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Characterization of Ca^{2+} and phosphocholine interactions with C-reactive protein using a surface plasmon resonance biosensor

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ABSTRACT

The interactions between Ca^{2+} and C-reactive protein (CRP) have been characterized using a surface plasmon resonance (SPR) biosensor. The protein was immobilized on a sensor chip, and increasing concentrations of Ca^{2+} or phosphocholine were injected. Binding of Ca^{2+} induced a 10-fold higher signal than expected from the molecular weight of Ca^{2+}. It was interpreted to result from the conformational change that occurs on binding of Ca^{2+}. Two sites with different characteristics were distinguished: a high-affinity site with K_D = 0.03 mM and a low-affinity site with K_D = 5.45 mM. The pH dependencies of the two Ca^{2+} interactions were different and enabled the assignment of the different sites in the three-dimensional structure of CRP. There was no evidence for cooperativity in the phosphocholine interaction, which had K_D = 5 μM at 10 mM Ca^{2+}. SPR biosensors can clearly detect and quantify the binding of very small molecules or ions to immobilized proteins despite the theoretically very low signals expected on binding, provided that significant conformational changes are involved. Both the interactions and the conformational changes can be characterized. The data have important implications for the understanding of the function of CRP and suggest that Ca^{2+} is an efficient regulator under physiological conditions.

C-reactive protein (CRP)[1] is a plasma protein and a component of the acute phase response in humans. The function of CRP is not completely clear, but it is likely that it plays an important role in host defense because it is able to activate the complement system and it is up-regulated during cytokine-mediated response to most forms of tissue injury, infection, and inflammation [1–3]. CRP belongs to the pentraxins, a protein family that has been highly conserved during evolution and that also includes the serum amyloid P component [4,5]. It consists of five identical subunits of approximately 23 kDa that are noncovalently attached and arranged symmetrically around a pore. Each protomer has two Ca^{2+} ions in a single combined binding site. The binding of Ca^{2+} to CRP induces a large conformational change whereby a loop moves to the main body of the molecule, hiding an otherwise exposed site of proteolysis [2,3]. Two groups of ligands have been reported to interact with CRP: one group that requires Ca^{2+} and a second group whose binding is inhibited by Ca^{2+}. Phosphocholine-containing compounds belong to the first group, and fibronectin and polycation compounds belong to the second group [1,6,7]. It has been assumed that CRP circulates in blood in a fully Ca^{2+}-loaded form given that the reported Ca^{2+} affinity is 0.06 mM [8]. Consequently, the only function of Ca^{2+} would simply be to stabilize the protein structure and to coordinate the binding of phosphocholine.

While setting up a surface plasmon resonance (SPR)-based assay for characterizing the interaction between CRP and modified peptides (manuscript submitted for publication), we explored optimal buffer conditions for the analysis, including the dependence on Ca^{2+} concentration. An unexpected finding was that the binding of Ca^{2+} gave a detectable signal despite the insignificant change in mass of the surface on binding. The phenomenon of detecting small molecules or even atoms by inducing conformational changes has been observed before but has rarely been used to determine binding affinities [9–13]. In the current study, we used the phenomenon to characterize the interaction between Ca^{2+} and CRP and obtained data that suggest a role for Ca^{2+} in the regulation of CRP function.

Materials and methods

Interaction studies

All studies were performed at 25 °C with a Biacore 2000 or a Biacore S51 instrument (GE Healthcare, Uppsala, Sweden). CRP (Millipore, Billerica, MA, USA) was immobilized on a CM5 chip by amine coupling according to standard procedures (GE Healthcare Biosciences, Uppsala, Sweden). Prior to immobilization, the buffer

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[1] *Abbreviations used:* CRP, C-reactive protein; SPR, surface plasmon resonance; RU, resonance units; EDTA, ethylenediaminetetraacetic acid.

