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# Mechanisms of the Anti-Pneumococcal Function of C-Reactive Protein

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Mechanisms of the Anti-Pneumococcal Function of C-Reactive Protein

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A dissertation

presented to

The faculty of the Department of Biomedical Sciences

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In partial fulfilment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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December 2013

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Pneumococcal C-polysaccharide, *Streptococcus pneumoniae*

ABSTRACT

Mechanisms of the Anti-Pneumococcal Function of C-Reactive Protein

by

Toh Boniface Gang

Human C-reactive protein (CRP) increases survival of and decreases bacteremia in mice infected with *Streptococcus pneumoniae*. Such protection of mice against pneumococcal infection is seen only when CRP is administered into mice 6 hours before to 2 hours after the injection of pneumococci, but not when CRP is given to mice at a later time. Our first aim was to define the mechanism of CRP-mediated initial protection of mice against infection. It was proposed that CRP binds to phosphocholine (PCh) moieties present in the cell wall and activates the complement system on the pneumococcal surface that kills the pathogen. We generated a CRP mutant F66A/T76Y/E81A incapable of binding to PCh. Mutant CRP did not protect mice from pneumococcal infection. Thus, the proposed hypothesis was correct; the PCh-binding property of CRP contributes to the protection of mice against pneumococcal infection. Our second aim was to investigate why CRP was not protective during the late stages of infection. Pneumococci are known to recruit an inhibitor of complement activation, factor H, from the host to their surface to escape complement attack. We considered the ability of CRP, in its nonative form, to bind to factor H, and generated a CRP mutant E42Q/F66A/T76Y/E81A capable of binding to factor H. *In vivo* experiments using the quadruple CRP mutant are in progress. We anticipate that the combination of wild-type and quadruple mutant CRP should be protective during the late stages of infection; wild-type CRP would bind to PCh and activate complement while mutant CRP would cover factor H to prevent its complement-inhibitory activity. Our long-term goal is to explore the possibility of developing a CRP-based strategy to treat pneumococcal infection.

DEDICATION

This manuscript is dedicated to my father Mr. Gang Solomon, who is of blessed memory; my mother Tebit Regina, who encourages me to be perseverant; and my wife and children. My wife Ngeche Rose Gang, who encourages and supports me in all my educational and professional development, occupies a special place in my heart. My children (Tebit Gang, Ngum Gang, Atuh Gang, and Gang-Afanwi Gang) have helped me build the strength and resilience that makes me succeed.

ACKNOWLEDGEMENTS

## ABBREVIATIONS

| | |
|---|---|
| BSA | Bovine serum albumin |
| CFU | Colony forming units |
| CHO | Chinese hamster ovary |
| CRP | C-reactive protein |
| ELISA | Enzyme-linked immunosorbent assay |
| EU | Endotoxin units |
| h | Hour |
| HRP | Horseradish peroxidase |
| i.v. | Intravenous |
| mAb | Monoclonal antibody |
| Min | Minute |
| OD | Optical density |
| PCh | Phosphocholine |
| PEt | Phosphoethanolamine |
| PnC | Pneumococcal C-polysaccharide |
| SAP | Serum amyloid P component |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TBS | Tris buffered saline |
| WT | Wild-type |

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CHAPTER 1

INTRODCUTION

C-reactive protein (CRP), a liver-expressed member of the pentraxin family, reacts with

cell wall C-polysaccharide (PnC) of *Streptococcus pneumoniae* in a $Ca^{2+}$-dependent manner (1-

3). During bacterial infection, inflammation and injury, its serum levels increase dramatically

(4). This acute surge in plasma levels is seen across the entire evolutionary spectrum, suggesting

that CRP has been evolutionarily conserved. A notable exception to this observation is the

mouse, in which CRP does not show an acute phase response to inflammatory stimuli (5). This

characteristic justifies the suitability of mice for *in vivo* studies involving the functional

characterization of CRP. CRP has binding specificity for the phosphocholine (PCh) residues

present in PnC (6, 7). CRP also binds to whole pneumococci in human and mouse sera and in

$Ca^{2+}$-containing buffers (8-11). Serum amyloid P component (SAP), another member of the

pentraxin family, is structurally similar to CRP, and displays $Ca^{2+}$-dependent binding specificity

for phosphoethannolamine (PEt) (11-13). Although CRP also binds to PEt, such binding is not as

avid as it binds to PCh (11-16). The innate immune properties of CRP seem to be conserved

throughout evolution

Structure of CRP

CRP is composed of  5 identical non-covalently attached subunits or monomers. Based

on the crystallographic data, each subunit has 206 amino acids and the molecular weight

(molecular weight) of each subunit is approximately 23 kDa.  All 5 subunits have the same

orientation in the pentamer, with a PCh-binding site located on the same face of each subunit

(17, 18). The PCh-binding site consists of a hydrophobic pocket formed by several amino acids

including Phe[66], Thr[76], and Glu[81] and 2 $Ca^{2+}$ ions that are bound to CRP by interactions with amino acids from other parts of the protein (16, 17, 19-20). The phosphate group of PCh directly coordinates with the 2 $Ca^{2+}$ ions. The choline group of PCh lies within the hydrophobic pocket. Phe[66] provides hydrophobic interactions with the 3-methyl groups of choline. Thr[76] is critical for creating the appropriately sized pocket to accommodate PCh. Glu[81] interacts with the positively charged nitrogen atom of choline. Previous mutational analyses of Thr[76] in CRP have confirmed the significance of the hydrophobic pocket for PCh-binding (21). In SAP, at the position corresponding to Thr[76] in CRP, it is a Tyr residue (Tyr[74]) (22, 23) (Fig. 1.1).
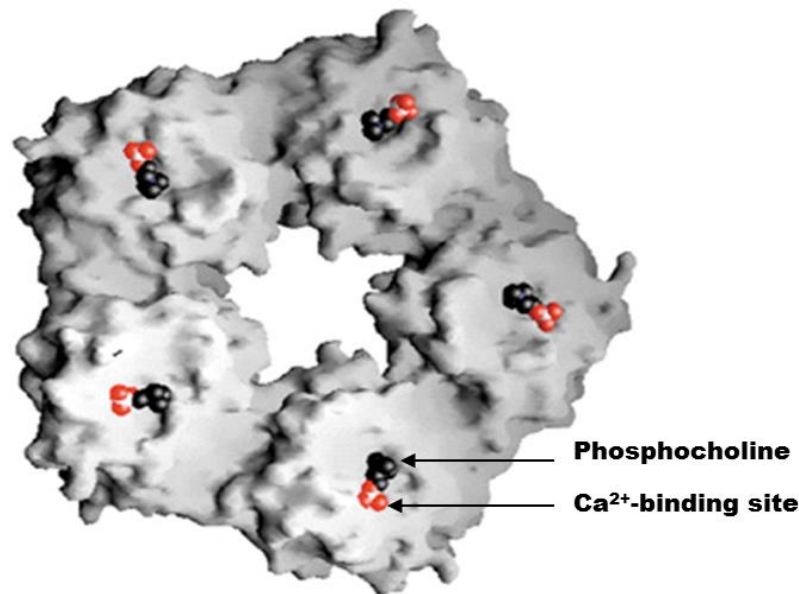


Figure 1.1. The binding of PCh to CRP. The adapted figure shows the positions of the 2 $Ca^{2+}$ (orange) and a molecule of PCh (Adapted from (16)).

The face opposite to the PCh-binding face of the CRP pentamer, also called the effector face, consists of a cleft that extends from the center of each monomer to the center of the pentamer. Key amino acid residues in this cleft are Asp[112] and Tyr[175], which are responsible for the binding of C1q, the first step in CRP-mediated complement activation (24, 25). After solving

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of the C1q structure, it became clear that the positively charged C1q head interacts with the

negatively charged central pore of the CRP protomer (26).

Pneumococcus

*S. pneumoniae*, commonly called pneumococcus, is the commonest cause of bacterial

community-acquired pneumonia, meningitis, otitis media, and bronchitis (27-29). It commonly

colonizes the upper respiratory tract of man and shows no symptoms of disease. From here it

adapts, proliferates and breaches host barriers to reach the circulatory system, lungs, spleen, and

brain where it causes disease. Children under the age of 5 years, the elderly, and

imunnocompromized individuals constitute the most vulnerable groups.

This vulnerability is related to the emergence of pneumococcal strains that are resistant to

common antibiotics and circumvents host immunity (30). Pneumococcus is a Gram-positive

bacterium surrounded by a cell wall that consists of teichoic acid-containing C-polysaccharide.

Attached to these molecules are PCh residues, which serve as the classical CRP-binding ligand.

Also present within the cell wall are cell wall-anchored pneumococcal surface proteins, choline-

binding proteins, and factor H inhibitor of complement, Hic (Fig. 1.2, next page) (31). Factor H

binds to pneumococci through Hic.

Pneumococcal polysaccharide vaccines have been developed against pneumococcus.

However, these have not been very successful due to the poor immunogenicity of the capsular

polysaccharides and lack of memory immune response in young children (32, 33). Capsular

polysaccharide-related strain variability also contributes to the lack of efficacy in these vaccines.

Conjugate vaccines that couple protein to capsular polysaccharides have been generated without

success due to high cost that would hinder implementation. Protein-based vaccines are being

explored using candidate pneumococcal surface proteins (34, 35).
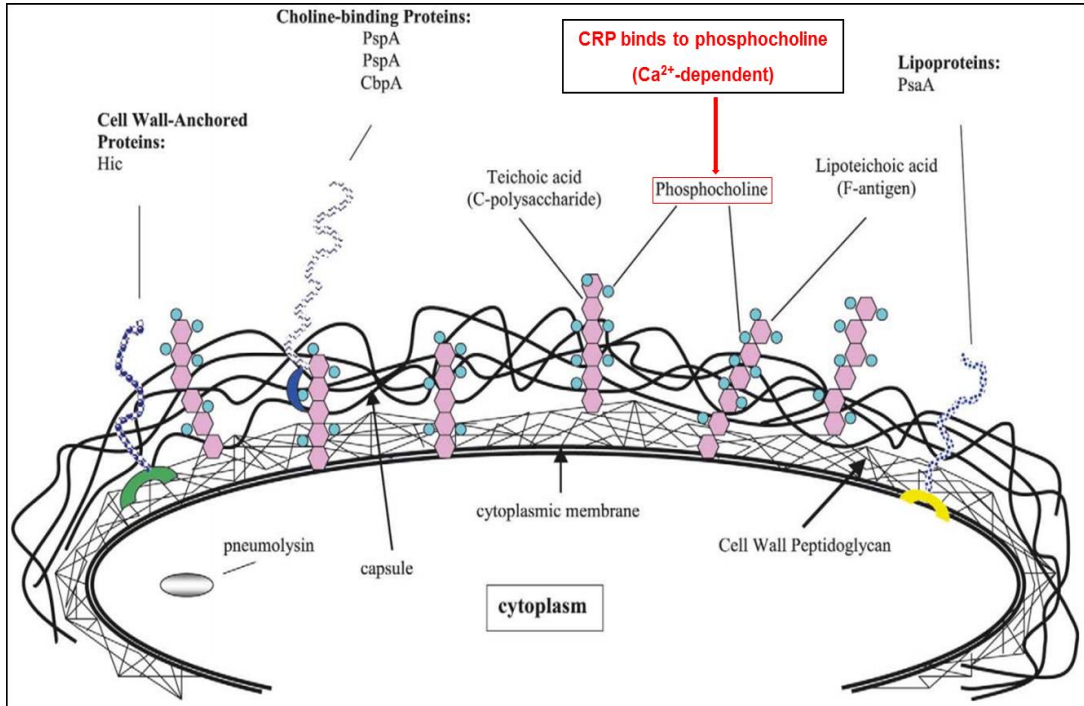


Figure 1.2. Structural relationships of surface structures and representation of selected immunogenic proteins of *S. pneumonia*. The figures was adapted to illustrate the binding of CRP to phosphocholine(Adapted from (66))

Functions of CRP

In broad terms, CRP has 2 functions: a recognition function and an effector function. The

recognition function involves the ability of CRP to recognize PCh-containing substances such as

PnC. The effector function involves the ability of PCh-complexed CRP to activate the

complement system. Previous data suggest that protection of mice results from the ability of

CRP to bind to pneumococci and activate complement (36). In mice, however, CRP is only a

trace serum component, not an acute phase protein (5). Mice have been used to explore the *in*

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*vivo* functions of human CRP. Passively administered human CRP is protective against lethal

pneumococcal infection, as determined by increased survival of and decreased bacteraemia in

infected mice (10, 36-37). Interestingly, CRP was most effective in protecting mice from

infection only when injected within the range of 6 h before to 2 h after administering

pneumococci into mice (38). The protective function of CRP was not observed when mice

received CRP 24 h or 36 h post infection (10, 38). Thus, the CRP-mediated protection of mice

requires the presence of CRP in the early stages of infection. Mice transgenic for human CRP

were also protected from lethal pneumococcal infection and showed both decreased bacteremia

and increased survival (39). However, the mechanism of CRP in pneumococcal infection has not

been elucidated (5, 40).

