deviated form (neoCRP or mCRP), was investigated using galactose-6-phosphate (Gal6P)- and Galβ3GalNAc-containing bovine serum albumin (BSA) derivatives. To this end, we synthesized glycosides of Gal6P and Galβ3GalNAc that can potentially generate a terminal aldehyde group. ω-Aldehydo glycosides were then conjugated to BSA *via* reductive amination. Using these neoglycoproteins, we showed that: (1) Gal6P-BSA and Galβ3GalNAc-BSA bound to both forms of CRP, the former with or without calcium and the latter only in the absence of calcium; (2) phosphate-containing ligands can be bound with or without calcium, but the binding is much stronger in the presence of calcium than in the absence, underscoring the importance of direct coordination of phosphate to two calcium ions observed in the X-ray structure of phosphorylcholine (PC)–CRP complex; (3) cross-inhibition studies further corroborated the hypothesis that binding sites of PC and sugar are contiguous; (4) while PC-BSA bound to the native CRP over a wide pH range of 4.5 to 9, all the carbohydrate ligands and protamine-BSA (poly-cation-based ligand) exhibited optimal binding at around pH 6 to 6.5; and (5) ligand-binding conformation of mCRP appears to be more fragile than that of the native CRP in the acidic media (pH < 6).

Keywords C-reactive protein · Europium fluorescence · Ligand-binding assay · Neoglycoproteins

Abbreviations

AIBN	2,2′-azobis(2-methylpropionitrile)
BCA	bicinchoninic acid
BSA	bovine serum albumin
Bn	benzyl
Bz	benzoyl
Bzl	benzylidene
Cbz	benzyloxycarbonyl
CRP	C-reactive protein
DCC	dicyclohexylcarbodiimide
DMF	*N*,*N*-dimethylformamide
DTPA	diethylenetriamine pentaacetic acid
EDC	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide
Gal6P	6-O-phospho-D-galactopyranose
α-GP	α-glycerophosphate
HRP	horseradish peroxidase
HSA	human serum albumin
Lac	lactose
MBP	mannose-binding protein
MES	2-(*N*-morpholino)-ethanesulfonic acid
HEPES	*N*-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
AMPSO	3-[*N*,*N*-dimethyl(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid
OPD	*o*-phenylenediamine
PC	phosphorylcholine
PEG	polyethyleneglycol
PBS	phosphate-buffered saline
TFAah	6-(*N*-trifluoroacetylamino)hexyl
TLC	thin-layer chromatography

R. T. Lee (✉) · Y. C. Lee
Department of Biology, The Johns Hopkins University 3400 N. Charles Street, Baltimore, MD 21218, USA
e-mail: reiko.lee@jhu.edu
Tel: +1-410-516-7322
Fax: +1-410-516-8716

Introduction

Human C-reactive protein (hCRP) is a prototype acute phase reactant that plays important roles in the defense against microbes and also in the clearance of the inflammation or

tissue injury sites [1,2]. The removal of microbes and apoptotic cells can be accomplished by CRP first binding to one of its ligands, phosphoryl choline (PC) groups, which are present on the pneumococcal cell-wall C-polysaccharide or the PC groups that became accessible on the apoptotic cells. This will trigger the classical complement cascade leading to eventual elimination of these cells [3]. In addition to PC, certain polysaccharides[4–6] and poly-cationic macromolecules, such as poly-L-lysine and protamine sulfate [7], are known to be bound by CRP and activate the complement system in a similar fashion.

hCRP in circulation, known as native CRP, is a non-covalently associating pentamer with each subunit interacting side by side with two neighboring subunits in a circular fashion to form a disc-like structure with a hole in the middle [8]. The two sides of the disc present very different surfaces. One side displays two closely located calcium binding sites on each subunit, and the phosphate group of PC is directly bound to the two calcium ions [9]. The opposite side is the site of interaction with the complement system. In addition to native CRP, a slightly denatured but physiologically significant form of CRP, known as neoCRP or mCRP, exists. mCRP is formed under mild denaturing conditions, such as acidic media below pH 4, heating at 65°, and coating on a plastic surface. mCRP presents new antigenic epitopes, and is thought to be a monomeric form, similar to the nascent subunit that can associate in a different fashion to form large aggregates [10,11]. mCRP is found deposited on the site of inflammation and is shown to exert immunomodulatory effects on lymphocytes [12–14].

A considerable knowledge exists with regard to the PC-based binding, including the PC-liganded CRP crystal structure [15]. As to the polycationic binding, we showed it to depend solely on the presence of multiple units of Lys-Lys or Arg-Arg sequences [16]. The carbohydrate-based binding, however, is much more complex and not well characterized. Although there are a few well-characterized structures of microbial polysaccharides that bind strongly to native CRP, the complexity and the varied structures of these polysaccharides do not shed much light on the minimum structural requirement for the binding to native CRP. While the lactosylated HSA or BSA binds to mCRP [17,18], the amount of Lac-BSA bound to native CRP was miniscule [16]. As it turns out, Lac-BSA was a poor choice as a binding probe, since the inhibition assay using mCRP showed that lactose itself was a poor inhibitor. The best, small carbohydrate inhibitor was a negatively charged Gal6P, which was ∼6-fold better inhibitor than the best neutral sugar inhibitors which include Galβ3GalNAc, and these sugars were in turn ∼8-fold better than lactose. Based on these results, we surmised that the neoglycoproteins containing Gal6P and Galβ3GalNAc may represent the best anionic and neutral sugar ligands for CRP. We synthesized such BSA derivatives and demonstrated their binding to both native CRP and mCRP. These neoglycoproteins allowed us to probe the carbohydrate-binding characteristics of native CRP, as well as to expand the characterization on the binding to mCRP.

Materials and methods

Materials and general methods

Chemicals were generally obtained from Sigma-Aldrich-Fluka (St. Louis, MO). Europium nitrate was from Alfa Aeser (Ward Hill, MA) and organic buffers were from Research Organics (Cleveland, OH). Protamine sulfate from salmon milt was from Calbiochem (La Jolla, CA), and horseradish peroxidase conjugate of goat anti-human CRP was from Bethyl Labs, Inc. (Montgomery, TX). Stable hydrogen peroxide buffer, *o*-phenylenediamine (OPD), SuperBlock and maleic anhydride activated microplates were from Pierce (Rockford, IL). The preparations of 6-trifluoroacetamidohexyl β-D-galactopyranoside (TFAah β-Gal) [19], PC-BSA [20], and Lac-BSA [21] have been reported. PC is attached to BSA amino groups *via* ethylene linkage, and lactosyl residues are attached as Lac-$SCH_2CONH(CH_2)_2$-protein. Recombinant human CRP was a gift of Dr. I. Takagahara (Oriental Bio-Service Kanto, Tsukuba, Japan).

The following colorimetric methods were used for the determination of concentrations: neutral sugar, phenol–sulfuric acid method [22], organic phosphate, pyrolysis–molybdate method [23], protein, bicinchoninic acid (BCA) assay [24]; and primary amino group, fluorescamine assay [25]. The detections of aromatic groups and carbohydrates on silica gel (F_{254}) TLC plates (E. Merck) were by UV absorption and by spraying with 15% sulfuric acid in 50% ethanol and heating. The ^{1}H-NMR spectra were recorded using a Varian Unity 400 MHz FT-NMR spectrometer. The europium fluorescence was determined after dissociating europium from the DTPA-chelate in a fluorescence enhancing milieu [26], in 96-well microplate, using a Wallac microplate fluorometer (Victor 1420 multilabel counter).