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of the protein was changed to 50 mM Mes (pH 6.0) using a Protein Desalting Spin Column (Pierce, Rockford, IL, USA), and the concentration was adjusted to 0.1 mg/ml. The immobilization resulted in a signal of approximately 3000 resonance units (RU). The running buffer consisted of 10 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM $CaCl_2$, and 0.005% surfactant P-20.

For the interaction studies with Ca^{2+}, running buffer without Ca^{2+} or a buffer consisting of 50 mM Mops, 50 mM Mes, and 50 mM Na–acetate (pH 5.0–8.0) was used. Calcium was injected in a concentration series ranging from 0.8 μM to 50 mM or from 6 μM to 100 mM. Both the association and dissociation phases were followed for 180s. As controls, a concentration series ranging from 0.025 to 0.4 mM of LiCl, KCl, CsCl, $ZnCl_2$, $MnCl_2$, $MgCl_2$, and $NiCl_2$ was injected. For the interaction studies with phosphocholine, a buffer consisting of 10 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM $CaCl_2$, and 0.005% surfactant P-20 was used. The association and dissociation phases were followed for 90 s each. A concentration series of phosphocholine ranging from 0.16 to 100 μM was used.

Signals were recorded in resonance units and presented graphically as a function of time in sensorgrams. At the beginning and end of a concentration series, a blank consisting of running buffer was injected. A step for regeneration of the surface was not necessary. A flow of 50 μl/min was used in all interaction studies.

Data evaluation

BIAevaluation 4.1 (GE Healthcare Biosciences) was used for the evaluation of sensorgrams. All sensorgrams were normalized with respect to the theoretical maximum signal estimated from the mass of Ca^{2+} or phosphocholine and the amount of immobilized protein. Signals from the reference surface (an unmodified flow cell) and a blank injection were subtracted. The steady-state values were calculated after 80 or 170 s. The K_D values were determined by global nonlinear regression analysis of the steady-state values using a single-site model for the phosphocholine interaction (Eq. 1) and a model for two independent binding sites for the Ca^{2+} interaction (Eq. 2):

$$[RL] = \frac{B_{max} * [L]}{K_D + [L]} \quad (1)$$

$$[RL] = \frac{B_{max\,1} * [L]}{K_{D\,1} + [L]} + \frac{B_{max\,2} * [L]}{K_{D\,2} + [L]} \quad (2)$$

Where B_{max} is the total binding capacity, RL is the amount of complex, $[L]$ is the concentration of the free analyte, and K_D is the equilibrium dissociation constant. The estimation of pK_a values for the Ca^{2+} binding sites was performed by fitting the K_D values at different pH values to a model for a singly ionizing system (Eq. 3) [14] by using GraFit 5.0.13 (Erithacus Software, Surrey, UK):

$$K_D = \frac{(K_{D\,max} + K_{D\,min}) * 10^{pH\text{-}pKa}}{10^{pH\text{-}pKa} - 1} \quad (3)$$

PyMOL (version 0.99, DeLano Scientific, San Carlos, CA, USA) was used to visualize the three-dimensional crystal structure of CRP. The coordinates for the three-dimensional structure were taken from PDB file 1B09.

Results

Immobilization of CRP

The immobilization of CRP by amine coupling gave a stable sensor surface. Although it was possible to immobilize amounts up to 10,000 RU, only approximately 3000 RU was immobilized so as to avoid mass transport limitations.

Interaction studies with phosphocholine

Phosphocholine interacted reversibly with CRP and exhibited fast association and dissociation (Fig. 1A). Saturation was reached at concentrations around 50 μM, indicating that the interaction corresponded to a well-defined stoichiometry. The binding of phosphocholine was calcium dependent, and no binding was observed for 100 μM phosphocholine in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA) (inset in Fig. 1A). To calculate the K_D value, the steady-state values were determined and plotted as a function of the phosphocholine concentration (Fig. 1B). The data could be satisfactorily described by a simple 1:1 interaction model without cooperativity, and the calculated K_D value was 5.0 ± 3.3 μM. The error was calculated as the standard deviation of three different measurements. The association and dissociation rate constants were faster than can be reliably determined, that is, in the order of (or faster than) the rate of diffusion.