CRP also plays a role in inflammatory diseases including atherosclerosis. It has been

shown that CRP prevents the uptake of low density lipoprotein by macrophages, although CRP

was not found to be atheroprotective *in vivo* (41-42). The plasma level of CRP, however, serves

as an indicator of infection or inflammation states in clinical practice (43).

Factor H and CRP

Factor H is a single chain plasma glycoprotein with molecular weight of 150 kDa. It is

not only constitutively expressed in the liver but is also produced by monocytes, fibroblasts,

endothelial cells, keratinocytes, and platelets (44-46). The plasma circulating concentration of

factor H in humans is between 200-300 µg/ml (47-78). It is composed of 20 domains called short

consensus repeats (SCR 1-20), each of which is made up of about 60 amino acids stabilized by 2

internal disulphide bonds. Factor H is the main regulator of the alternative complement pathway

and helps to maintain an anti-inflammatory milieu (49-50). It does this by inhibiting the

assembly of C3 and C5 convertase enzymes through competition with factor B for C3b binding. It also facilitates the breakdown of the convertase by displacing bound factor Bb and acting as a cofactor for factor I in the cleavage and inactivation of C3b (51-52). It is the property of factor H to polyanionic surface molecules such as glycosaminoglycans and sialic acid that allows it to regulate complement activation on self surfaces (53).

Recent data suggest that CRP undergoes subtle structural modification under conditions of acidic pH, high salt concentration, and oxidation (54, 55), and that structurally altered CRP binds to FACTOR H. There is no evidence of an interaction between CRP and factor H under conditions of infection and/or inflammation in vivo (56). It was demonstrated that some proteins can acquire new functions when exposed to different environmental conditions, including post translational modifications and denaturation. Like immunoglobulins, CRP appears to acquire new functions based on structural modifications (57, 58). An important gain of function property is the ability of acidic pH-modified CRP to bind to factor H (54).

<u>Site-Directed Mutagenesis of CRP</u>

Exposure to low pH and oxidation were used to modify CRP and convey new characteristics/functions. The limitation of these strategies is that they are difficult to replicate *in vivo* in order to investigate the functional role of CRP in a homeostatic environment. Thus, our strategy was to use site-directed mutagenesis to modify CRP to generate CRP with functions not exhibited by the native protein (Fig. 1.3, next page). The advantage of this approach is that the CRP mutant generated can be used for *in vivo* studies.

Unlike other methods, which may result in transitional state structural change, CRP modified by mutagenesis undergoes a permanent change that can be verified by sequencing and assessing properties that were targeted for elimination or introduction.
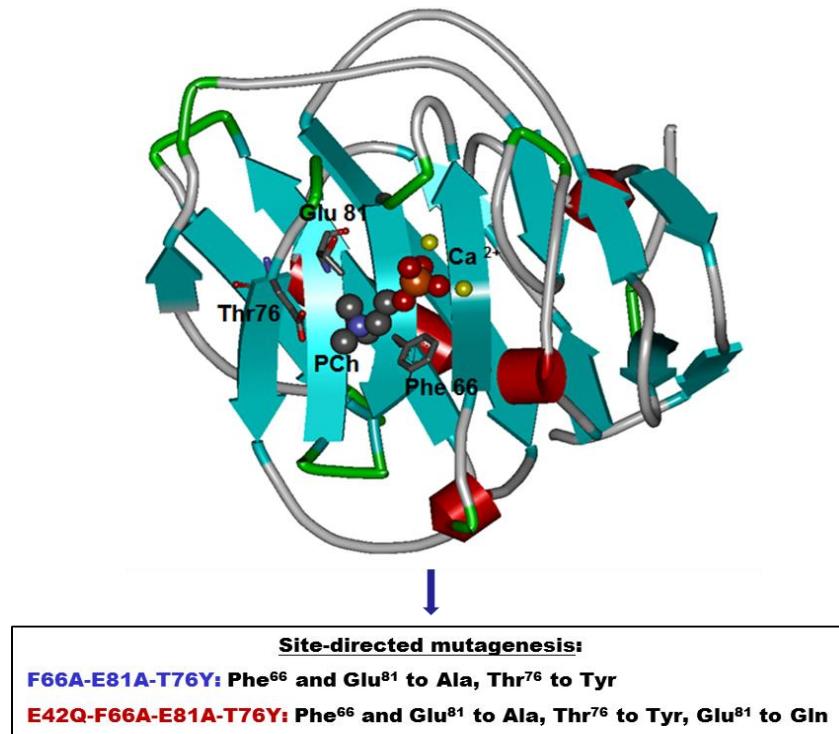


Figure 1.3. Site-directed mutagenesis of CRP. Based on the crystallographic structure of CRP, F66A/E81A/T76Y (**triple**) mutant CRP cDNA was generated using F66A/E81A cDNA as template. F66A/E81A/T76Y cDNA was in turn used for the generation of E42Q/F66A/E81A/T76Y (**quadruple**) mutant CRP cDNA in conjunction with that of E42Q. The figure was generated using PyMOL (65).

Rationale and Hypotheses

Because PnC-complexed CRP activates the classical complement pathway in both human and mouse sera (40), it was proposed that CRP is protective through a mechanism in which CRP binds to pneumococci through PCh groups present on their surface. The pathogen-bound CRP activates the complement system, and bacteremia is then reduced through complement-dependent opsonophagocytosis (59-61). It has also been shown that CRP enhances uptake and

presentation of pneumococcal antigens through FcγRs on dendritic cells and stimulates protective adaptive immunity (62). So we hypothesized that if the binding of CRP to PCh is required for the protection of mice against pneumococcal infection, then a CRP mutant that does not bind to PCh would not be protective against pneumococcal infection.

Pneumococci have been demonstrated to recruit factor H onto their surface. We postulated that the bacteria recruit factor H *in vivo* and exploit its complement regulatory property to prevent killing through complement activation. In the meantime, it has been determined that the administration of native CRP into mice later during infection does not provide protection. Factor H binds to modified forms of CRP (47, 54, 55, 63, and 64). E42Q CRP mutant that acquired factor H binding ability has been described (54). We suggest that a CRP mutant that does not bind to PCh but binds to factor H would permit the investigation of the involvement of factor H in bacterial serum resistance. We used site-directed mutagenesis to generate a CRP mutant that combines the property of not binding to PCh and binding to factor H and used it to demonstrate the role of factor H in bacterial infection. Based on this hypothesis, we proposed that the CRP mutant will bind to factor H on the bacterial surface and prohibit its complement regulating activity. This would allow WT CRP bound to the bacteria to facilitate complement activation and deposition.

This project is unique because it provides the opportunity to understand how CRP accomplishes its protective function against bacterial infection. Data from this study will provide an insight into how this evolutionarily conserved plasma protein has retained innate properties. Data generated would contribute to further understanding of how innate immunity evolved and may provide leads on targeting of community-acquired pneumonia and other pneumococcus-associated diseases.

## Specific Aims

1. To determine whether the binding of CRP to PCh on pneumococci is required for the protection of mice against initial stages of pneumococcal infection

   a. To generate a CRP mutant that does not bind to PCh

   b. To determine the protective ability of the CRP mutant generated in aim 1a in a mouse model of pneumococcal infection

      - We published the findings and reproduced in chapter 2.

2. To investigate the effects of a CRP mutant capable of binding to factor H on the protection of mice against late stages of pneumococcal infection

   a. To confirm bacterial recruitment of factor H

   b. To generate a CRP mutant that binds to factor H

   c. To assess binding of the CRP mutant to factor H-coated bacteria

   d. To use the CRP mutant in mouse protection experiments

      - The findings are reported in Chapter 3.

CHAPTER 2


The Phosphocholine-Binding Pocket on C-reactive Protein is Necessary for Initial Protection of Mice against Pneumococcal Infection

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Running Title: CRP, PCh , Pneumococcal infection

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Abstract

Human CRP protects mice from lethal *S. pneumoniae* infection when injected into mice 6 h before to 2 h after the administration of pneumococci. Given that CRP binds to PCh - containing substances and subsequently activates the complement system, it has been proposed that the anti-pneumococcal function of CRP requires the binding of CRP to PCh moieties present in PnC. To test this proposal experimentally, in this study, we utilized a new mutant CRP incapable of binding to PCh. Based on the structure of CRP-PCh complexes which showed that Phe$^{66}$, Thr$^{76}$ and Glu$^{81}$ formed the PCh -binding pocket, we constructed a mutant CRP F66A-T76Y-E81A in which the pocket was blocked by substituting Thr$^{76}$ with Tyr. Compared to wild-type CRP, the mutant CRP bound more avidly to PEt and could be purified by affinity chromatography using PEt-conjugated sepharose. The mutant CRP did not bind to PCh , PnC or pneumococci. The mutant CRP was free in the mouse serum and its rate of clearance *in vivo* was not faster than that of wild-type CRP. When either 25 µg or 150 µg of CRP was administered into mice, then unlike wild-type CRP, the mutant CRP did not protect mice from lethal pneumococcal infection. Mice injected with mutant CRP had higher mortality rates than mice that received wild-type CRP. Decreased survival was due to the increased bacteremia in mice treated with the mutant CRP. We conclude that the PCh -binding pocket on CRP is necessary for CRP-mediated initial protection of mice against pneumococcal infection.

<center>Introduction</center>

CRP, a member of the pentraxin family of proteins, reacts with PnC of *S. pneumoniae* in a $Ca^{2+}$-dependent manner (1-3). The binding specificity of CRP is for the PCh moieties present in PnC (4). CRP also binds to whole pneumococci in human and mouse sera and in $Ca^{2+}$-containing buffers (5-7). Another member of the pentraxin family, serum amyloid P (SAP), which is structurally similar to CRP, displays $Ca^{2+}$-dependent binding specificity for PEt (8-10). CRP also binds to PEt but not as avidly as it does to PCh (4, 8-13).