General synthetic methods

O-Benzoylation was by reacting the substrate with benzoyl chloride in pyridine. After the reaction, the mixture was evaporated, dissolved in chloroform or methylene chloride, and washed with water. Organic layer was dried and evaporated. *O*-Benzylation was carried out in DMF by first reacting the available OH groups with NaH, followed by addition of benzyl bromide. The reaction mixture was diluted with chloroform, extracted with water, and the chloroform solution was dried and evaporated. De-*O*-acylation was by 10 mM NaOMe in dry methanol for 2 h at room temperature, followed by

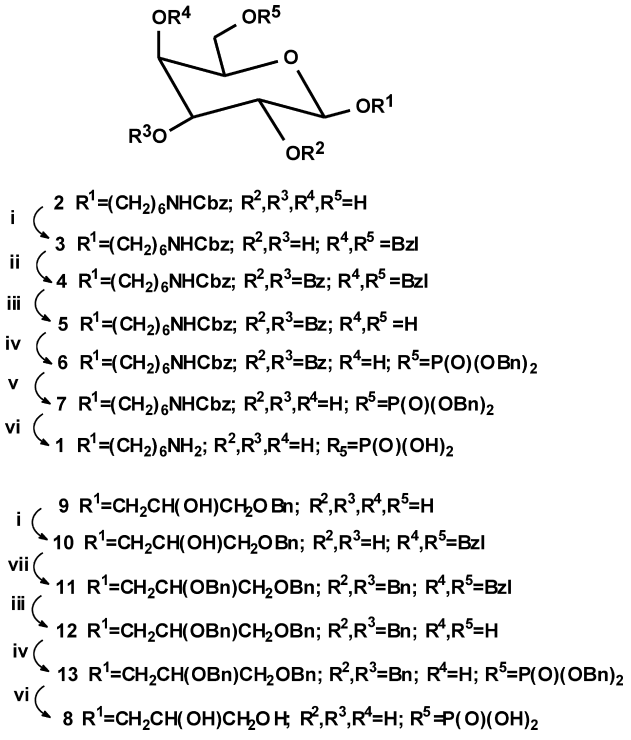

Fig. 1 Syntheses of Gal6P glycosides. Conditions: (i) 1,1-dimethoxytoluene, *p*-toluenesulfonic acid; (ii) benzoyl chloride/pyridine; (iii) 80–90% acetic acid, 80°; (iv) DIDBP, tetrazole, then 3-chloroperoxybenzoic acid; (v) 10 mM sodium methoxide in methanol; (vi) H_2 with Pd/C; (vii) NaH, benzylbromide.

neutralization (Dowex 50, H^+ form) and evaporation. *O*-Benzylidenation was carried out in *N*, *N*-dimethylformamide (DMF) with 2–4 fold excess 1,1-dimethoxytoluene and a catalytic amount of *p*-toluenesulfonic acid at 50 to 60° for 4 h. For de-*O*-benzylidenation, the compound in 80 to 90% acetic acid was heated at 80° for 1 h and evaporated. Hydrogenation was carried out in a Brown hydrogenator [27] using H_2 at atmospheric pressure and 10% Pd on carbon as catalyst. The formation of glycosidic linkage was by reacting an OH-containing compound with per-*O*-acetylated 1-bromo sugar (abbreviated as acetobromo sugar hereafter) in 1.1 to 1.5-fold molar excess over the alcohol in toluene–nitromethane mixture (1:1) with $Hg(CN)_2$ (equimolar to 1-bromo sugar) and Drierite for overnight at room temperature. The reaction mixture as chloroform solution was washed with NaCl (1 M) and KBr (1 M), and its ethanolic solution was fractionated on a column of Sephadex LH-20 (5 × 195 cm) using 95% ethanol as eluant. Fractions were monitored with TLC.

Preparation of glycosides of Gal6P and Galβ3GalNAc for conjugation to BSA

We synthesized two β-glycosides of Gal6P: a long form having $-O(CH_2)_6NHCO(CH_2)_2CONHCH_2CH(OCH_3)_2$ as aglycon and a short form having glyceryl aglycon. Reaction schemes for these preparations are shown in Fig. 1. Both preparations start with β-galactosides with properly protected aglycon (compounds **2** and **9**), which are converted to the respective 4, 6-*O*-benzylidene derivatives, followed by protection of remaining hydroxyls with benzyl or benzoyl group, removal of the benzylidene group to expose 4- and 6-OH groups, and mono-phosphorylation at the 6-position by use of a bulky phosphorylating agent, dibenzyl diisopropylphosphoramidite, in a slight excess of the molar equivalent amount [28,29]. In the case of the long form of Gal6P glycoside, the final product shown in (Fig. 1), 6-aminohexyl β-Gal6P (**1**), is conjugated with $HOOC(CH_2)_2CONHCH_2CH(OCH_3)_2$ to form an ω-acetal glycoside as described [18].

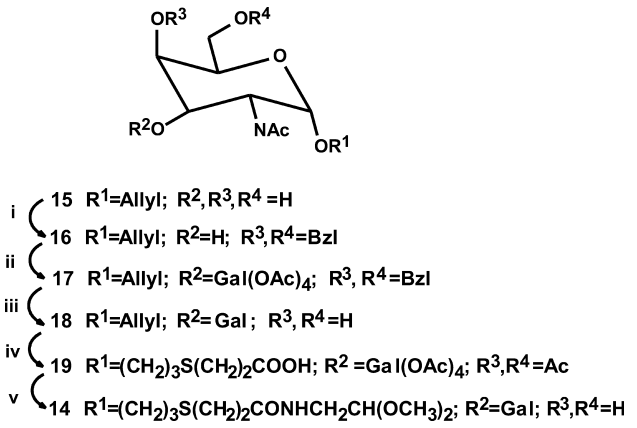

Fig. 2 Synthesis of ω-acetal glycoside of Galß3GalNAc. Conditions: (i) 1,1-dimethoxytoluene, *p*-toluenesulfonic acid; (ii) acetobromoGal, $Hg(CN)_2$; (iii) 10 mM sodium methoxide in methanol; then 80% acetic acid, 80°; (iv) acetic anhydride/pyridine, then 3-thiopropionic acid, AIBN, UV irradiation; (v) 2-aminoacetaldehyde dimethyl acetal, 1-hydroxybenzotriazole, DCC; then 10 mM sodium methoxide in methanol.

Synthesis of glycoside of Galβ3GalNAc, **14**, as shown in Fig. 2 starts with allyl α-GalNAc, which was prepared from per-*O*-acetylated α-GalNAc [30], by refluxing it with allyl alcohol in DMF in the presence of BF_3-etherate. After deacetylation followed by benzylidenation, the product, **16**, was galactosylated to yield **17**. After de-protection and per-*O*-acetylation, the aglycon was elongated first with thiopropionic acid via Michael addition (UV irradiation in the presence of AIBN), then by conjugating 2-aminoacetaldehyde dimethylacetal to the newly generated carboxyl group using dicyclohexyl carbodiimide as coupling agent in the presence of 1-hydroxybenzotriazole.

Throughout the syntheses, reactions were monitored by TLC and the products were purified by crystallization. If necessary, silica gel or gel filtration chromatography was performed before crystallization. Most of the intermediates and final products were characterized by ^{1}H-NMR. Here we present only the ^{1}H-NMR data of the final products or the intermediates immediately before the final de-protection.