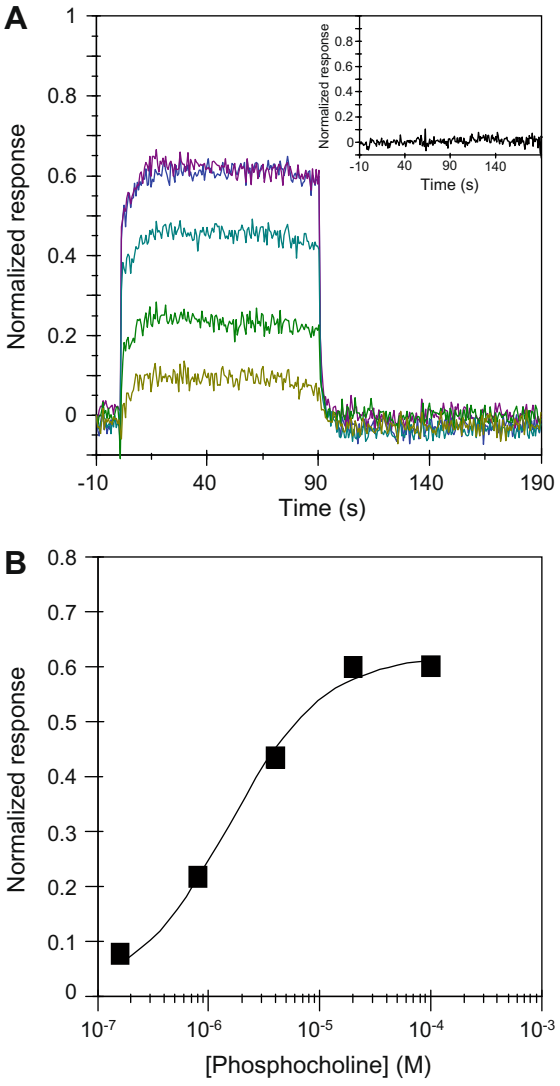

Fig. 1. Interaction between phosphocholine and CRP. Shown are sensorgrams for the interaction between phosphocholine (0.16–100 μM) and CRP (A) and the control in the presence of 1 mM EDTA (inset). The steady-state values were determined after 90 s and plotted as a function of phosphocholine concentration (B). The sensorgrams were normalized with respect to the theoretical maximum signal estimated from the mass of phosphocholine and the amount of immobilized protein. A model representing an interaction with one binding site (Eq. 1) was fitted to the steady-state values and is shown as a solid line in panel B.

Interaction studies with Ca^{2+}

The injection of Ca^{2+} over the CRP surface resulted in a signal that was concentration dependent. The maximum signal was approximately 10-fold higher than expected from the change in mass on the binding of two Ca^{2+} ions (Fig. 2A). Because the dissociation of calcium was fast, regeneration of the surface was not necessary. The sensorgrams showed two apparent saturation levels: one around 2.5 normalized response units and one around 10 normalized response units. Due to the fast kinetics and the strong spikes in the beginning and end of the injection, especially at high concentrations, the association and dissociation rate constants could not be determined. Therefore, the steady-state binding levels were determined and plotted as a function of the concentration as for phosphocholine above (Fig. 2B). The double sigmoidal shape indicated a complex interaction involving at least two distinct binding events. Different models were applied to the dataset, and a model for two independent binding sites with different affinities (Eq. 2) was the simplest one that described the data satisfactorily. The calculated K_D values in Hepes buffer at pH 7.4 was 0.03 ± 0.011 mM for the high-affinity site and 5.45 ± 0.52 mM for the low-affinity site. As a control, other ions with one or two positive charges were injected (Fig. 3). Only divalent ions induced a response, approximately half of that of Ca^{2+}. The monovalent cations did not give a detectable signal.