CRP is composed of 5 identical noncovalently attached subunits. Each subunit has 206 amino acids and the molecular weight of each subunit is approximately 23 kDa (14). All 5 subunits have the same orientation in the pentamer, with a PCh-binding site located on the same face of each subunit (14, 15). The PCh-binding site consists of a hydrophobic pocket formed by several amino acids including $Phe^{66}$, $Thr^{76}$ and $Glu^{81}$, and 2 $Ca^{2+}$ ions, which are bound to CRP by interactions with amino acids from other parts of the protein (14, 16). The phosphate group of PCh directly coordinates with the 2 $Ca^{2+}$ ions. The choline group of PCh lies within the hydrophobic pocket. $Phe^{66}$ provides hydrophobic interactions with the 3 methyl groups of choline. $Thr^{76}$ is critical for creating the appropriately sized pocket to accommodate PCh. $Glu^{81}$ interacts with the positively charged nitrogen atom of choline (Fig. 2.1*A*). Previous mutational analysis of $Thr^{76}$ in CRP has confirmed the significance of the hydrophobic pocket for PCh-binding (17). In SAP, at the position corresponding to $Thr^{76}$ in CRP, it is a Tyr ($Tyr^{74}$) (18, 19).

Pneumococci remain the most common cause of community-acquired pneumonia world-wide (20-22). In humans, CRP is an acute phase protein, that is, its serum concentration is increased several hundred-fold in response to pneumococcal infection (2). However, the functions of CRP in pneumococcal infection are not known (3). In mice, CRP is only a trace

<center>23</center>

serum component and is not an acute phase protein (23). Therefore mice are used to explore the

*in vivo* functions of human CRP. In mouse models of infection, passively administered human

CRP has been shown to be protective against lethal pneumococcal infection, as determined by

increased survival of and decreased bacteremia in the infected mice (24, 25). Interestingly, CRP

was most effective in protecting mice from infection only when injected within the range of 6 h

before to 2 h after administering pneumococci into mice (26). The protective function of CRP

was not observed when mice received CRP 24 h or 36 h post infection (7, 26). Thus, the CRP-

mediated protection of mice requires the presence of CRP in the early stages of infection. Mice

transgenic for human CRP were also protected from lethal pneumococcal infection and showed

both decreased bacteremia and increased survival (27).

Because PnC-complexed CRP activates the complement system, in both human and mouse

sera (3, 28), it has been proposed that CRP is protective through a pathway in which CRP binds

to pneumococci through PCh groups present on their surfaces, the pathogen-bound CRP

activates the complement system, and bacteremia is then reduced through complement-

dependent opsonophagocytosis (29-31). The aim of this study was to determine whether the

binding of CRP to PCh on pneumococci was required for the protection of mice against

pneumococcal infection. Employing site-directed mutagenesis, we generated a new mutant CRP,

F66A-T76Y-E81A, incapable of binding to PCh, and used the mutant CRP in mouse protection

experiments (Fig. 2.1*B*). We hypothesized that if the binding of CRP to PCh was required for the

protection of mice against pneumococcal infection, then the mutant CRP should not be

protective.

**A. WT CRP complexed with PCh**
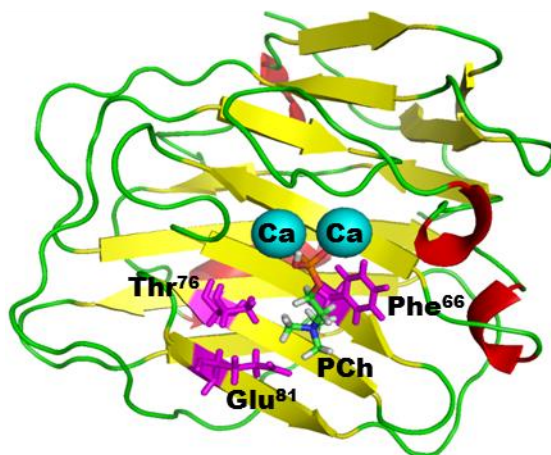
**B. Triple mutant CRP**
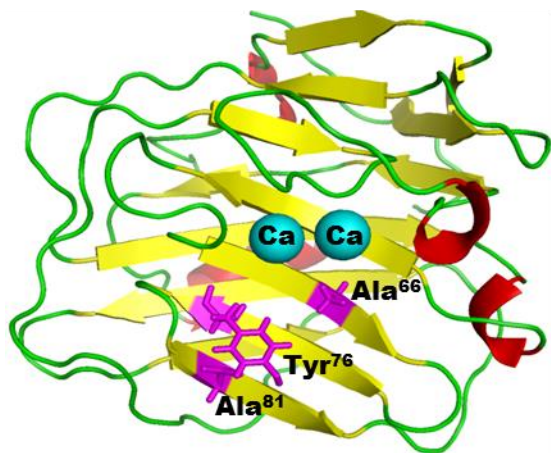
Figure 2.1. A subunit of CRP. *A*, structure of 1 of the 5 subunits of WT CRP bound to PCh (Protein Data Bank (PDB) ID 1B09) is shown. The side chains of Phe[66], Thr[76] and Glu[81] involved in the formation of the PCh-binding pocket are highlighted. Calcium ions ($Ca^{2+}$) are shown as cyan balls. *B*, molecular modeling of triple mutant CRP. The PDB coordinates of mutant CRP were generated from the PDB file 1B09) using SYBYL (Tripos, Inc.). The side chains of Phe[66], Thr[76] and Glu[81] are substituted with Ala, Tyr, and Ala, respectively. 1 of the 5 subunits is shown. The figures were rendered using PyMOL (66).

<u>Materials and Methods</u>

<u>Construction and expression of the CRP triple mutant F66A/T76Y/E81A</u>

The construction of the F66A-E81A mutant CRP cDNA has been described earlier (13). The F66A-E81A mutant CRP cDNA was used as the template for construction of the triple

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mutant CRP cDNA (substitution of Phe[66] and Glu[81] with Ala and of Thr[76] with Tyr). Mutagenic oligonucleotides, 5'-GGATACAGTTTT*TAC*GTGGGTGGGTCTG-3' and 5'-CAGACCCACCCAC*GTA*AAAACTGTATCC-3', to substitute Thr[76] with Tyr (codons shown in bold and italicised letters), were designed according to the sequence of F66A-E81A mutant CRP cDNA template and obtained from Integrated DNA Technologies. Mutagenesis was conducted using the QuikChange site-directed mutagenesis kit (Stratagene). Mutations were verified by nucleotide sequencing, utilizing the services of the Molecular Biology Core Facility of our university. Stable transfection of triple mutant CRP cDNA was carried out in CHO cells, as described previously (7). A CHO cell line expressing the Triple mutant CRP was isolated by a series of sub-cloning steps.

Preparation of PEt-conjugated sepharose

PEt-conjugated Sepharose was prepared as described previously (32). In brief, 25 ml of packed ECH-Sepharose 4B beads (GE Healthcare) was first washed with water (pH 4.5) and subsequently with 0.5 M NaCl. Then, 180 mg of PEt (Sigma-Aldrich, P0503) was dissolved in 25 ml water (pH 4.5) and added to the washed beads. 500 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma-Aldrich, E6383) was added to the mixture of PEt and sepharose beads, and stirred for 1 h at room temperature. After monitoring the pH for 1 h to ensure that the pH stayed at 4.5, the mixture was left overnight at 4 ℃ with slow stirring. The beads were then washed with 0.1 M acetate buffer, pH 4.0, followed by washing with 100 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl. Washing was repeated 3 times alternating between the acetate and Tris buffers. Finally, the beads were washed with water and then with TBS containing 2 mM $CaCl_2$.

Purification of native WT CRP

WT CRP was purified from discarded human pleural fluid by affinity chromatography on a PCh-sepharose column (Pierce) followed by ion-exchange chromatography on a MonoQ column (GE Healthcare) and gel filtration chromatography on a Superose12 column (GE Healthcare), as described previously (33), and stored frozen. On the day of the experiments, CRP was re-purified by gel filtration chromatography on a Superose12 column to remove any form of modified CRP which might have been generated due to storage of CRP. Re-purified CRP was stored in TBS containing 2 mM $CaCl_2$ at 4 ºC and was used within a week. The purity of CRP was confirmed by using denaturing SDS-PAGE.

Purification of triple mutant CRP

Purification of mutant CRP from the cell culture supernatants involved 2 steps: $Ca^{2+}$-dependent affinity chromatography on a PEt-conjugated sepharose column followed by gel filtration chromatography on a Superose12 column. For affinity chromatography, the culture media containing CRP was diluted (1:1) in 0.1 M borate buffer saline, pH 8.3, containing 3 mM $CaCl_2$ and passed through the PEt-conjugated sepharose column. After collecting the flow-through and washing the column with the same buffer, bound CRP was eluted with 0.1 M borate buffer saline, pH 8.3, containing 5 mM EDTA. Eluted CRP was concentrated and further purified by gel filtration chromatography on a Superose12 column. Gel filtration chromatography was carried out as described for WT CRP, except that the column was equilibrated and eluted with TBS containing 5 mM EDTA. It was necessary that the gel filtration chromatography of the mutant CRP be performed in the presence of EDTA because, in the presence of $Ca^{2+}$, the mutant CRP bound to Superose beads (data not shown). Eluted CRP was

immediately dialyzed against TBS containing 2 mM $CaCl_2$, stored at 4 ºC, and was used within a week. The purity of CRP was confirmed by using denaturing SDS-PAGE.

The concentration of purified WT and mutant CRP was determined by measuring the OD at 280 nm and using the extinction coefficient value of 19.5. For mouse protection experiments, both purified WT and mutant CRP were treated with the Detoxi-Gel Endotoxin Removing Gel according to manufacturer's instructions (Thermo). The concentration of endotoxin in CRP was determined by using the Limulus Amebocyte Lysate kit QCL-1000 according to manufacturer's instructions (Lonza).


PCh-binding assay

Binding activity of CRP for PCh was evaluated by using PCh-conjugated BSA and PnC (Statens Serum Institut) as the ligands, as described previously (7, 33). Microtiter wells (96-well plates) were coated with 10 □g/ml of PCh-BSA or PnC in TBS, overnight at 4 ºC. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS containing 5 mM $CaCl_2$, 0.1% gelatin and 0.02% Tween-20 (TBS-Ca buffer) was added in duplicate wells. After incubating the plates for 2 h at 37 ºC, the wells were washed with TBS-Ca buffer. The assays were performed in duplicate plates. In 1 plate, anti-CRP mAb HD2.4, diluted in TBS-Ca buffer, was used (1 h at 37 ºC) to detect bound CRP. In the other plate, rabbit polyclonal anti-CRP Ab (Sigma-Aldrich), diluted in TBS-Ca buffer, was used (1 h at 37 ºC) to detect bound CRP. HRP-conjugated goat anti-mouse IgG and HRP-conjugated donkey anti-rabbit IgG (Thermo), diluted in TBS-Ca buffer, were used (1 h at 37 ºC) as secondary Ab. Color was developed and the $OD_{405}$ read in a microtiter plate reader (Molecular Devices).

Anti-CRP mAb-binding assay

The anti-CRP mAb HD2.4- and EA4.1-binding assays were performed as described previously (13). Microtiter wells (96-well plates) were coated with 10 □g/ml of anti-CRP mAb HD2.4 (34, 35) or EA4.1 (34) in TBS, overnight at 4 ℃. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates for 2 h at 37 ℃, the wells were washed with TBS-Ca buffer. Rabbit polyclonal anti-CRP Ab (Sigma-Aldrich), diluted in TBS-Ca buffer, was used (1 h at 37 ℃) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (Thermo), diluted in TBS-Ca buffer, were used (1 h at 37 ℃) as secondary Ab. Color was developed and the $OD_{405}$ read in a microtiter plate reader (Molecular Devices).