^{1}H-NMR of 6′-(benzyloxycarbonylamino)hexyl-6-*O*-(di-*O*-benzylphosphoryl)-β-D-galactopyranoside (**7**) in CD_3OD: δ 7.473–7.28 (m, 15 H, aromatic Hs); 5.138, 5.118, 5.060 (s, 2 H, benzyl CH_2); 4.657 (d, J = 2.81 Hz, 1 H, H4); 4.44–4.33 (m, 2 H, 2 H6s); 4.298 (d, J = 7.6 Hz, 1 H, H1); 3.872 (m, 1 H, H5); 3.693 (broad s, 1 H, 1/2 CH_2O); 3.622 (m, 1 H, H3); 3.58–3.527 (m, 2 H, H2 and 1/2 CH_2O); 3.108 (t, J = 7.2 Hz, 2 H, CH_2N); 1.636 (m, 2 H, CH_2); 1.505 (m, 2 H, CH_2); 1.435–1.29 (m, 4 H, 2 CH_2). Comparison of ^{1}H-NMR spectra of **7** with the non-phosphorylated counterpart indicated that phosphate group is at the 6-position. Product **7** and the final product, ω-acetal glycoside, both showed large down-field shifts of H4 and H6 signals, and only small shifts or no shift for other H signals. These tendencies are similar to ^{1}H-signals of Gal6P versus galactoside. See also ^{1}H-NMR characterization of **8** below.

^{1}H NMR of **8** in D_2O: δ 4.49 (d, J = 2.8 Hz, 1H, H4); 4.378 and 4.374 (d, J = 8 Hz, 1H, H1); 4.303 (broad d, J = 12.8 Hz, 1H, H6); 4.158 (d, d, J = 12 and 21.8 Hz, 1H, H6); 3.88 (d, d, J = 3.6 and 10.4 Hz, 1H, H3); 3.845–3.783 (m, 2H, H2 and H5); 3.672–3.451 (m, 5H, aglycon Hs). For the position assignment of *O*-phospho group, ^{1}H-NMR spectrum of **8** in D_2O was compared to that of 2,3-dihydroxypropyl β-D-galactopyranoside, which was obtained from **9** by hydrogenolysis. There were small down-field shifts in H1, H2, H5 and aglycon H signals, major down-field shifts of H4 and H6 signals, and no change in H3 signal, indicating that *O*-phospho group is at 6-position. Signal for H4 of non-phosphorylated glycoside was at 3.796 (d. J = 2.8 Hz, 1H) and those of H6s were at 3.825–3.771 (m, 2H).

^{1}H-NMR of **14** in D_2O: There are 6 methylene groups in the aglycon. They are labeled in alphabetical order from the one closest to the sugar as "a" and the farthest as "f".δ 4.756 (d, J = 3.6 Hz, 1H, H1); 4.419 (t, J = 5.2 Hz, 1H, methine in aglycon); 4.336 (d, J = 8.0 Hz, 1H, H1′); 4.189 (dd, J = 2.8 and 11.2 Hz, 1H, H3′); 3.857 (d, J = 6.4 Hz, 1H, unknown); 3.776 (d, J = 3.2 Hz, 1H, H4); 3.693–3.578 (m, 5H, unknown); 3.528 (dd, J = 3.4 and 4.4 Hz, 1H, H6 or H6′); 3.494 (dd, J = 3.4 and 10 Hz, 1H, H6 or H6′); 3.452–3.35 (m, 2H, unknown); 3.305 (s, 6H, O-methyl Hs); 3.241 (d, J = 5.2 Hz, 2H, f); 2.694 (t, J = 7 Hz, 2H, e); 2.564 (t, J = 7 Hz, 2H, d); 2.443 (t, J = 7 Hz, 2H, c); 1.894 (s, 3H, N-acetyl methyl Hs); 1.778 (m, 2H, b). Unassigned signals for 8 Hs should represent H2′, H3, 2 H5, 2 H6 or H6′, and 2 "a" methylene Hs.

Preparations of neoglycoproteins and Eu labeling

The glycosides to be conjugated to protein contained either a glyceryl group or a terminal acetal group. The aldehydo group was generated from ω-acetal glycosides by heating at 100 °C in 0.05 M HCl for 20 min, followed by neutralization and evaporation [21], and from the glyceryl aglycon by periodate oxidation ($NaIO_4$ at 2 mM initially, and additional amounts introduced every 30 min until equimolar amount is reached). The periodate-oxidized product was evaporated and purified on a column of Sephadex G-15 in water. The aldehydo glycosides were then conjugated to BSA at pH 7.2 with pyridine borane as the reducing agent [21]. The BSA derivatives were stored as lyophilized products after successive dialysis against PBS, 0.15 M NaCl (twice) and water (twice). Contents of Gal6P and Galβ3GalNAc in the BSA derivatives were determined as organic phosphate and by the phenol-sulfuric acid method using proper standards. The structure of linking arms between the sugar and BSA and the assigned abbreviations of BSA derivatives are shown below. BSA derivative containing the long form of Gal6P: $-(CH_2)_6NHCO(CH_2)_2CONH(CH_2)_2-$, Gal6P-ASA-BSA; the short form of Gal6P: $-(CH_2)_2-$, Gal6P-ET-BSA; Galβ3GalNAc-containing BSA: $-(CH_2)_3S(CH_2)_2CONH(CH_2)_2-$, Gal$\beta$3GalNAc-PEE-BSA.

To attach europium to proteins, neoglycoproteins (0.5 to 1 mg) were derivatized with the acetal-containing DTPA-labeling reagent as described [18,31]. Europium nitrate was then added, and the BSA derivative containing Eu-chelate was purified by gel filtration.

Conjugation of protamine to BSA

Protamine sulfate from salmon milt is a mixture of highly basic polypeptides with average molecular weight of ~6000. It contains a high percentage (>50%) of arginine, but no lysine or acidic amino acids, and N-terminus is frequently occupied by a proline. For this reason, we utilized the C-terminus of protamine for conjugation to BSA. Protamine sulfate (30 mg), 1,8-diaminooctane (45 mg, ~60-fold molar excess over protamine) and EDC methiodide (30 mg, ~20-fold molar excess over protamine) in 5 ml of 0.2 M NaCl at pH 5.5 was kept at room temperature overnight, and the mixture was separated on a column (2 × 140 cm) of Sephadex G-15 in water, collecting 5 ml/fraction. The monitoring of eluted fractions by microBCA and fluorescamine assays showed that reproducibly only the earliest eluting fractions of protamine (~ 1/4 of the whole protamine peak) contained free amino group. It may be that only the largest protamine molecules possess the easily modifiable carboxylate group. The amino-containing protamine fractions were combined and lyophilized.

In order to increase the efficiency of coupling to BSA, the amino-containing protamine was conjugated to partially succinylated BSA, which had been prepared by reacting BSA in 0.2 M sodium borate buffer, pH 8.5, with succinic anhydride (0.66 equivalent of total amino group), followed by dialysis and lyophilization. Conjugation of the protamine derivative

(3 mg) and succinyl-BSA (25% of amino group had been succinylated, 13 mg) was carried out in 0.1 M cacodylate buffer, pH 6.5, containing 0.1 M NaCl using EDC methiodide (6 mg, added in 2 portions) as the coupling agent. The mixture was fractionated on a column (2.5 × 110 cm) of Sephadex G-100 using 10 mM HEPES buffer, pH 7.2, as eluant. Fractions containing protein were combined, lyophilized and labeled with DTPA as described above.