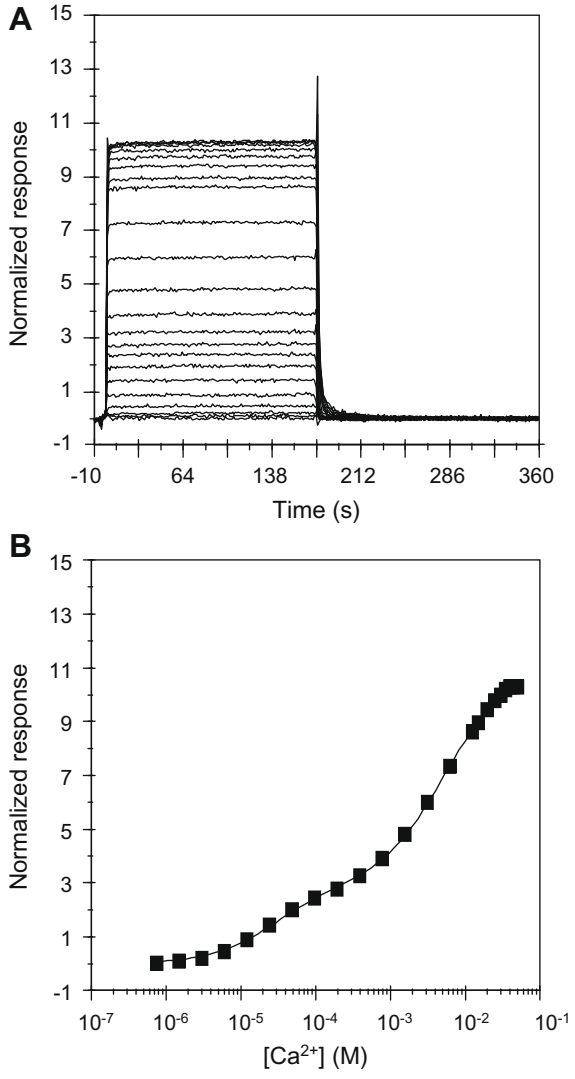

Fig. 2. Interactions between Ca^{2+} and CRP. Shown are sensorgrams for the interaction between Ca^{2+} (8 μM–50 mM) and CRP (A) and steady-state values after 170s as function of Ca^{2+} concentration (B). The sensorgrams were normalized to the theoretical maximum signal estimated from the mass of two Ca^{2+} ions and the amount of immobilized protein. A model representing the interaction with two independent binding sites (Eq. 2) was fitted to the steady-state values and is shown as a solid line in panel B.

pH dependence of Ca^{2+} interaction

Because the two Ca^{2+} ions in CRP are coordinated by different combinations of Glu, Gln, Asp, and Asn residues, it was expected that they could differ in their pH dependencies. To distinguish between the two sites, the influence of pH on the Ca^{2+} affinity was investigated. Therefore, a buffer containing Mes, Mops, and Na–acetate and covering the pH range 4.0 to 8.0 was used. These buffer compounds were chosen because it has been reported that they do not interact with calcium ions and because they have previously been used to study Ca^{2+}–protein interactions [15,16]. A series of Ca^{2+} concentrations were injected at different pH values, and the steady-state values were determined (Fig. 4). The K_D values were calculated (Table 1) by fitting a model for two independent binding sites with different affinities to these data (as above). The clear double sigmoidal shape of the steady-state curves was reduced at lower pH values and disappeared at pH 5.0 because the K_D values of both binding sites became more similar.

Both binding sites showed a strong but opposite pH dependency (Fig. 5). With increasing acidity, the K_D values for the high-affinity site increased, whereas those for the low-affinity site decreased. The data could be described by a model representing a singly ionizing system. Thus, it was possible to estimate the apparent pK_a value for the high-affinity site, which was 4.25 ± 0.12. The fitted curve for the low-affinity binding site had an inflexion point at pH 5.93 ± 0.12. The denaturation of the protein in an acidic environment did not allow reliable measurements below pH 5.0.