Pneumococci

*S. pneumoniae* type 3 strain WU2, were made virulent by sequential i.v. passages in mice, and were stored in aliquots at -80°C in Todd-Hewitt broth containing 0.5% yeast extract and 10% glycerol, as described previously (7, 28). For each experiment, a separate aliquot of frozen pneumococci was thawed. Pneumococci were then grown in Todd-Hewitt broth containing 0.5% yeast extract and collected from mid-log phase cultures. Pneumococci were washed and resuspended in normal saline ($OD_{600}$ = 0.35 = 2.5 x $10^8$ CFU/ml). The concentration, purity and viability of pneumococci were confirmed by plating on blood agar plates.


Pneumococci-binding assay

Microtiter wells (96-well plates) were coated with pneumococci in TBS ($10^7$ CFU/100 μl/well) overnight at 4 ℃. The unreacted sites in the wells were blocked with TBS containing

0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates for 2 h at 37 ºC, the wells were washed with TBS-Ca buffer. The assays were performed in duplicate plates. The plates were then processed exactly as described for the PCh-binding assay.

The binding of CRP to pneumococci in the fluid phase was investigated as follows. Pneumococci (2 x $10^7$ CFU; final concentration $10^8$ CFU/ml) were incubated with CRP (10 $\Box$g; final concentration 50 $\Box$g/ml), in TBS containing 2 mM $CaCl_2$ and 0.02% Tween-20, at 37 ºC in a shaking water bath. After 30 min, pneumococci were pelleted, washed 3 times with the same buffer, resuspended in TBS, and subjected to denaturing SDS-PAGE.

PEt-binding assay

Binding activity of CRP for PEt was evaluated by using 1-oleoyl-2(1,2-biotinyl (amidodecanoyl)-*sn*-glycero-3-PEt (biotinylated-PEt) (Avanti Polar Lipids, 193053) as the ligand. Stock biotinylated-PEt (1 mg/ml in choloroform) was nitrogen-bubbled for 5 min to evaporate chloroform and then air-dried for 1 h at room temperature to evaporate residual chloroform. Biotinylated-PEt was resuspended in 1 ml ethanol, aliquoted, and stored at -20 ºC.

Microtiter wells (96-well plates) were coated with 10 $\Box$g/ml of avidin (Sigma-Aldrich, A9275) in TBS, for 2 h at 37 ºC. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. After washing the wells with TBS, biotinylated-PEt diluted in TBS (10 $\Box$g/ml) was added to the wells for 2 h at 37 ºC. After washing the wells with TBS-Ca buffer, CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates overnight at 4 ºC, the wells were washed with TBS-Ca buffer.

The assays were performed in duplicate plates. The plates were then processed exactly as described for the PCh-binding assay.

Streptavidin could not be used to capture biotinylated PEt on the wells because, in preliminary experiments, CRP was found to bind to streptavidin (data not shown). This was not the case with avidin coating (data not shown).

Mice

Male C57BL/6J mice (Jackson ImmunoResearch Laboratories) were brought up and maintained according to protocols approved by the University Committee on Animal Care. Mice were 8-10 week old when used in experiments.

Mouse protection experiments

Two separate mouse protection experiments were performed using 2 batches of purified WT and mutant CRP. Mice were first injected i.v. with either 25 μg or 150 μg of WT or triple mutant CRP in 150 μl TBS containing 2 mM $CaCl_2$. The endotoxin content in 25 μg and 150 μg WT CRP was 0.18±0.09 EU and 1.08±0.52 EU, respectively. The endotoxin content in 25 μg and 150 μg mutant CRP was 0.16±0.09 EU and 0.93±0.54 EU, respectively. After 30 min, mice were injected i.v. with 5 x $10^7$ CFU (based on $OD_{600}$) of pneumococci in 100 μl of saline. The actual number of pneumococci injected, based on the plating results obtained next day, was 5.15±0.13 x $10^7$ CFU. Survival of mice was recorded 3 times per day for 7 d. Survival curves were generated using the GraphPad Prism 4 software. To determine $p$ values for the differences in the survival curves among various groups, the survival curves were compared using the software's Logrank test. To determine bacteremia (CFU/ml) in the surviving mice, blood was

collected daily for 5 d from the tip of the tail vein, diluted in normal saline, and plated on blood agar plates for colony counting. Bacteremia values for dead mice was taken as $>10^8$ CFU/ml, because mice died when the bacteremia exceeded $10^8$ CFU/ml. The plotting and statistical analyses of the bacteremia data were done using the GraphPad Prism 4 software and Mann-Whitney two-tailed $t$-test.

Clearance of CRP from mouse circulation

Mice were injected i.v. with 100 µg of CRP in TBS containing 2 mM $CaCl_2$ through the tail. Blood was collected from the tip of the tail vein at various times up to 30 h. The concentration of CRP in the serum was measured by ELISA (13). The statistical analysis of the data was performed using the Mann-Whitney two-tailed $t$-test.

Repurification of CRP from purified triple mutant CRP-spiked mouse serum

Purified triple mutant CRP (1 mg) was added to 2 ml C57BL/6 mouse serum (Innovative Research) and incubated for 30 min at 37 ºC. The final volume was then increased to 10 ml by adding 0.1 M borate buffer saline, pH 8.3, containing 3 mM $CaCl_2$. The mutant CRP was repurified by $Ca^{2+}$-dependent affinity chromatography on the PEt-conjugated sepharose column, as described above. After collecting the flow-through and washing the column with the same buffer, bound CRP was eluted with 0.1 M borate buffer saline, pH 8.3, containing 5 mM EDTA. To control the experiment, mouse serum alone (2 ml), without spiking with purified mutant CRP, was used. The EDTA eluates were subjected to denaturing SDS-PAGE.

Results

## Characterization of the CRP triple mutant

The mutant CRP cDNA was successfully expressed in CHO cells and could be purified

by PEt-affinity chromatography followed by gel filtration chromatography. The elution profiles

of WT CRP and mutant CRP from the gel filtration column were almost overlapping; the elution

volume of the mutant CRP was only 250 µl less than that of WT CRP (Fig. 2.2*A*). Denaturing

SDS-PAGE analysis of purified WT CRP and mutant CRP showed single bands and the

molecular weight of the subunits of mutant CRP was same as that of WT CRP (Fig. 2.2*B*). We

also evaluated the epitope for the anti-CRP mAb HD2.4. The mAb HD2.4 is a pentameric CRP-

specific Ab and its epitope is located on the face opposite to the PCh-binding face of the CRP

pentamer (34, 36). As shown in Fig. 2.2*C*, both WT and mutant CRP recognized the mAb HD2.4

equally well. These data demonstrated that the substitution of Phe$^{66}$, Thr$^{76}$ and Glu$^{81}$ with Ala,

Tyr and Ala, respectively, did not affect the overall structure of CRP and that the mutant CRP

was pentameric.


## The CRP triple mutant  binds neither PCh nor pneumococci

The PCh-binding activity of the mutant CRP was assessed by using 2 different PCh-

containing ligands, PCh-BSA and PnC. WT CRP bound to both ligands in a CRP concentration-

dependent manner, but the mutant CRP neither bound to PCh-BSA (Fig. 2.3*A*) nor to PnC (Fig.

2.3*B*). We characterized the PCh-binding site using the anti-CRP mAb EA4.1 also. The binding

of this mAb to CRP is $Ca^{2+}$-dependent, and can be inhibited by PCh, indicating that EA4.1 binds

at or near the PCh-binding site (34). As shown in Fig. 2.3*C*, the mutations decreased the binding

of CRP to EA4.1 by approximately 99%; for equivalent binding to EA4.1, 100 ng/ml of mutant

CRP was required compared to 1 ng/ml of WT CRP, indicating that the EA4.1-binding epitope, and hence the PCh-binding site, on the mutant CRP was lost.
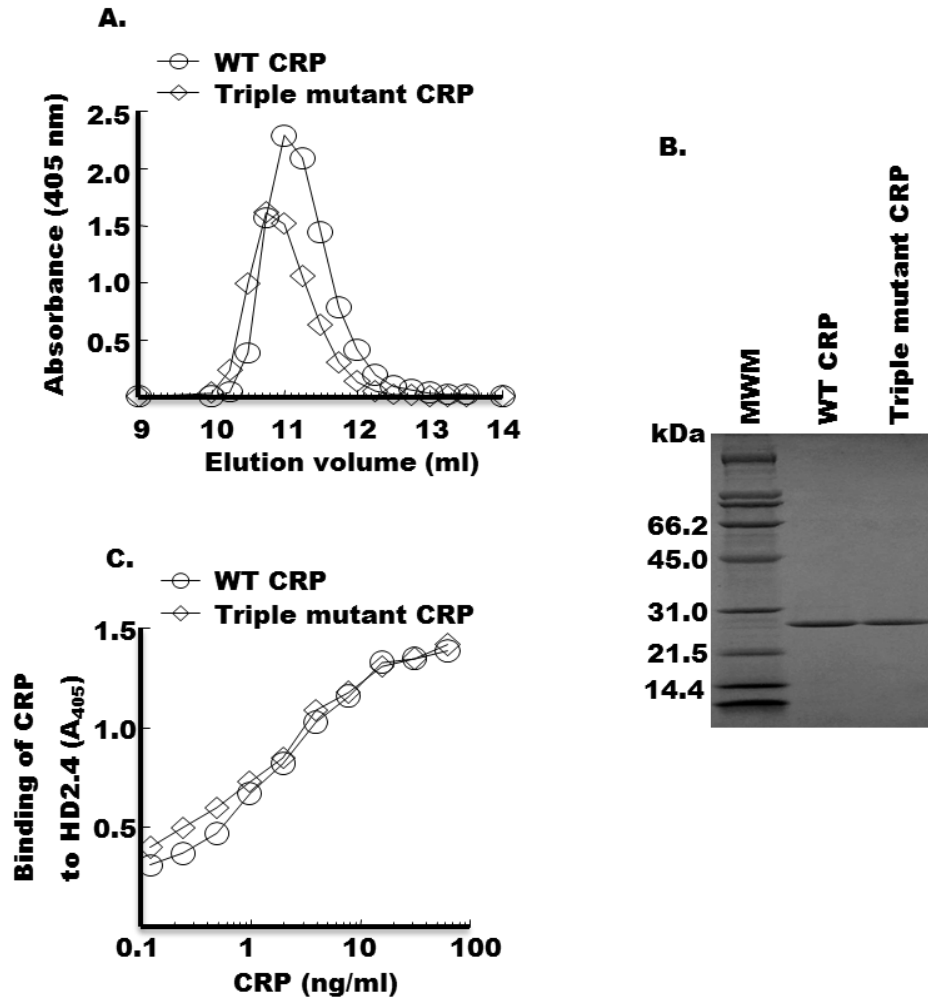