Ligand-binding assay for mCRP

The assay method has been described in detail [18]. It involves plating CRP in the wells of a high protein-binding 96-well microplate [17], incubating the CRP-surface with an Eu-ligand with or without inhibitor, and the subsequent release of Eu from the ligand–CRP complex for fluorescence measurement. The standard assay is done in a MES [2-(*N*-morpholino)-ethanesulfonic acid] buffer at pH 6.5 containing 0.15 M NaCl and 0.1% BSA. For blanks, either BSA was immobilized in lieu of CRP or Gal6P or α-glycerophosphate (α-GP) was included in the incubation at 100 mM. Inhibition assays were carried out at 50 nM Eu-Gal6P-BSA or 100 nM Eu-Galβ3GalNAc-BSA, and the results were plotted in semi-logarithmic fashion (Fig. 6) to obtain the IC_{50} (concentration that causes 50% reduction in the binding) values. For the pH studies, the following buffering salts (at 25 mM) were used: pH 4.5, acetate; pH 5.0 to 6.5, MES; pH 7.0 to 8.0, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES); and pH 8.5 to 9.0, 3[*N*,*N*-dimethyl(2-hydroxyethyl)amino]-2-hydroxylpropanesulfonic acid (AMPSO).

PEG-Assisted precipitation–filtration assay for native CRP

The assay method which is based on precipitating ligand–CRP complex with PEG, followed by filtration through a Durapore filter plate using MultiScreen filtering system (Millipore) has been described in detail [16].

ELISA-based native CRP assay

The native CRP assay based on the PEG-assisted precipitation and filtration described above produced a very high non-specific background above pH 7, which we attributed to non-specific binding mainly to the filter. To overcome this problem, we devised an assay method that does not involve filtration. A maleic anhydride-activated 96-well plate was reacted with PC-BSA or neogycoproteins according to the manufacturer's protocol. For control, BSA was similarly immobilized. After blocking twice with SuperBlock, the wells were washed and then incubated with a CRP solution for 1h with shaking. The incubation buffers at various pHs contained 25 mM buffering salt, 0.15 M NaCl, and 0.1% BSA. Washing buffers were as above except they contained 0.05% Tween 20 instead of BSA. The wells were washed and then incubated with a goat anti-human CRP HRP conjugate (500–1000 dilution of 1 mg/ml stock solution) for 1 h, then with an OPD–peroxide solution per manufacturer's instruction. The developed color was determined at 490 nm at 15 min and 30 min using BioRad microplate reader.

Results

Binding of Eu-Gal6P-BSA and Eu-Galβ3GalNAc-BSA to mCRP

We have shown earlier that binding of Eu-PC_{40}-BSA and Eu-Lac_{40}-BSA had opposite calcium requirement, *i.e.*, the former bound in the presence of calcium while the latter bound in the absence of calcium [18]. The newly synthesized Eu-$Gal6P_{22}$-ASA-BSA, on the other hand, was bound throughout the calcium range of 0–10 mM (not shown), which agrees well with the earlier results of Gal6P inhibiting both Eu-PC_{40}-BSA and Eu-Lac_{40}-BSA equally well, and suggests that Gal6P binds between the PC and the neutral sugar binding areas. As to the binding of neoglycoprotein containing a neutral sugar, Eu-Galβ3GalNAc$_{41}$-PEE-BSA, binding occurred only in the absence of calcium (Fig. 3) in agreement with the earlier result of Eu-Lac_{40}-BSA [18].

The pH dependence of Eu-$Gal6P_{22}$-ASA-BSA binding to mCRP was determined from pH 5 to 9 at 5 mM calcium or without added calcium. Figure 4 shows the results from several experiments which were compiled by setting the net bound fluorescence at pH 6.5 as 100%. The optimal binding occurred at pH 6.5 with or without calcium, but the pH range of binding seems to be narrower in the presence of calcium than in the absence. Figure 5 shows that the pH dependence of Eu-Galβ3GalNAc-BSA was similar to that of Eu-Lac-BSA

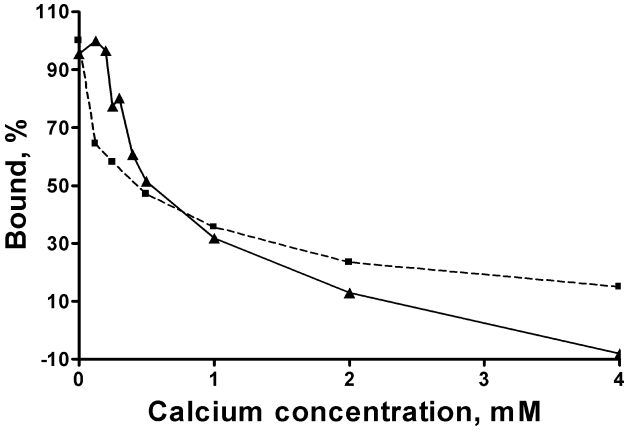

Fig. 3 Dependence of binding of Eu-Galß3GalNAc$_{41}$-PEE-BSA on calcium concentration. ■, mCRP; ▲, native CRP.

Table 1 Inhibition constants of small inhibitors in mCRP assay using Eu-PC_{40}-BSA, Eu-Gal6P_{22}-ASA-BSA, and Eu-Lac_{40}-BSA as reference ligands

	IC_{50}, mM			
	Eu-PC_{40}-BSA[a]	Eu-Gal6P_{22}-BSA		Eu-Lac_{40}-BSA[a]
Inhibitor	+Ca	+Ca	−Ca	−Ca
PC	0.017	1.5	15	36
Pi	3.6	0.45	3.8	2.7
α-GP	1.35	0.72	22	2.0
Gal6P	1.1	0.25	0.8	1.35
GalA	8.7	10	10	3.8
TFAah β-Gal	>30	~50	~33	4.2
Galβ3GalNAcα-All	ND[b]	>50	~45	8

[a]Data for Eu-PC_{40}-BSA and Eu-Lac_{40}-BSA are from reference [18].
[b]ND = not determined.

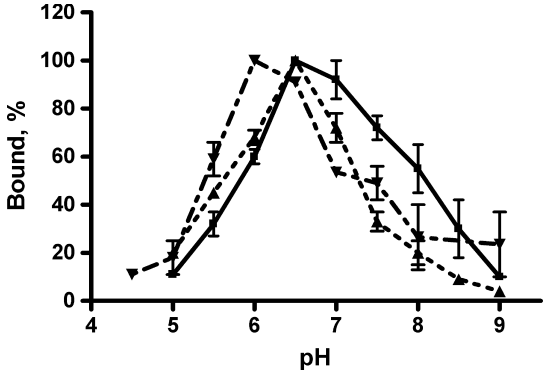

Fig. 4 Dependence on pH of binding of Eu-Gal6P_{22}-ASA-BSA to mCRP: ■, no calcium added; ▲, in the presence of 5 mM calcium. Dependence on pH of binding of Eu-Gal6P_{38}-ET-BSA to native CRP: ▼, in the presence of 5 mM calcium.

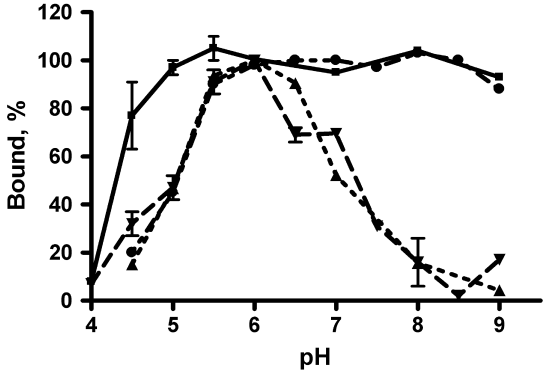

Fig. 5 Dependence on pH of binding of various Eu-labeled ligands. ●, Eu-PC_{40}-BSA to mCRP; ▲, Eu-Galß3GalNAc$_{41}$-PEE-BSA to mCRP; ▼, Eu-protamine-BSA to mCRP; ■, Eu-PC_{40}-BSA to native CRP. Data for Eu-labeled PC_{40}-BSA to native CRP and mCRP are from references [16] and [18], respectively.