Discussion

The binding of phosphocholine to immobilized CRP was calcium dependent; this is well known for the protein and indicates that it was immobilized in a functional manner. Because no cooperativity was observed, it can be assumed that all subunits have the same affinity for phosphocholine with a K_D value of 5 μM, which is in agreement with a previous study [17]. This K_D value is approximately 1000-fold higher than the concentration of CRP in a healthy human [2,18]. Hence, CRP would bind only a low amount of free phosphocholine under physiological conditions, also in the case of up-regulation during inflammation. In contrast, due to the avidity effect of multiple binding sites, the K_D value of the pentameric CRP for a membrane rich in phosphocholine lipids would be far below the blood concentration provided that the head groups are arranged in a way that pentameric CRP can bind to several at a time. It has been shown previously that also the structure of a membrane is important to induce binding of CRP and that CRP binding increases with membrane fluidity [19–21]. Preliminary SPR biosensor experiments confirmed that CRP does not bind to membranes consisting only of phosphocholine lipids (data not shown). Thus, it is likely that the pentameric structure and the low affinity for soluble phosphocholine have an important role in the specificity of CRP for membranes that expose phosphocholine.

There have been previous reports that SPR signals are not strictly mass induced but also can arise as a result of conformational changes and that the binding of small molecules or ions such as Ca^{2+} can be detected by this technology [9–13,22]. The binding of Ca^{2+} to CRP induces a strong conformational change in which a loop (residues 138–150) moves into the protein and hides an

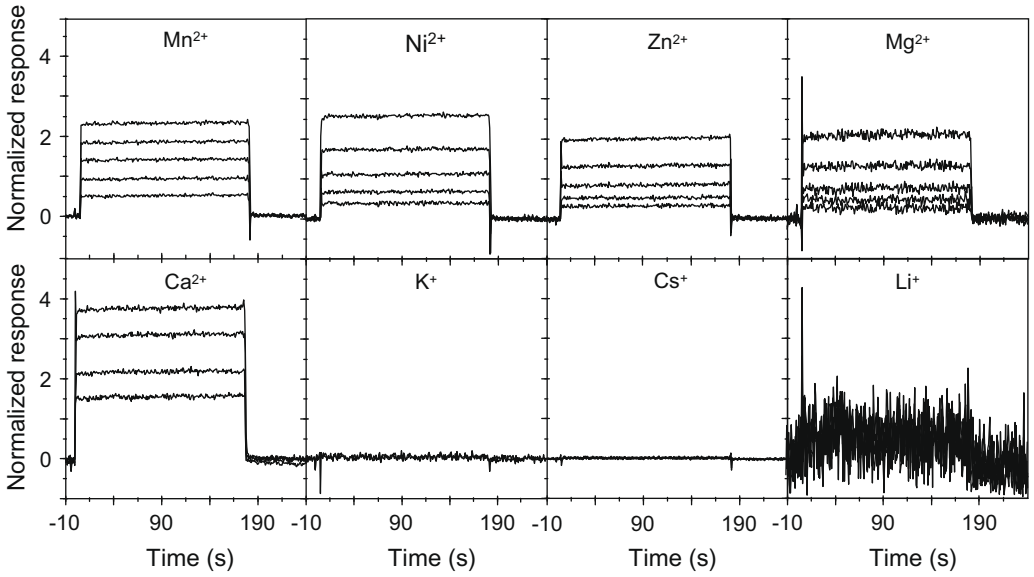

Fig. 3. Interaction between different metal ions and CRP. Shown are sensorgrams for the interaction between different metal ions (0.02–0.4 mM) and CRP. The sensorgrams were normalized to the theoretical maximum signal estimated from the mass of the ions and the amount of immobilized protein.