Figure 2.2. Overall pentameric structure of the CRP triple mutant F66A/T76Y/E81A. *A*, Elution profiles of WT and mutant CRP from the superose12 gel filtration column are shown. WT CRP (1.0 mg) in TBS containing 2 mM $CaCl_2$ was applied to the equilibrated column and eluted with the same buffer. Mutant CRP (0.95 mg) in TBS containing 5 mM EDTA was applied to the equilibrated column and eluted with the same buffer. Sixty fractions (0.25 ml) were collected and protein measured ($OD_{280}$) to determine the elution volume of CRP from the column. A representative of 3 experiments is shown. *B,* Denaturing SDS-PAGE (4%-20% gel) of CRP (5 mg). A representative gel, stained with Coomassie brilliant blue, is shown. *C*, microtiter wells were coated with anti-CRP mAb HD2.4. The unreacted sites in the wells were blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP was detected by using rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the absorbance was read at 405 nm. A representative of 3 experiments is shown.
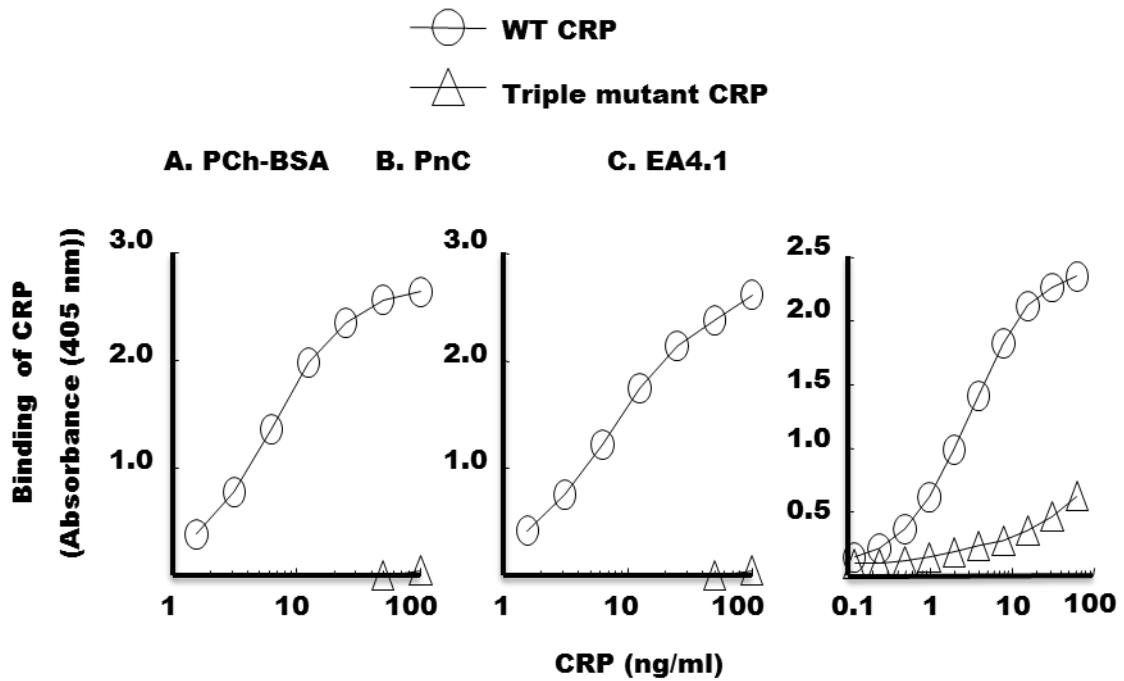
Figure 2.3. PCh-binding site of CRP. *A* and *B*, microtiter wells were coated with PCh-BSA (*A*) and PnC (*B*). The unreacted sites in the wells were blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was developed, and the absorbance was read at 405 nm. A representative of 4 experiments is shown. Similar results were obtained when polyclonal anti-CRP antibody was used to detect ligand-bound CRP (data not shown). *C*, microtiter wells were coated with anti-CRP mAb EA4.1. The unreacted sites in the wells were blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP was detected by using rabbit polyclonal anti-CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed, and the absorbance was read at 405 nm. A representative of 3experiments is shown.

We also determined the binding of mutant CRP to whole pneumococci that were used in the mouse protection experiments. In the solid phase pneumococci-binding assay, the mutant CRP did not bind to whole pneumococci (Fig. 2.4*A*). In the fluid phase binding assay also, the mutant CRP did not bind to pneumococci (compare lanes 4 and 5 in Fig. 2.4*B*). Thus, the Triple mutant CRP binds neither PCh nor pneumococci.
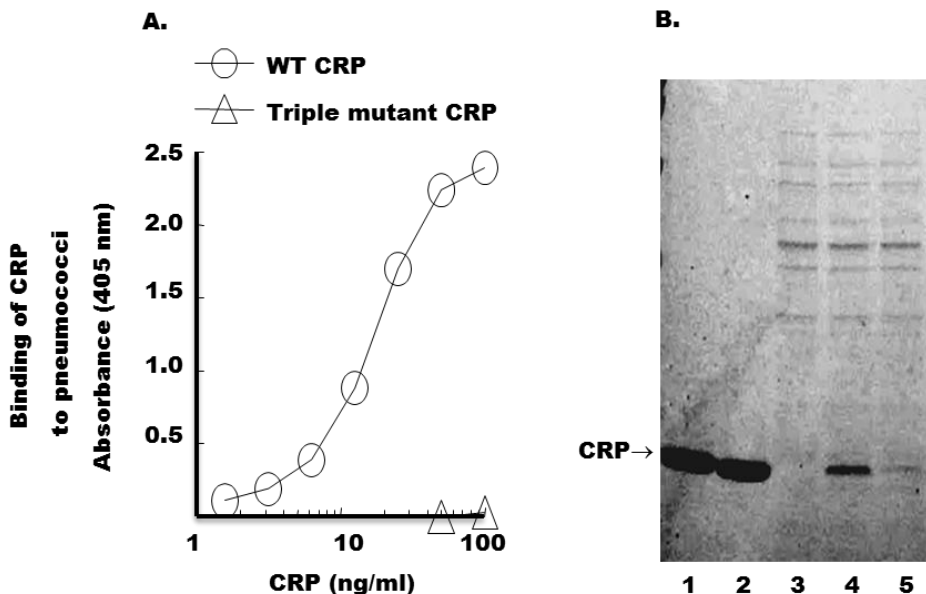
Figure 2.4. Binding of CRP to pneumococci. *A*, microtiter wells were coated with pneumococci. The unreacted sites in the wells were blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was developed, and the absorbance was read at 405 nm. A representative of 4 experiments is shown. Similar results were obtained when polyclonal anti-CRP antibody was used to detect ligand-bound CRP (data not shown). *B*, a representative denaturing SDS-PAGE gel (4–20%) stained with Coomassie Brilliant Blue is shown. *Lane 1*, purified WT CRP (5 μg); *lane 2*, purified mutant CRP (5 μg); *lane 3*, pneumococci alone; *lane 4*, pneumococci after mixing with WT CRP; *lane 5*, pneumococci after mixing with mutant CRP.

Because the substitution of Thr[76] to Tyr was based on the structure of SAP, and SAP binds to PEt (8-10), we next evaluated the effects of the mutations on the binding of CRP to PEt. Both, WT CRP and mutant CRP bound to PEt in a CRP concentration-dependent manner (Fig. 2.5). However, the mutant CRP was much more efficient than WT CRP in binding to PEt. For equivalent binding to PEt, 10 μg/ml of WT CRP was required compared to 170 ng/ml of mutant CRP. By repeating the PEt-binding assays 4 times, we found that approximately 98% less of mutant CRP was required compared to WT CRP for equivalent binding to PEt. Thus, the triple mutant CRP binds to PEt more avidly than WT CRP does.

36
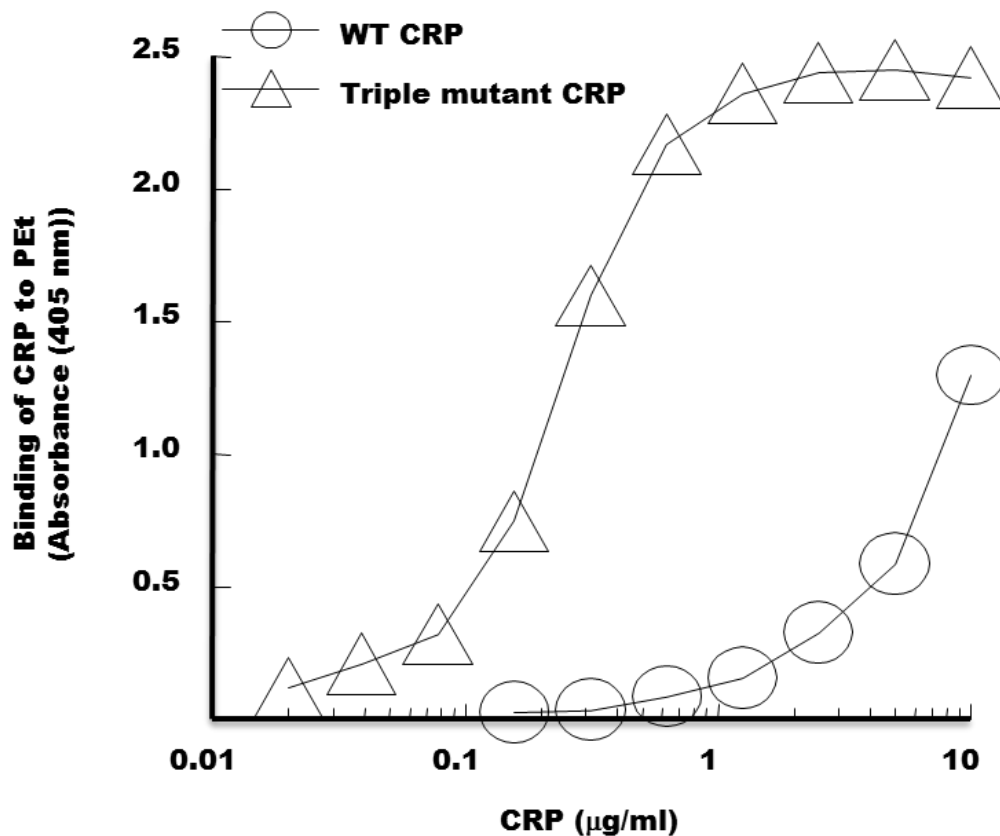
Figure 2.5. Binding of CRP to PEt. Microtiter wells were first coated with avidin in TBS. The unreacted sites in the wells were blocked with gelatin. Biotinylated PEt diluted in TBS was then added to the wells. After washing the wells with TBS, purified CRP diluted in TBS-Ca buffer was added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was developed and the OD was read at 405 nm. A representative of 4 experiments is shown. Similar results were obtained when polyclonal anti-CRP Ab was used to detect PEt-bound CRP (data not shown).

In the ligand-binding assays (Figs. 2*A*, 2*B*, 3*A*, and 4), similar results were obtained irrespective of which anti-CRP Ab, the anti-CRP mAb HD2.4 (data shown) or polyclonal anti-CRP Ab (data not shown), was used to detect ligand-bound CRP. Because the mAb HD2.4 is a pentameric CRP-specific Ab (34, 36), the ligand-binding data further suggested that the overall structure of the mutant CRP was not different from that of WT CRP.

<u>The CRP triple mutant does not protect mice from pneumococcal infection</u>

Fig. 2.6*A* shows the combined results of 2 separate protection experiments using 25 µg of CRP and 6 mice in each group in each experiment. The median survival time (the time taken for the death of 50% of mice) for mice injected with bacteria alone (control group A) was 40 h. The median survival time for mice injected with bacteria and WT CRP (group B) was 88 h. The median survival time for mice injected with bacteria and the mutant CRP (group C) was 44 h. In WT CRP-treated group, no deaths occurred in 44 h, and 25% mice survived up to 7 d. By the end of the 3rd day, all mice died in the mutant CRP-treated group while the survival was 58% in WT CRP-treated group. Fig. 2.6*B* shows the combined results of 2 separate protection experiments using 150 µg of CRP and 6 mice in each group in each experiment. The median survival time for mice injected with bacteria and WT CRP (group D) was 92 h. The median survival time for mice injected with bacteria and the mutant CRP (group E) was 46 h. In WT CRP-treated group, no deaths occurred in 46 h, and 17% mice survived up to 7 d. By the end of the 3rd day, 75% mice survived in WT CRP-treated group compared to only 25% in the mutant CRP-treated group. By the end of the 4th day, all mice died in the mutant CRP-treated group while the survival was 25% in WT CRP-treated group. Thus, similar results were obtained when mice were given either 25 µg CRP or 150 µg CRP; in contrast to WT CRP, the mutant CRP did not decrease mortality and did not prolong survival of infected mice. Because 25 µg of WT CRP was protective and the mutant CRP was not protective even when used at 150 µg that is, at 6-times more than the protective dose, we conclude that the CRP-mediated protection of mice from infection is dependent upon the PCh-binding activity of CRP.