[18]. At pH 6.5, binding of both Gal6P- and Galβ3GalNAc-BSAs increased in a dose-dependent manner up to 150 nM, former in the presence of 5 mM calcium and the latter without added calcium (not shown). Non-specific binding of the Eu-ligands in these assays ranged from 13 to 20% of the total bound amount in the absence of a competitor.

The inhibition studies using Eu-Gal6P_{22}-ASA-BSA was carried out at pH 6.5 with 5 mM calcium or without. Figure 6 shows as example inhibition curves of Gal6P and α-GP with and without calcium. The inhibition constants of small inhibitors are presented in Table 1, and those for neoglycoproteins in Table 2. Table 1 also lists for comparison the earlier inhibition data of Eu-PC_{40}-BSA and Eu-Lac_{40}-BSA. The inhibition data using Eu-Galβ3GalNAc-BSA were not included, since the results were similar to those obtained with Eu-Lac_{40}-BSA. Because Eu-Gal6P-BSA (both ASA and ET type) can bind with or without calcium, we were able to make a side-by-side comparison of the effect of calcium on the inhibition potencies of various compounds, which was not possible using either Eu-PC_{40}-BSA or Eu-Lac_{40}-BSA. Results in Table 1 (under the Eu-Gal6P_{22}-BSA subheading) show that all the phosphate-containing inhibitors, *i.e.*, PC, α-GP, and Pi, with the exception of Gal6P, had 8-fold or higher affinity in the presence of calcium than in the absence (see also Fig. 6).

In general, inhibition of the self was most effective; for instance PC (IC_{50} = 0.017 mM) was the best inhibitor of Eu-PC-BSA and Gal6P (IC_{50} = 0.25 mM) was the best inhibitor of Eu-Gal6P-BSA. However, this is not true for Eu-Lac-BSA or Eu-Galβ3GalNAc-BSA, since Gal6P was much better inhibitor than the best neutral sugar inhibitors, *e.g.*, TFAah β-Gal and α-allyl (all) glycoside of Galβ3GalNAc. This demonstrates the dominant role the phosphate group plays in binding the carbohydrate ligand. This is also manifested in the inhibition constants of the two neutral glycosides being much higher against Eu-Gal6P-BSA (IC_{50} = 30–50 mM) than against Eu-Lac-BSA (IC_{50} = 4–8 mM). These facts emphasize the strong affinity-enhancing effect of the 6-*O*-phospho group of Gal6P even in the absence of calcium.

Table 2 IC_{50} values of neoglycoproteins obtained by mCRP assay using Eu-Gal6P_{22}-ASA-BSA (Ligand 1) and Eu-Galβ3GalNAc$_{41}$-PEE-BSA (Ligand 2) as reporter ligands

	Sugar coupling level	IC_{50}, μM		
		Ligand 1		Ligand 2
Inhibitor	mol/mol	+calcium	−calcium	−calcium
Gal6P-ASA-BSA	21	9.5	12.5	11
	30	7.6	ND[a]	ND
Lac-BSA	19	NT[b]	35	ND
	40	NT	24	28
Galβ3GalNAc- PEE-BSA	41	NT	ND	16

[a]ND = not determined.
[b]NT = Not tested, since these inhibitors do not bind in the presence of calcium.

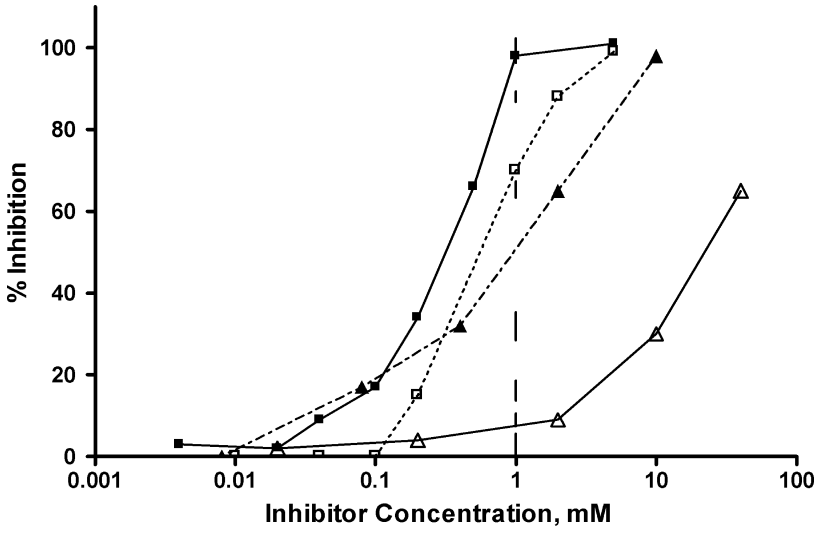

Fig. 6 Inhibition of Eu-Gal6P_{38}-ET-BSA binding to mCRP. Solid symbols: binding in the presence of 5 mM calcium chloride; open symbols: binding in the absence of calcium. Squares: Gal6P; triangles: α-GP.

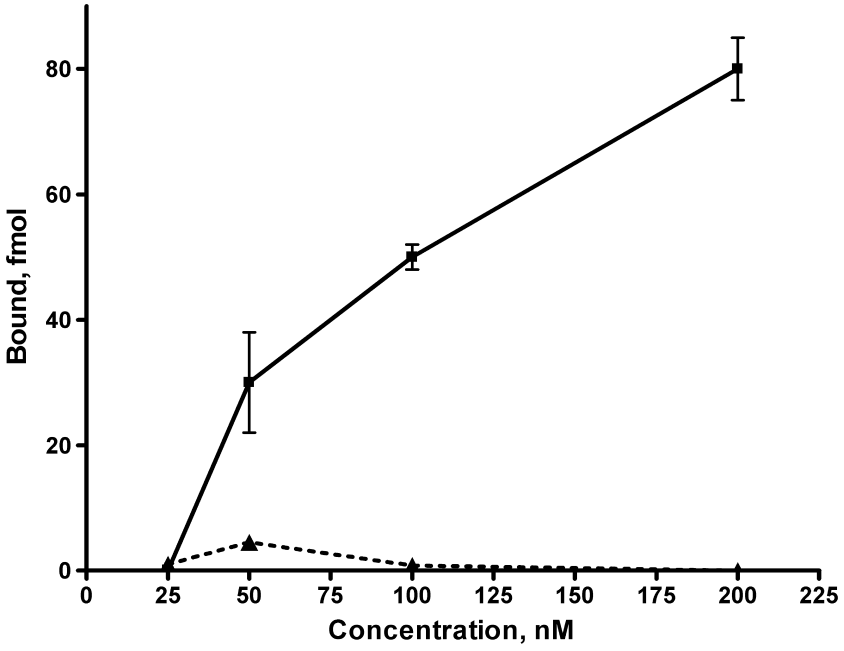

Fig. 7 Concentration dependence of binding of Eu-neoglycoproteins to native CRP. ■, Eu-Galß3GalNAc$_{41}$-PEE-BSA; ▲, Eu-Lac$_{40}$-BSA.