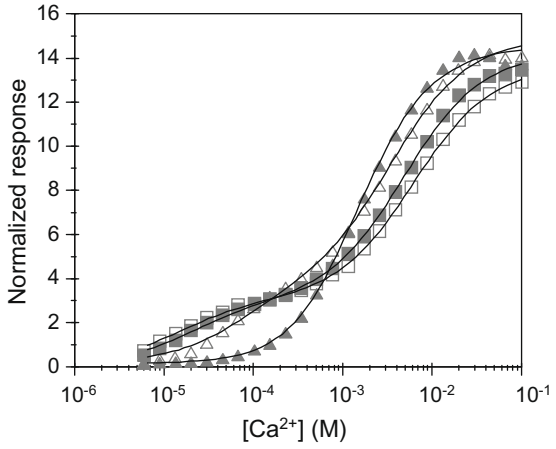

Fig. 4. pH dependence of Ca^{2+} binding to CRP. Ca^{2+} concentrations between 0.006 and 100 mM were injected at pH 5.0 (▲), 6.0 (Δ), 7.0 (■), and 8.0 (□). The sensorgrams at the different pH values were of the type shown in Fig. 2A, and the steady-state values were determined after 170s. A model for two independent binding sites (Eq. 2) was fitted to the steady-state values and is shown as solid lines.

Table 1
Affinities for the interaction between Ca^{2+} and CRP at different pH values.

pH	$K_{D\,1}$ (mM)	$K_{D\,2}$ (mM)
5.0	0.68 ± 0.083	1.01 ± 0.03
6.0	0.07 ± 0.009	3.85 ± 0.47
7.0	0.03 ± 0.014	5.98 ± 1.55
8.0	0.01 ± 0.003	6.85 ± 0.84

Note. K_D values were calculated by a global nonlinear regression analysis of the data in Fig. 4 using an interaction model for two independent binding sites. Errors were calculated as the standard deviation of two different measurements.

otherwise exposed proteolytic site [3,23,24]. The large positive SPR signal, induced by calcium ions, cannot be explained by an increase of mass alone and, therefore, is assumed to be related to this conformational change.

Moreover, it has been reported that both Ca^{2+} binding sites of CRP have the same affinity with a K_D value of 0.06 mM [8]. In

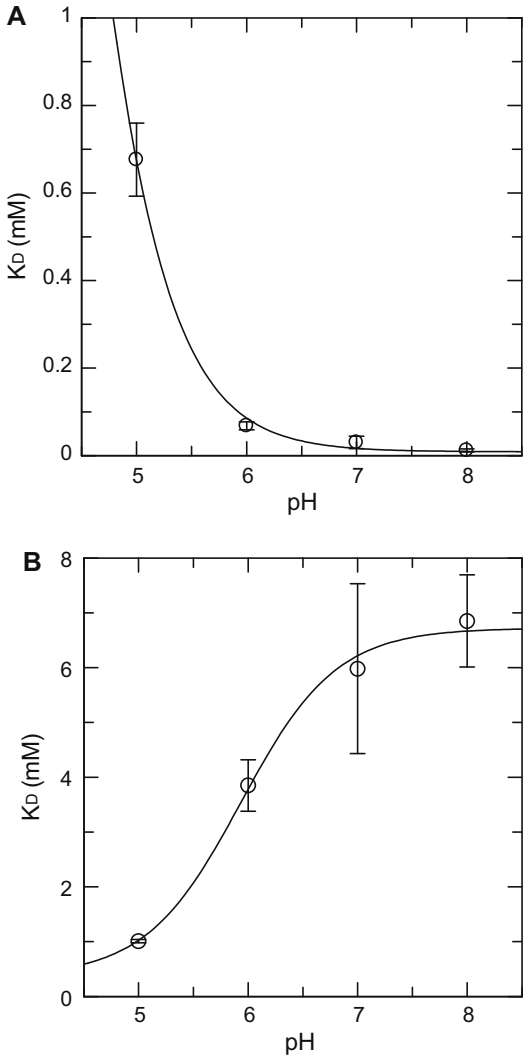

Fig. 5. Ca^{2+} affinity as a function of pH. The K_D values for the high-affinity (A) and low-affinity (B) binding sites were plotted against the pH (data from Table 1). The solid lines are theoretical curves obtained from fitting a model representing a singly ionizing system to the data (Eq. 3).