**A.**



Group A: Bacteria alone (*n* =12)
Group B: Bacteria + 25 µg WT CRP (*n* =12)
Group C: Bacteria + 25 µg Triple mutant CRP(*n* =11)
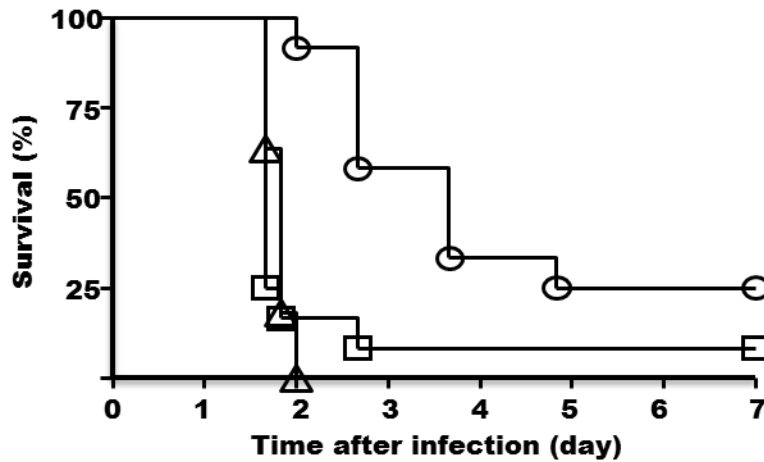
**B.**



Group A: Bacteria alone (*n* =12)
Group D: Bacteria + 150 µg WT CRP (*n* =12)
Group E: Bacteria + 150 µg Triple mutant CRP (*n* =12)
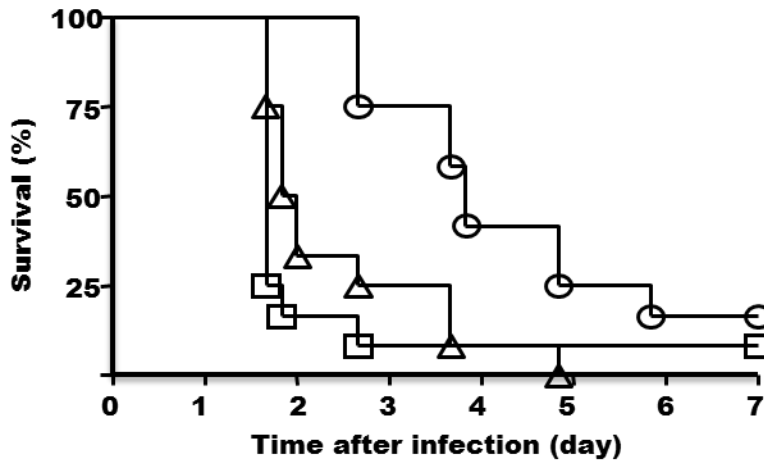
Figure 2.6. Survival curves of mice infected with *S. pneumoniae* with 150 μg of CRP. Mice were injected with 5 x $10^7$ CFU pneumococci, with or without 150 mg of either WT or mutant CRP. CRP was injected first; bacteria were injected 30 min later. Deaths were recorded 3 times a day for 7 days. The data are combined from 3 separate experiments with 6-8 mice in each group in each experiment. The *p* values for the differences in the survival curves among groups A/B, A/C, and B/C are <0.0001, <0.0001 and 0.49, respectively.

Fig. 2.7 shows the bacteremia values in mice from the protection experiment shown in Fig. 5.

Based on the median bacteremia values, in mice injected with bacteria alone (group A), 1 day

post infection, bacteremia was approximately $5.8 \times 10^4$ CFU/ml of blood. In mice injected with

bacteria and 25 μg WT CRP (group B), bacteremia was $8.4 \times 10^2$ CFU/ml 1 day post infection.

In mice injected with bacteria and 150 μg WT CRP (group D), bacteremia was $2.5 \times 10^3$ CFU/ml

1day post infection. However, in mice injected with bacteria and 25 μg mutant CRP (group C),

1 day post infection, bacteremia was $5.9 \times 10^5$ CFU/ml. In mice injected with bacteria and 150

μg mutant CRP (group E), 1day post infection, bacteremia was $2.3 \times 10^5$ CFU/ml. In groups A,

C and E, bacteremia increased dramatically after day 1, and mice died once bacteremia exceeded

$10^8$ CFU/ml. In mice administered with WT CRP, there was an increase in bacteremia past day

1, but it took another 2 days to exceed $10^8$ CFU/ml when those mice died, compared to <1 day

for the WT CRP-treated group. Statistically significant differences in bacteremia were observed

between the control (group A) and WT CRP-treated groups (groups B and D), and between the

WT CRP-treated and the mutant CRP-treated groups (groups C and E), until day 2. These results

indicated that the increased resistance to infection in WT CRP-treated mice was associated with

slower increase in bacteremia and that the PCh-binding activity of CRP was critical in this

process.

The CRP triple mutant stays free in the mouse serum and its clearance rate *in vivo* is not faster
than that of WT CRP

Although the mutant CRP did not protect mice from infection even when used at an amount

which was 6-times higher than the protective dose for WT CRP, we determined the rate of

clearance of mutant CRP from mouse circulation and compared it with that of WT CRP to

confirm that the mutant CRP was not protective because of its inability to bind pneumococci and

not because of its faster clearance *in vivo*.
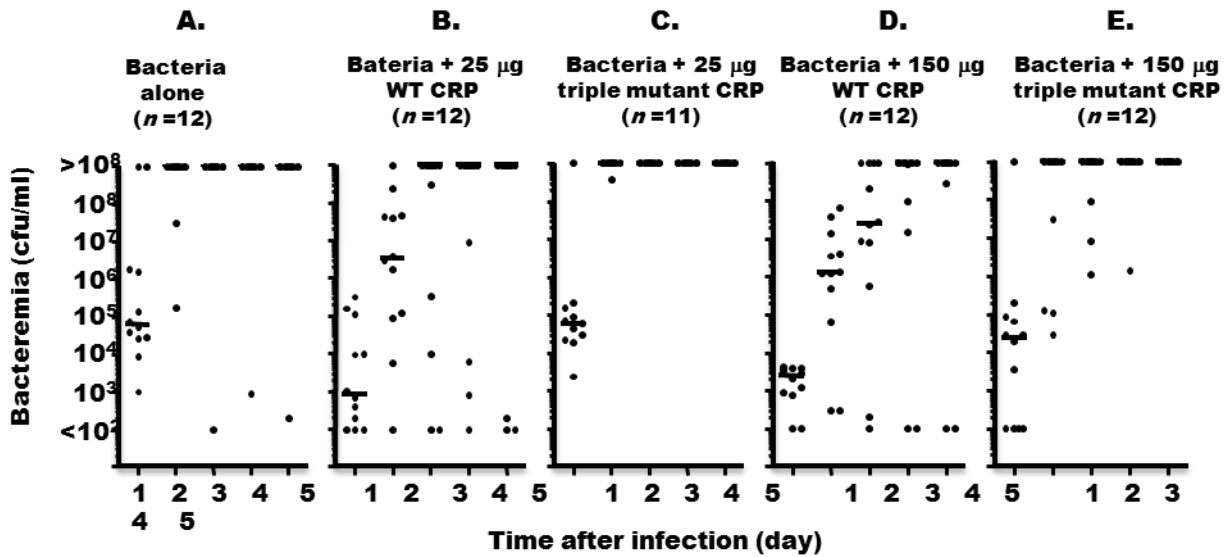


Figure 2.7. Bacteremia in mice treated with or without 150 μg of either WT or triple mutant CRP. Blood samples were collected from each surviving mouse shown in Fig. 7 for the first 5 days post-infection. Bacteremia was determined by plating. Each dot represents 1 mouse. The horizontal line in each group of mice represents the median value of bacteremia in that group. A bacteremia value of $>10^8$ indicates a dead mouse. The *p* values for the differences between groups A/B, A/C, and B/C, on day 1, are <0.0001, <0.0001 and 0.44, respectively. The *p* values for the differences between groups A/B, A/C, and B/C, on day 2, are <0.0001, <0.0001 and 0.19, respectively.

The average slope of the 4 clearance curves for WT CRP was -0.67±0.02 (Fig. 2.8*A*) and the

average slope of the 4 clearance curves for mutant CRP was -0.33±0.03 (Fig. 2.8*B*). Although

the rate of clearance of mutant CRP (0.33 μg/ml/h) was significantly different (*p* = 0.03) from

that of WT CRP (0.67 μg/ml/h), the clearance of mutant CRP was not faster than that of WT

CRP. These data suggested that the mutations did not confer instability to the mutant CRP *in*

*vivo*.

41
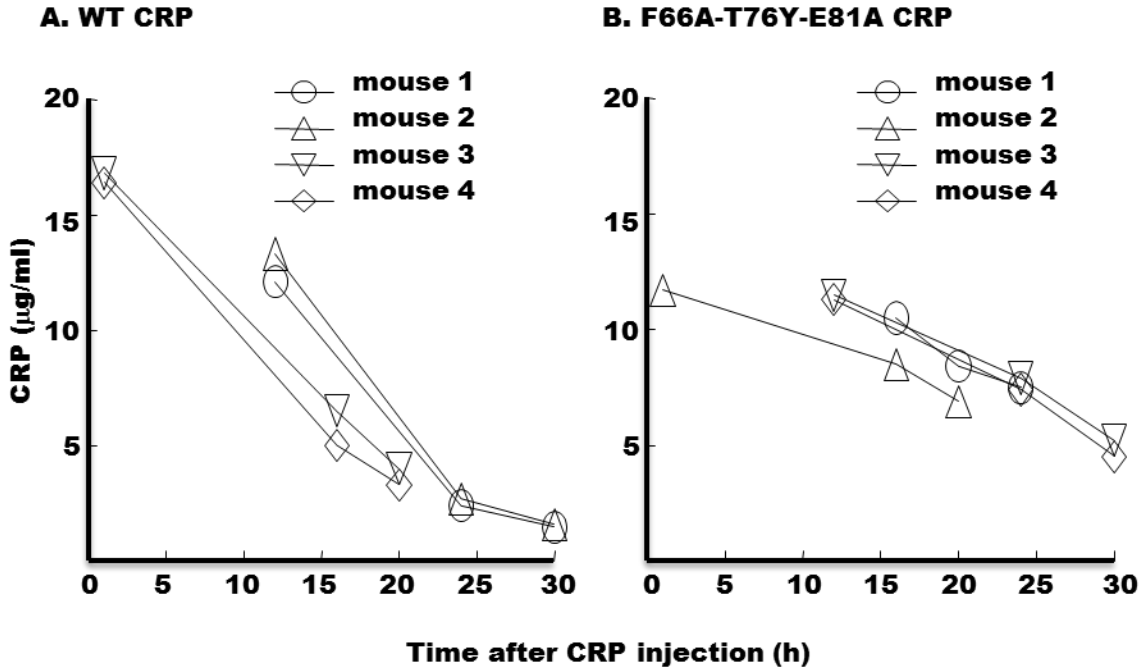
Figure 2.8. Clearance of CRP from mouse circulation. Mice were injected with 100 mg of CRP in TBS containing 2 mM $CaCl_2$. Blood was collected at various time points, sera separated, and the concentration of CRP measured. The time at which the CRP concentration in serum was at its peak was considered as zero time and the CRP concentration at this time was taken as 100% injected CRP; clearance of CRP was then followed at various time intervals.