Binding to native CRP by the precipitation–filtration assay

While the long form of Gal6P-BSA conjugate, *i.e.*, Gal6P-ASA-BSA, produced good binding data for the mCRP assay, it produced rather high non-specific background binding (∼80%) in the native CRP assay that utilizes PEG precipitation and filtration. On the assumption that the long linker is largely responsible for the high non-specific binding, we synthesized the short form, Gal6P-ET-BSA, which has an ethylene group as the linker. Under the standard assay conditions at pH 6.5, Eu-Gal6P$_{38}$-ET-BSA did give a much lower background of ∼30%. As in the case of mCRP, native CRP bound Eu-Gal6P$_{38}$-ET-BSA with or without calcium in a dose-dependent manner (not shown), and the inhibitory potency of Gal6P was comparable whether calcium was present or not (IC_{50} = 0.07 mM in the presence of 5 mM calcium, and 0.09 mM in the absence). Figure 7 shows the binding of Eu-Galβ3GalNAc$_{41}$-PEE-BSA in the absence of calcium, which increased in a dose-dependent manner, while Eu-Lac$_{40}$-BSA failed to bind under the same conditions, as was observed in an earlier study [16]. Similar to mCRP, the binding of Eu-Galβ3GalNAc-BSA to native CRP was inhibited in the presence of high concentrations of calcium. However, unlike mCRP, the binding to native CRP seemed to be quite favorable in the low range of calcium concentration (0 to 0.2 mM) (Fig. 3).

ELISA-based native CRP assay

As mentioned in the Methods section, the precipitation–filtration assay presented a problem of very high non-specific binding for neoglycoproteins above pH 7, although this was not the case for PC-BSA, perhaps due to its binding affinity being very much higher than those of neoglycoproteins. The ELISA-based assay did eliminate the problem of large background increases in the alkaline pH range. Surprisingly, the specific binding of CRP to the PC-BSA surface (*i.e.*, the amount of CRP bound to the PC-BSA surface minus the amount bound to the BSA surface) reached a plateau at a very low CRP concentration of 0.5 nM, which is about 1000-fold lower than that observed in the precipitation–filtration assay. Presumably, highly clustered PC residues on the surface enhanced the binding affinity as compared to the one-to-one interaction in the solution phase. In contrast, the binding to the Gal6P-BSA-immobilized surface required much higher CRP concentration of 0.25 μM to produce a good signal for specific binding, whereas even at this concentration binding of CRP to Galβ3GalNAc-BSA-immobilized surface was quite weak (non-specific background ∼80%). As monomers, PC has ∼40-fold and ∼200-fold higher affinity than Gal6P and Galβ3GalNAc, respectively. It appears that these affinity differences were greatly magnified in the surface-immobilization assay. To confirm if the ELISA assay truly exhibits the characteristics of native CRP, the pH dependence of binding to the PC-BSA surface was carried out (Fig. 5). The pH-profile was almost exactly the same as seen with the precipitation–filtration assay [16], in that the binding was maintained in a wide pH range of 4.5 to 8.5. Maintaining the high binding capacity at pH 4.5 is indicative of native CRP, since mCRP steadily loses its binding capacity below pH 6. In addition, Gal6P was much better inhibitor than other sugar phosphates (data not shown), which is another characteristic for native CRP. These results indicated that CRP in the ELISA assay behaved as native CRP. (See Discussion for detail). Using this assay, we were able to generate the pH-profile of CRP binding to the Gal6P-BSA surface (Fig. 4), which showed that the pH-profile was quite similar to that of mCRP, with the exception of the pH optima of native CRP being at a slightly more acidic region than that of mCRP.

Binding of Eu-protamine-BSA to mCRP

In the past, we used Eu-labeled poly-L-lysine (PL) as ligand for the study of polycation-dependent binding to CRP [16]. While binding of Eu-PL in the native CRP assay (precipitation–filtration) was robust, miniscule amounts of Eu-PL were bound (both specific and non-specific) in the mCRP assay as compared to Eu-PC-BSA and Eu-Lac-BSA, suggesting that perhaps the highly cationic nature of PL was refractive to binding to the hydrophobic surface. To overcome this problem, we prepared a BSA-based polycationic ligand by conjugating a well-known polycationic ligand, protamine sulfate, to BSA. Eu-protamine-BSA was bound in a dose-dependent manner to mCRP in the absence of calcium (not shown). As in the case of Eu-PL binding to native CRP [16], the binding of Eu-protamine-BSA to mCRP occurred with or without calcium, and more was bound in the absence of calcium than in the presence. The bound amount of Eu-protamine-BSA at 5 mM calcium was about 70% of that in the absence of calcium.

The pH dependence of protamine-BSA binding to mCRP is included in (Fig. 5). The binding was optimal at pH 6, which is somewhat different from that of native CRP binding Eu-PL [16]. Eu-PL was bound to native CRP in a wide pH range of 4.5 to 9, although the bound amount was highest around pH 6 to 7.

Summary of binding dependence of CRP to calcium and pH

Results on the calcium-dependence of various ligands binding to native CRP and mCRP from this and earlier studies are compiled in Table 3. The calcium requirement was the same

Table 3 Calcium requirement for the binding of various ligands

	Binding	
Eu-Ligand	+calcium	−calcium
PC-BSA	+	−
Gal6P-BSA	+	+
Galβ3GalNAc-BSA	−	+
PL, protamine-BSA	+	+

for native CRP and mCRP for all the ligands examined. PC and neutral sugars have the opposite calcium requirement, the former binding in the presence and the latter in the absence of calcium, whereas Gal6P was bound under both conditions. Polycationic compounds also bound with or without calcium. The accumulating data on the pH dependence of CRP seem to suggest that CRP, whether native or m-form, has highest binding capacity slightly below the neutral pH, regardless of the nature of ligand. It is possible that PC-BSA, which shows a broad binding range of 4.5 to 9 for native CRP and from 6 to 9 for mCRP (Fig. 5), may actually have the highest binding capacity at pH near 6, but its strong binding affinity obscures the presence of such binding optima.

Discussion

hCRP is composed of five identical monomers, with each monomer associating non-covalently with two neighboring monomers in a circular fashion to form a disc-shaped structure. Two closely placed calcium-binding sites per monomer are all located on one side of the disc, the side where the best known ligand, PC, is bound *via* the two calcium atoms [9]. The opposite side is thought to be the site of interaction with complement components. In addition to PC, CRP is known to bind two other classes of ligand: poly-cationic compounds, such as poly-lysine and protamine, and a variety of polysaccharides containing galactose and related structures as a component (Table 4).

In the previous studies, we demonstrated that polycations appear to bind on the same side of the disc where PC is bound,

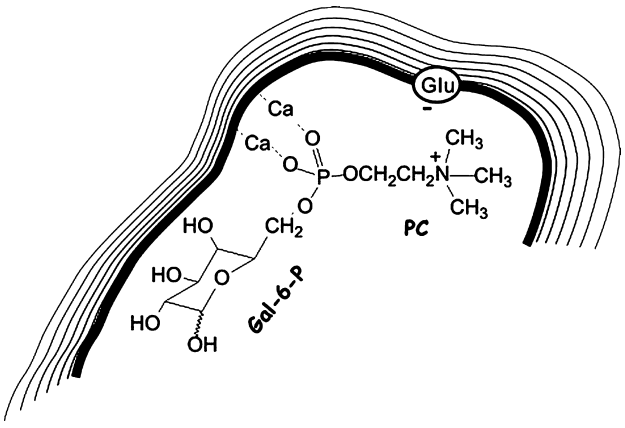

Fig. 8 Schematic presentation of PC–carbohydrate binding area of CRP.