contrast, our data show that CRP has a high-affinity binding site with a K_D value of 0.03 mM and a low-affinity binding site with a K_D value of 5.45 mM at physiological pH. Furthermore, the differences in saturation levels indicate that binding to the high-affinity site induces only a small change in the structure and that binding to the low-affinity site is necessary to induce the major change. The coordination of the two Ca^{2+} ions is known from the three-dimensional crystal structure of CRP. Binding site 1 coordinates Ca^{2+} via Glu138, Asp140, Glu147(×2), and Gln150 and binding site 2 via Asp60, Asn61, Glu138, Asp140, and the main chain carbonyl of Gln139 (Fig. 6) [3,23,24]. The Ca^{2+} in binding site 1 is coordinated only by amino acids that are part of the mobile loop; therefore, it is likely that Ca^{2+} binding to this site induces the conformational change. Furthermore, in binding site 2, the arrangement of the oxygen atoms, which coordinate Ca^{2+}, are more closely oriented to an ideal octahedron. This indicates that binding site 1 is the low-affinity site and binding site 2 is the high-affinity site. Both binding sites showed large differences in pH dependency. The high-affinity binding site showed a decrease in affinity with increasing acidity, in accordance with the loss of interaction on protonation of the calcium-coordinating Asp and Glu carboxylates. It is likely that the protonation of one carboxylic acid causes a strong decrease in affinity. The estimated pK_a value probably reflects only one of the carboxylic acids, and the pK_a of the remaining carboxylic acids would be lower than pH 5.0. The low-affinity site showed increasing affinity with higher acidity; this is not possible to explain by titration of acidic residues. Another explanation, therefore, could be that the protonation of Glu138 or Asp140 reduces the interaction with the Ca^{2+} at the high-affinity site, thereby making the residues more flexible and allowing tighter binding to the Ca^{2+} at the low-affinity site. The change in affinity could also be attributed to some other amino acids or electrostatic interactions that are part of the conformational change and influenced by changes in pH.

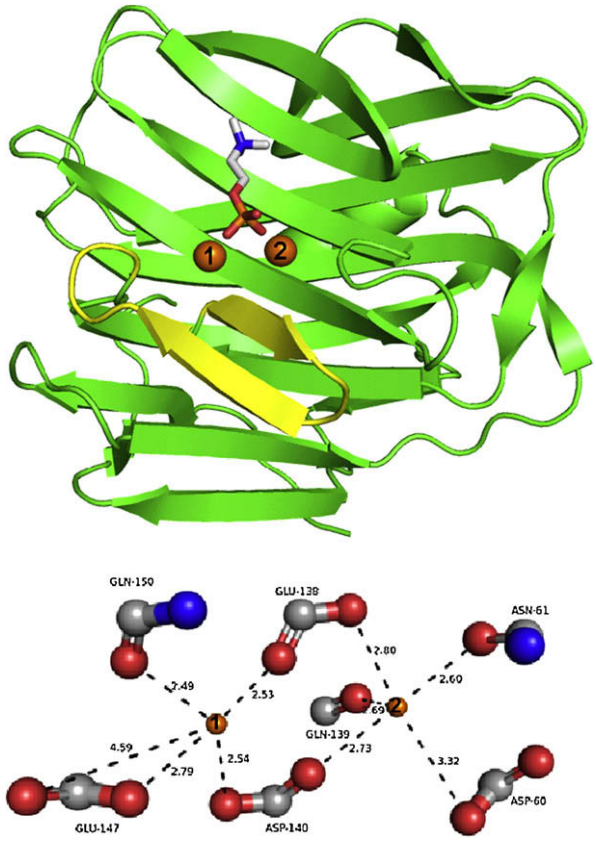

Fig. 6. Calcium binding sites of CRP. The structure for CRP (PDB ID: 1B09) shows the two Ca^{2+} ions (orange) and the bound phosphocholine. The mobile loop is colored in yellow. The lower figure shows close-ups of the binding sites. Both Ca^{2+} ions are coordinated by six oxygen atoms, but the oxygen atoms in binding site 2 appear to be better oriented for ideal octahedral coordination. Binding site 1 is proposed to be the low-affinity site, and binding site 2 is proposed to be the high-affinity site.