Although the clearance data also suggested that the mutant CRP was free in circulation and available for functions because it reacted with anti-CRP mAb HD2.4, we used another approach to confirm that the mutant CRP was free in the mouse serum and was not sequestered by any other serum protein. As shown in Fig. 2.9, the mutant CRP present in the mouse serum bound to PEt in a $Ca^{2+}$-dependent manner and could be eluted with EDTA (lane 3). Besides CRP, no additional bands were seen when compared with the nonspecific bands seen with the serum alone control (compare lanes 3 and 4). Successful repurification of the mutant CRP from the mutant CRP-spiked mouse serum further suggested that the mutant CRP was free in the mouse serum and was not sequestered by any other serum protein.

42
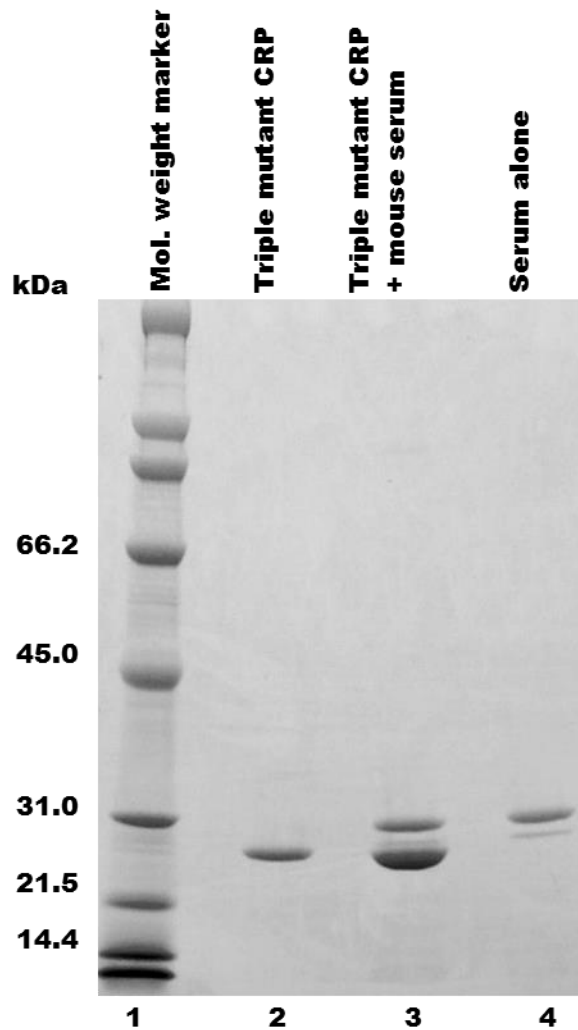
Figure 2.9. Repurification of CRP triple mutant from purified mutant CRP-spiked mouse serum. A representative denaturing SDS-PAGE gel (4–20%) stained with Coomassie Brilliant Blue is shown. *Lane 1*, molecular mass markers; *lane 2*, purified mutant CRP (5 μg); *lane 3*, EDTA eluate (25 μl, $A_{280}$ 1.13) from the PEt affinity chromatography column through which mouse serum containing mutant CRP was passed in the presence of $CaCl_2$; *lane 4*, EDTA eluate (25 μl, $A_{280}$ 0.29) from the PEt affinity chromatography column through which mouse serum alone was passed in the presence of $CaCl_2$.

In this study, we tested the hypothesis that the CRP-mediated protection of mice from pneumococcal infection is dependent upon the PCh-binding activity of CRP. We mutated 3 amino acids in the PCh-binding pocket of CRP in order to generate a mutant CRP incapable of binding to PCh. We then compared the protective ability of WT CRP with that of mutant CRP. Our major findings were: 1. Substitution of Phe$^{66}$, Thr$^{76}$ and Glu$^{81}$ with Ala, Tyr and Ala, respectively, abolished the PCh-binding, PnC-binding and pneumococcus-binding activity of CRP. 2. The triple mutant CRP was more efficient than WT CRP in binding to PEt. 3. Mutations in the PCh-binding pocket of CRP did not affect the overall pentameric structure of CRP. 4. The triple mutant CRP stayed free in the mouse serum and its clearance rate *in vivo* was not faster than that of WT CRP. 5. At both 25 µg and 150 µg the triple mutant CRP did not protect mice from pneumococcal infection.

Previously, we tested the same hypothesis that the CRP-mediated protection of mice from pneumococcal infection is dependent upon the PCh-binding activity of CRP (7). The mutant CRP used in the earlier investigation was a CRP double mutant, F66A-E81A, which also does not bind to PCh, PnC or pneumococci (7, 13). We reported that the F66A-E81A mutant CRP was as capable as WT CRP in protecting mice from pneumococcal infection, which was a surprising finding (7). This and the following 3 points prompted us to make another mutant CRP incapable of binding to PCh and repeat the mouse protection experiments. First, we wanted to use a mutant CRP, incapable of binding to PCh, with more drastic changes in the PCh-binding pocket. We wanted to block the pocket. In the earlier F66A-E81A double mutant, although 2 residues were mutated, the pocket was not blocked. Second, we wanted to use freshly purified CRP in all experiments. Third, because we were now successful in establishing a mouse model of

pneumococcal infection in which 25 µg CRP was protective, as has been shown by others (25, 26), we wanted to use 2 different doses of CRP (25 µg and 150 µg) in protection experiments. Previously (7), we only used 1 dose of CRP, 150 µg.

As shown in Fig. 9$A$, Phe[66], Thr[76] and Glu[81] participate in CRP-PCh interaction (16). To generate a mutant CRP incapable of binding to PCh, our mutagenesis plan included mutating all 3 amino acids. Based on the structure of SAP (18), we mutated Thr to Tyr, and added the T76Y mutation to our previously published mutant CRP, F66A-E81A. Molecular modelling of the triple mutant CRP showed that the large side chain of Tyr could partially block the PCh-binding pocket (Fig. 2.1$B$). Loss of binding of triple mutant CRP to PCh, but dramatically enhanced binding to PEt, further indicated that the PCh-binding pocket was blocked in the mutant CRP. Also, it was easier to purify this mutant CRP by affinity chromatography due to its strong avidity for PEt. Interestingly, like SAP (9, 10, 37, 38), the mutant CRP exhibited carbohydrate-binding property; in the presence of $Ca^{2+}$, the mutant CRP bound to agarose beads used for gel filtration chromatography (data not shown).

CRP binds to FcγR (39-41), the FcγR have been implicated in the protection against pneumococcal infection in mice (42), and CRP has been shown to enhance uptake and presentation of pneumococcal antigens through FcγR on dendritic cells and stimulate protective adaptive immunity (43). However, we did not characterize the mutant CRP for binding to FcγR because FcγR do not participate in CRP-mediated protection of mice against pneumococcal infection; CRP was equally protective in WT and FcγR knockout mice (44). We did not characterize the mutant CRP for activating the complement system because CRP activates complement only when ligand-complexed (30), but the mutant CRP was unable to complex with PCh-BSA, PnC or pneumococci.

We used a high dose, $5 \times 10^7$ CFU, of pneumococci in mouse protection experiments. This dose of bacteria was necessary, because endogenous mouse CRP might have been protective against lower doses of bacteria. It has been reported that endogenous mouse CRP is also protective against pneumococcal infection in mice; the $LD_{50}$ of pneumococci was drastically reduced in CRP knockout mice (45). We used 25 µg and 150 µg CRP in mouse protection experiments, however, we also found that even 10 µg of WT CRP was protective (data not shown), as has been reported earlier (24-26). Our finding that WT CRP was protective while mutant CRP was not protective even at 150 µg dose clearly indicated that the PCh-binding pocket in CRP was critical for the initial protection of mice from pneumococcal infection. Our findings are consistent with other reports which showed that the PCh on pneumococci mediated the function of CRP to block the attachment of pneumococci to platelet-activating factor receptors on human pharyngeal epithelial cells and that the PCh-binding activity of CRP was required for the protection of mice from challenge with platelet-activating factor (46, 47).

Because CRP protects mice from infection only when injected within the range of 6 h before to 2 h after administering pneumococci, and not when injected 24 h or 36 h post infection (7, 26), we conclude that CRP is able to use the PCh-binding-based mechanism for the protection of mice only during the early stages of infection. It has been shown that native CRP undergoes structural transformations under several different experimental conditions (3, 33, 48-53). It has also been shown that structurally altered, or slightly-to-completely denatured, CRP is capable of binding to immobilized factor H (3, 33, 51-56). Factor H is a regulator of complement activation and has been implicated in the resistance of pneumococci to complement attack (3, 57-59). It remains to be investigated whether such CRP-factor H interaction plays any role in the CRP-mediated protection of mice from pneumococcal infection. However, the combined data indicate

that the CRP-mediated protection of mice requires the presence of CRP during the early stages of infection.

The outcome of the *in vivo* experiments obtained with the use of the triple mutant CRP in this investigation were different from the results obtained with the use of the CRP double mutant reported earlier (7); the reasons behind this difference are not clear. Both mutants do not bind to PCh, PnC or pneumococci. The only differences in the ligand-binding properties of the 2 mutants were toward their binding avidity for PEt and mAb EA4.1 (supplemental Fig. 2.1), which do not explain the differences in the outcome of the *in vivo* experiments. Whether the difference in the results of the 2 *in vivo* experiments was due to the difference in the protective ability of WT CRP in the 2 animal model systems, or due to the difference in the ages of purified CRP, remains to be explored. Development of infection models involving passively administered human WT and mutant CRP in CRP knockout mice (45, 60-62) and SAP knockout mice (63-65), and also the development of mice transgenic for mutant CRPs may provide more information on the mechanisms of anti-pneumococcal function of CRP in protecting mice against early and late stages of infection.

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CHAPTER 3


Generation of a C-reactive protein mutant that binds to factor H: Evaluation of the effects of

mutant CRP during late-stage pneumococcal infection in mice

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Abstract

Human CRP protects mice from lethal *S. pneumoniae* infection when injected into mice 6 h before to 2 h after the administration of pneumococci. However, when injected at 36 h after infection, CRP does not offer any protection against pneumococcal infection in mice. Because pneumococci have been shown to recruit factor H, we proposed that the 36 h serum resistance observed with pneumococci is due to the complement-inhibitory property of pneumococci-bound factor H. To investigate the role of factor H in pneumococcal serum resistance, we employed site-directed mutagenesis of CRP to generate a mutant which binds to factor H but does not bind to PCh. Based on the factor H-binding property of a previously reported CRP mutant E42Q, we added the E42Q mutation to CRP triple mutant F66A/T76Y/E81A incapable of binding to PCh. We constructed and expressed CRP quadruple mutant E42Q/F66A/T76Y/E81A. Unlike wild-type CRP, mutant CRP bound avidly to factor H immobilized on microtiter wells but did not bind to PCh-BSA, PnC, or pneumococci. Mutant CRP also bound to *in vitro*-prepared factor H-coated pneumococci as well as to factor H-coated pneumococci isolated from the infected mice. The rate of clearance of mutant CRP from mouse circulation was similar to that of wild-type CRP. These results suggest that quadruple mutant CRP may be able to bind to serum resistant pneumococci through immobilized factor H and render them susceptible by neutralizing the complement inhibitory properties of factor H. *In vivo* experiments using the quadruple CRP mutant are in progress. We anticipate that the combination of wild-type and quadruple mutant CRP should be protective during the late stages of infection; wild-type CRP would bind to PCh and activate complement while mutant CRP would cover factor H to prevent its complement-inhibitory activity. Our long-term goal is to explore the possibility of developing a CRP-based strategy to treat pneumococcal infection.