but the binding sites of two ligands do not overlap [16]. The carbohydrate ligands, on the other hand, bind next to the PC site, as depicted in (Fig. 8). The best known small carbohydrate ligand, Gal6P, shares the phosphate binding site with PC, and the galactosyl group occupies the site opposite from the choline site. Interestingly, however, PC and lactose have opposite calcium requirement; *i.e.*, PC binds in the presence of calcium, whereas lactose binds in the absence of calcium [18]. Inhibition assays suggested that Gal6P, which straddles the PC and the sugar sites, can bind both in the presence and absence of calcium. In order to probe the carbohydrate-binding area in more detail, we synthesized neoglycoproteins containing Gal6P and Galβ3GalNAc. Gal6P was chosen since, in addition to being the best carbohydrate inhibiter, it bridges the binding areas of PC and neutral sugars, and Galβ3GalNAc was chosen as one of the best small neutral sugar ligand structures. Using Eu chelate as the reporter group, we showed that both neoglycoproteins bound to mCRP as well as to native CRP. Thus, we showed for the first time that BSA derivatives containing multiple copies of simple sugar structures can be bound by native CRP. Demonstrating binding of neoglycoproteins to native CRP has been elusive thus far, although binding to mCRP has been demonstrated by us and others using lactosylated BSA and HSA [17,18]. Our failure to demonstrate the lactosylated BSA to

Table 4 Repeating unit of microbial polysaccharides that bind to CRP

Source	Repeating unit structure	Reference
S. pneumoniae C-polysaccharide		
type 1	-6Glcβ-3AAT[a]Galα-4GalNAcα-3(6-PC)GalNβ-1ribitol-5-P-	[40]
type 4	-4ManNAcβ-3-L-FucNAcα-3GalNAcα-4CEtGalα-	[6]
Rough	-4GlcNAcβ-4(6-P)GalNAcα-4GlcNAc-	[6]
Rough	-6GalNAcβ-P-	[4]
Leishmania donovani	-6Galβ-4Manα-P-	[39]

[a]Abbreviations used in the table are: AATGal, 2-acetamido-4-amino-2,4,6-trideoxygalactose; CEtGal, 2, 3-*O*-carboxyethylidene-galactose.

bind to native CRP, despite it binding well to mCRP, probably stems from the difference in sensitivity of the two assay systems, rather than the difference in binding affinity. Assays that involve immobilized binding proteins used for mCRP are known to show higher affinity than the solution assays, due presumably to the clustering of binding sites, as well as to a much slower dissociation of ligand from the binding surface [32]. In addition, our solution-phase assay for native CRP involves dilution of the reaction mixture and a separation step (filtration) which cannot be carried out as expeditiously as the assays using microplate.

In accordance with the earlier inhibition data, Eu-Gal6P-BSA bound almost equally well to both forms of CRP whether calcium is present or not. This fact allowed us to directly compare and examine the effect of calcium on the binding characteristics of various inhibitors, which has hitherto been not possible. As shown in Table 1 under the sub-heading of Eu-Gal6P$_{22}$-BSA, phosphate-containing compounds, with exception of Gal6P, are bound 8-fold or more strongly in the presence of calcium than in the absence. The X-ray structure of CRP complexed with PC shows that the two oxygen molecules of phosphate are coordinating directly to two calcium ions, which are 4 Å apart in the PC-binding site [9]. Our results suggest that phosphate-containing ligands can occupy the binding site with or without calcium, but the calcium-coordinated binding of phosphate greatly enhances the binding strength. Gal6P, however, was bound almost equally well with or without calcium. This is understandable because calcium has the opposite effects on the binding of galactosyl and phosphate groups: *i.e.*, the galactosyl residue binds only in the absence of calcium and the phosphate group binds much stronger in the presence of calcium than in the absence. These two effects seem more or less to cancel each other, so that the resulting affinity of Gal6P is not too different with or without calcium. Results of cross inhibition using Eu-labeled PC-, Gal6P-, and neutral sugar (Galβ3GalNAc) containing BSA derivatives (Table 1) concur well with the postulated contiguous binding area (Fig. 8), since PC and Gal6P inhibited each other's binding site very effectively, and Gal6P, not PC, was the best inhibitor for the neutral sugar-dependent binding. The latter result further stresses the affinity-enhancing effect of phosphate group on carbohydrate ligands, even in the absence of calcium.

mCRP is a slightly denatured form of CRP that presents a set of new epitopes in addition to the native CRP epitopes [10]. We found that the binding characteristics of two forms of CRP did not differ much in terms of calcium requirement, pH optima and majority of inhibition data, suggesting that the ligand-binding area of mCRP is similar to that of native CRP. One significant difference is in the ligand-binding capacity at pH below 6, as seen in (Figs. 4 and 5). Binding of all ligands to mCRP, regardless of its nature being zwitter ionic (PC-BSA), negatively charged (Gal6P-BSA), neutral (Galβ3GalNAc-BSA), or positively charged (protamine-BSA), steadily decreased below pH 6, whereas PC-BSA still possessed a good deal of capacity to bind to native CRP even at pH 4.5. This suggests that a slight denaturation accompanying the production of mCRP made the protein structure more susceptible to acid. However, both forms of CRP appear to be quite stable on the alkaline side up to pH 9. Another difference is that the binding specificity of mCRP is less stringent than that of native CRP with respect to the carbohydrate structure. For instance, two assay methods for native CRP both showed that Gal6P is a much better inhibitor than any other sugar phosphates, *e.g.*, Man6P and Galα1-P, whereas Gal6P was only a marginally better inhibitor than other sugar phosphates in the mCRP assay. It may be that the conformational change which accompanies the formation of mCRP affects the neutral sugar binding area in such a way as to allow accommodation of varied sugar structures.

Lectins of both plant and animal origin generally bind target sugar residue(s) at the non-reducing terminus, and this interaction is in some cases further augmented by interaction with the penultimate sugar residue(s). Mannose-binding protein (MBP) is no exception, but it is also capable of binding to sugar residues in the middle of polysaccharide chains [33]. This is understandable from the fact that MBP has a shallow binding area with a spacious opening in both the C1 and C6 ends of the mannose residue [34], and that the binding strength is generated essentially by a coordinated interaction at the C3–C4 region [35]. Neoglycoproteins we used in the present study contain non-reducing terminal sugar structures. However, as shown in Table 4, many straight-chain bacterial polysaccharides and a lipophosphoglycan of *Leishmania donovani* [36], bind strongly to CRP. The name CRP is derived from its interaction with the C-polysaccharide of type 1 pneumococcus (entry 1 in Table 4), which contains a PC side chain in its repeating unit [37], and presumably owe its strong interaction with CRP to these PC side chains. In the remaining polysaccharides, the likely target structures for CRP are GalNAc residues and Gal6P/GalNAc6P residues in monoester or diester forms, all imbedded within the sugar chain, suggesting that the carbohydrate-binding area of CRP is constructed in such a way as to allow easy access to the target sugars located in the polysaccharide chain.

Acknowledgment We thank Dr. I. Takagahara for a generous supply of human rCRP and Ms. M. Hughes for recording ^{1}H-NMR spectra.