So far, it has been believed that CRP circulates in blood in a completely Ca^{2+}-bound form, with a free Ca^{2+} concentration of approximately 1.2 mM and an affinity in a micromolar range [8,25]. The results of the current study suggest that in the bloodstream only 22% of the proteins are saturated with two Ca^{2+} ions and, therefore, that only one monomer of the pentameric CRP is in a condition to bind phosphocholine. On the other hand, in areas of inflammation where the Ca^{2+} concentration can be several-fold higher, nearly 100% of the protein would be occupied by two Ca^{2+} ions. In these areas, CRP can bind with high affinity to lipid membranes because the binding to several phosphocholine head groups is necessary. In contrast, binding to fibronectin or polycationic compounds is inhibited by high Ca^{2+} concentrations, and existing results indicate that one Ca^{2+} binding site should be vacant for these interactions [6]. We propose that Ca^{2+} has a regulatory effect for CRP and that the preferred ligands in blood and in inflamed areas are different. The Ca^{2+} binding to the low-affinity site induces the major conformational change, not only preventing the protein from proteolytic cleavage but also changing the binding specificity.

The serum amyloid P component is another member of the pentraxin family with approximately 50% homology to CRP. This protein has also been reported to have high- and low-affinity binding sites for Ca^{2+}, and it has been suggested that this protein circulates in blood only partly Ca^{2+} loaded [26–28]. Several Ca^{2+} binding sites are known to bind different divalent cations, as expected given that they have similar properties as Ca^{2+} [29]. But these ions typically either have a low affinity compared with Ca^{2+} or cannot induce a sufficient conformational change for functionality. CRP was also found to bind different divalent ions, but the induced responses were lower than those for Ca^{2+}, indicating that the binding affinity and/or the induced conformational change are weaker. Nevertheless, the physiological relevance of these interactions is also dependent on the concentration of the ions. Because the Mn^{2+} concentration in blood is in a nanomolar range, this binding has no physiological relevance [30]. In contrast, the Mg^{2+} and Zn^{2+} interactions have been observed in a physiological relevant concentration range given that their blood concentrations are much higher at approximately 0.6 and 0.02 mM, respectively [31,32]. But even under normal conditions in blood (i.e., where the Ca^{2+} ion concentration is low), the high-affinity site will be occupied by calcium and the free Ca^{2+} binding sites probably have too low affinity for Zn^{2+} and Mg^{2+} to bind. The strong Ca^{2+}-induced conformational change of CRP makes the protein more compact and decreases the hydrodynamic radius [33]. It has been suggested that such a change in density close to the surface should be associated with a negative change in response units [9]. Our data show that the binding of calcium to CRP is associated with a positive signal increase. This indicates that the signal shift is influenced not only by an increase or decrease of hydrodynamic radius but also by other factors such as charge and density distribution on the chip surface.

Conclusions

This study has shown that SPR-based biosensor interaction analysis can be used to investigate interactions between proteins and ions provided that a major conformational change is induced. Such interaction studies could give new insights into the relation-

ship between structure and properties of ion binding motifs. The determined affinities for the interaction between CRP and phosphocholine or Ca^{2+} were in a physiologically relevant range; thus, the data suggest that Ca^{2+} has a regulatory effect for CRP. A continuation of the study would be to investigate the interaction between CRP and other ligands such as fibronectin and polycationic compounds. Furthermore, it would be interesting to study how the affinity of CRP to a membrane is influenced by the membrane structure. This information could help to elucidate the role of CRP during immune response and different diseases such as atherosclerosis and Alzheimer's disease.

Acknowledgments

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