References

1. Volanakis, J.E.: Human C-reactive protein: expression, structure, and function. Mol. Immunol. **38**, 189–97 (2001)
2. Gershov, D., Kim, S., Brot, N., Elkon, K.B.: C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate

immune response: implications for systimic autoimmunity. J. Exp. Med. **192**, 1353–63 (2000)
3. Szalai, A.J.: The antimicrobial activity of C-reactive protein. Microbes Infect. **4**, 201–5 (2002)
4. Gotschlich, E.C., Liu, T.Y.: Structural and immunological studies on the pneumococcal C polysaccharide. J. Biol. Chem. **242**, 463–70 (1967)
5. Higginbotham, J.D., Heidelberger, M., Gotschlich, E.C.: Degradation of a pneumococcal type-specific polysaccharide with exposure of group-specificity. Proc Natl Acad Sci USA **67**, 138–42 (1970)
6. Gotschlich, E.C., Liu, T.Y., Oliveira, E.B.: Binding of C-reactive protein to C-carbohydrate and PC-substituted protein. Ann. NY Acad. Sci. **389**, 163–71 (1982)
7. Potempa, L.A., Siegel, J.N., Gewurz, H.: Binding reactivity of C-reactive protein for polycations. II. Modulatory effects of calcium and phosphocholine. J. Immunol. **127**, 1509–14 (1981)
8. Shrive, A.K., Cheetham, G.M., Holden, D., Myles, D.A., Turnell, W.G., Volanakis, J.E., Pepys, M.B., Bloomer, A.C., Greenhough, T.J.: Three dimensional structure of human C-reactive protein. Nat. Struct. Biol. **3**, 346–54 (1996)
9. Thompson, D., Pepys, M.B., Wood, S.P.: The physiological structure of human C-reactive protein and its complex with phosphocholine. Structure Fold Des. **7**, 169–77 (1999)
10. Potempa, L.A., Siegel, J.N., Fiedel, B.A., Potempa, R.T., Gewurz, H.: Expression, detection and assay of a neoantigen (Neo-CRP) associated with a free, human C-reactive protein subunit. Mol. Immunol. **24**, 531–41 (1987)
11. Ying, S.C., Gewurz, H., Kinoshita, C.M., Potempa, L.A., Siegel, J.N.: Identification and partial characterization of multiple native and neoantigenic epitopes of human C-reactive protein by using monoclonal antibodies. J. Immunol. **143**, 221–8 (1989)
12. Potempa, L.A., Zeller, J.M., Fiedel, B.A., Kinoshita, C.M., Gewurz, H.: Stimulation of human neutrophils, monocytes, and platelets by modified C-reactive protein (CRP) expressing a neoantigenic specificity. Inflammation **12**, 391–405 (1988)
13. Rees, R.F., Gewurz, H., Siegel, J.N., Coon, J., Potempa, L.A.: Expression of a C-reactive protein neoantigen (neo-CRP) in inflamed rabbit liver and muscle. Clin. Immunol. Immunopathol. **48**, 95–107 (1988)
14. Diehl, E.E., Haines, G.K., 3rd, Radosevich, J.A., Potempa, L.A.: Immunohistochemical localization of modified C-reactive protein antigen in normal vascular tissue. Am. J. Med. Sci. **319**, 79–83 (2000)
15. Szalai, A.J., Agrawal, A., Greenhough, T.J., Volanakis, J.E.: C-reactive protein: structural biology, gene expression, and host defense function. Immunol. Res. **16**, 127–36 (1997)
16. Lee, R.T., Takagahara, I., Lee, Y.C.: Mapping the binding areas of human C-reactive protein for phophorylcholine and polycationic compounds. Relationship between the two types of binding sites. J. Biol. Chem. **277**, 225–32 (2002)
17. Kottgen, E., Hell, B., Kage, A., Tauber, R.: Lectin specificity and binding characteristics of human C-reactive protein. J. Immunol. **149**, 445–53 (1992)
18. Lee, R.T., Lee, Y.C.: Carbohydrate-binding properties of human neo-CRP and its relationship to phosphorylcholine-binding site. Glycobiology **13**, 11–21 (2003)
19. Weigel, P.H., Naoi, M., Roseman, S., Lee, Y.C.: Preparation of 6-aminohexyl D-aldopyranosides. Carbohydr. Res. **70**, 83–91 (1979)
20. Stults, N., Lee, Y.C., Hoppe, C.A., Kawaguchi, K., Kohda, S., Takagahara, I., Koishi, T., Liu, T.Y.: Preparation of phosphorylcholine derivatives of bovine serum albumin and their application to the affinity chromatography of C-reactive protein. Anal. Biochem. **161**, 567–73 (1987)
21. Lee, R.T., Lee, Y.C.: Preparation and some biochemical properties of neoglycoproteins produced by reductive amination of thioglycosides contianing an ω -aldehydoaglycon. Biochemistry **19**, 156–63 (1980)
22. McKelvy, J.F., Lee, Y.C.: Microheterogeneity of the carbohydrate group of Aspergillus oryzae α-amylase. Arch. Biochem. Biophys. **132**, 99–110 (1969)
23. Ames, B.N., Dubin, D.T.: The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**, 769–75 (1960)
24. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C.: Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76–85 (1985)
25. Naoi, M., Lee, Y.C.: A fluorometric measurement of ligands incorporated into BrCN-activated polysaccharides. Anal. Biochem. **57**, 640–4 (1974)
26. Hemmila, I.A., Dakubu, S., Mukkala, V.-M., Siitarl, H., Lovgren, T.: Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. **137**, 335–43 (1984)
27. Brown, C.A., Brown, H.C.: Catalytic hydrogenation II. A new, convenient technique for laboratory hydrogenations. A simple, automatic device for atmospheric pressure hydrogenations. J. Org. Chem. **31**, 3989–95 (1966)
28. Yu, K.-L., Fraser-Reid, B.: A novel reagent for the synthesis of myo-inositol phosphates: N, N-diisopropyl dibenzyl phosphoramidite. Tetrahedron Letts. **29**, 979–82 (1988)
29. Tegge, W., Ballou, C.E.: Chiral syntheses of D- and L-myo-inositol 1,4,5-triphosphate. Proc. Natl. Acad. Sci. USA **86**, 94–8 (1989)
30. Lee, R.T., Wong, T.C., Lee, Y.C.: Synthesis of 6′-aminohexyl 2-acetamido-2-deoxy-D-galactoside isomers and a unique isomerization catalyzed by ion exchange resin. J. Carbohydr. Chem. **5**, 343–57 (1986)
31. Lee, R.T., Lee, Y.C.: A derivative of diethylentriaminepentaacetic acid for europium labeling of proteins. Bioconjugate J. **12**, 845–9 (2001)
32. Butler, J.E.: Solid phases in immunoassay. In Immunoassay, vol., edited by Christopoulos TK (Academic Press, San Diego, 1996), pp. 205–25.
33. Lee, R.T., Shinohara, Y., Hasegawa, Y., Lee, Y.C.: Lectin-carbohydrate Interactions: Fine specificity difference between two mannose-binding proteins. Bioscience Reports **19**, 283–92 (1999)
34. Lee, R.T., Ichikawa, Y., Fay, M., Drickamer, K., Shao, M.C., Lee, Y.C.: Ligand-binding characteristics of rat serum-type mannose-binding protein (MBP-A). J. Biol. Chem. **266**, 4810–5 (1991)
35. Ng, K.K.-S., Drickamer, K., Weis, W.I.: Structural analysis of monosaccharide recognition by rat liver mannose-binding protein. J. Biol. Chem. **271**, 663–74 (1996)
36. Culley, F.J., Bodman-Smith, K.B., Ferguson, M.A., Nikolaev, A.V., Shantilal, N., Raynes, J.G.: C-reactive protein binds to phosphorylated carbohydrates. Glycobiology **10**, 59–65 (2000)
37. Jennings, H.J., Lugowski, C., Young, N.M.: Structure of the complex polysaccharides C-substance from Streptococcus pneumoniae type 1. Biochemistry **19**, 4712–9 (1980)