Introduction

C-reactive protein is a member of the pentraxin family of proteins which reacts with PnC of *S. pneumoniae* in a $Ca^{2+}$-dependent manner (1, 2, 3). The binding specificity of CRP is for the PCh residues present in PnC (4). CRP also binds to whole pneumococci in humans (5, 6, 7). Another member of the pentraxin family, serum amyloid P component (SAP), which is structurally similar to CRP, displays $Ca^{2+}$-dependent binding specificity for PEt (8, 9, 10). CRP also binds to PEt but not as avidly as it binds to PCh (8, 9, 10, 11, 12, and 13).

CRP is composed of 5 identical non-covalently attached subunits. Each subunit has 206 amino acids and the molecular weight of each subunit is approximately 23 kDa (14). All 5 subunits have the same orientation in the pentamer, with a PCh-binding site located on the same face of each subunit (14, 15). The PCh-binding site consists of a hydrophobic pocket formed by several amino acids including Phe[66], Thr[76] and Glu[81], and 2 $Ca^{2+}$ ions which are bound to CRP by interactions with amino acids from other parts of the protein (14, 16). The phosphate group of PCh directly coordinates with the 2 $Ca^{2+}$ ions. The choline group of PCh lies within the hydrophobic pocket. Phe[66] provides hydrophobic interactions with the 3 methyl groups of choline. Thr[76] is critical for creating the appropriately sized pocket to accommodate PCh. Glu[81] interacts with the positively charged nitrogen atom of choline. Previous mutational analyses of Thr[76] in CRP have confirmed the significance of the hydrophobic pocket for PCh-binding (17). In SAP, at the position corresponding to Thr[76] in CRP, it is a Tyr residue (Tyr[74]) (18, 19). The Tyr may play a role in the high binding avidity of SAP to PEt.

In humans, CRP is an acute phase protein, that is, its serum concentration is increased several hundred-fold in response to pneumococcal infection (2). The functions of CRP, however, in pneumococcal infection are not known (20, 21). In mice, however, CRP is only a trace serum

component, not an acute phase protein (22). Mice have been used to explore the *in vivo* functions

of human CRP. Passively administered human CRP has been shown to be protective against

lethal pneumococcal infection, as determined by increased survival of and decreased bacteremia

in the infected mice (7, 23, 24). Interestingly, CRP was most effective in protecting mice from

infection only when injected within 6 h before and 2 h after administering pneumococci into

mice (25). The protective function of CRP was not observed when mice received CRP 36 h post

infection (7, 25). Thus, the CRP-mediated protection of mice requires the presence of CRP in the

early stages of infection. The failure of CRP to protect mice during late stage of infection is

inexplicable. Mice transgenic for human CRP were also protected from lethal pneumococcal

infection and showed both decreased bacteremia and survival (26).

Pneumococci, like many bacteria, have been demonstrated to recruit factor H onto their

surface (27, 28, 29, 30). The possible involvement of pneumococci-recruited factor H in bacterial

serum resistance is a question that warrants investigation. Factor H is a single chain plasma

glycoprotein with molecular weight of 150 kDa. It is not only constitutively expressed in the

liver but is also in monocytes, fibroblasts, endothelial cells, keratinocytes, and platelets (31, 32,

33). The plasma circulating concentration of factor H in humans is between 200-300 µg/ml (34,

35). It is composed of 20 domains called short consensus repeats (SCR 1-20), each of which is

made up of about 60 amino acids stabilized by 2 internal disulphide bonds. Factor H is the main

regulator of the alternative complement activation pathway and helps to maintain an anti-

inflammatory milieu (36, 37). It does this by inhibiting the assembly of C3 and C5 convertase

enzymes through competition with factor B for C3b binding. It also facilitates the breakdown of

the convertase by displacing bound factor Bb and acting as a cofactor for factor I in the cleavage

and inactivation of C3b (38, 39).

There is limited evidence of an interaction between pentameric CRP and factor H under conditions of infection and/or inflammation (40). However, modified forms of CRP have been shown to bind readily to factor H (34, 40, 41, 42, 43, 44). The E42Q mutant CRP, with factor H binding ability has been described (45). PnC-complexed CRP activates the classical complement pathway in both human and mouse sera (21). We have demonstrated earlier that CRP is protective through a mechanism in which CRP binds to pneumococci through PCh groups present on their surface only during the early stages of infection (46). It has also been shown that CRP enhances uptake and presentation of pneumococcal antigens through FcγRs on dendritic cells and stimulates protective adaptive immunity (47). The observation that administration of CRP to mice infected with pneumococci at 36 h does not offer protection suggests that there is an undefined mechanism that operates during the later stages of pneumococcal infection. We hypothesized that the bacteria recruit factor H *in vivo* and exploit its complement regulatory ability to prevent complement activation by the host. We suggested that a mutant CRP that does not bind to PCh but binds to factor H would allow us to investigate the involvement of factor H in bacterial serum resistance. We employed site-directed mutagenesis to generate a mutant CRP that combines the property of not binding to PCh and binding to factor H and used it to demonstrate the role of factor H in bacterial infection. Based on the above hypothesis, we proposed that the mutant CRP would bind to factor H on the bacterial surface and neutralize its complement regulating activity. This would allow WT CRP bound to the bacteria to facilitate complement activation and deposition when administered to mice during late stage pneumococcal infection.

Construction and expression of the CRP quadruple mutant E42Q/F66A/T76Y/E81A

The construction of the triple mutant CRP cDNA has been described earlier (46). The triple mutant CRP cDNA was used as the template for construction of the quadruple mutant quadruple mutant CRP cDNA (substitution of $Glu^{42}$ with Gln, $Phe^{66}$ & $Glu^{81}$ with Ala and of $Thr^{76}$ with Tyr). Mutagenic oligonucleotides, 5'-C CAC TTC TAC ACG *CAA* CTG TCC TCG ACC-3' and 5'-GGT CGA GGA CAG *TTG* CGT GTA GAA GTG G-3', to substitute $Glu^{42}$ with Gln (codons shown in bold and italicized letters), were designed according to the sequence of triple mutant CRP cDNA template and obtained from Integrated DNA Technologies. Mutagenesis was conducted using the Quick Change site-directed mutagenesis kit (Stratagene). Mutations were verified by nucleotide sequencing, utilizing the services of the Molecular Biology Core Facility of the university. Stable transfection of the quadruple mutant CRP cDNA was carried out in CHO cells, as described previously (7). A CHO cell line expressing the Quadruple mutant CRP was isolated by a series of sub-cloning steps.

Purification of WT CRP

WT CRP was purified from discarded human pleural fluid by affinity chromatography on a PCh-sepharose column (Pierce) followed by ion-exchange chromatography on a MonoQ column (GE Healthcare) and gel filtration chromatography on a Superose12 column (GE Healthcare), as described previously (44), and stored frozen. On the day of the experiments, CRP was re-purified by gel filtration on a Superose12 column to remove any form of modified CRP which might have generated due to storage of CRP. Re-purified CRP was stored in TBS

containing 2 mM $CaCl_2$ at 4 ºC and was used within a week. The purity of CRP was confirmed by using denaturing SDS-PAGE.

Purification of CRP quadruple mutant

Purification of mutant CRP from the cell culture supernatants involved 2 steps: $Ca^{2+}$-dependent affinity chromatography on a PEt-conjugated sepharose column followed by gel filtration on a Superose12 column. For affinity chromatography, the culture media containing CRP was diluted (1:1) in 0.1 M borate buffer saline, pH 8.3, containing 3 mM $CaCl_2$ and passed through the PEt-conjugated sepharose column. After collecting the flow-through and washing the column with the same buffer, bound CRP was eluted with 0.1 M borate buffer saline, pH 8.3, containing 5 mM EDTA. Eluted CRP was concentrated and further purified by gel filtration on a Superose12 column. Gel filtration was carried out as described for WT CRP, except that the column was equilibrated and eluted with TBS containing 5 mM EDTA. It was necessary that the gel filtration of mutant CRP be performed in the presence of EDTA because, in the presence of $Ca^{2+}$, the mutant CRP bound to Superose beads (data not shown). Eluted CRP was immediately dialyzed against TBS containing 2 mM $CaCl_2$, stored at 4 ºC, and was used within a week. The purity of CRP was confirmed by using denaturing SDS-PAGE.

For mouse protection experiments, both purified WT and mutant CRP were treated with the Detoxi-Gel Endotoxin Removing Gel according to manufacturer's instructions (Thermo). The concentration of endotoxin in CRP preparations and buffers was determined by using the Limulus Amebocyte Lysate kit QCL-1000 according to manufacturer's instructions (Lonza).

<u>PCh-binding assay</u>

Binding activity of CRP for PCh was evaluated by using PCh-conjugated BSA and PnC (Statens Serum Institut) as the ligands, as described previously (7, 44). Microtiter wells (96 well plates) were coated with 10 µg/ml of PCh-BSA or PnC in TBS, overnight at 4 ℃. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS containing 2 mM $CaCl_2$, 0.1% gelatin and 0.02% Tween-20 (TBS-Ca buffer) was added in duplicate wells. After incubating the plates for 2 h at 37 ℃, the wells were washed with TBS-Ca buffer. The assays were performed in duplicate plates. In 1plate, anti-CRP mAb HD2.4, diluted in TBS-Ca buffer, was used (1 h at 37 ℃) to detect bound CRP. In the other plate, rabbit polyclonal anti-CRP Ab (Sigma-Aldrich), diluted in TBS-Ca buffer, was used (1 h at 37 ℃) to detect bound CRP. HRP-conjugated goat anti-mouse IgG and HRP-conjugated donkey anti-rabbit IgG (Thermo), diluted in TBS-Ca buffer, were used (1 h at 37 ℃) as secondary antibodies. Color was developed and the $OD_{405}$ read in a microtiter plate reader (Molecular Devices).

<u>PEt-binding assay</u>

Binding activity of CRP for PEt was evaluated by using 1-oleoyl-2(1, 2-biotinyl (amidodecanoyl)-*sn*-glycero-3-PEt (biotinylated-PEt) (Avanti Polar Lipids, 193053) as the ligand. Stock biotinylated-PEt (1 mg/ml in choloroform) was nitrogen-bubbled for 5 min to evaporate chloroform and then air-dried for 1 h at room temperature to evaporate residual chloroform. Biotinylated-PEt was resuspended in 1 ml ethanol, aliquoted, and stored at -20 ℃.

Microtiter wells (96 well plates) were coated with 10 µg/ml of avidin (Sigma-Aldrich, A9275) in TBS, for 2 h at 37 ℃. The unreacted sites in the wells were blocked with TBS

containing 0.5% gelatin for 45 min at room temperature. After washing the wells with TBS, biotinylated-PEt diluted in TBS (10 µg/ml) was added to the wells for 2 h at 37 ºC. After washing the wells with TBS-Ca buffer, CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates overnight at 4 ºC, the wells were washed with TBS-Ca buffer. The assays were performed in duplicate plates. The plates were then processed exactly as described for the PCh-binding assay.

Streptavidin could not be used to capture biotinylated PEt on the wells because, in preliminary experiments, CRP was found to bind to streptavidin (data not shown). This was not the case with avidin coating (data not shown).

Factor H binding assay

The binding activity of CRP for factor H was evaluated using mouse factor H (R & D Systems) and human factor H (Complement Technology) as the ligands in solid phase ELISA. Microtiter wells were coated with 10 µg/ml of mouse factor H and human factor H in TBS, overnight at 4 ºC. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates for 2 h at 37 ºC, the wells were washed with TBS-Ca buffer. Rabbit polyclonal anti-CRP Ab (Sigma-Aldrich), diluted in TBS-Ca buffer, was used (1 h at 37 ºC) to detect bound CRP.  HRP-conjugated donkey anti-rabbit IgG (Thermo), diluted in TBS-Ca buffer, were used (1 h at 37 ºC) as secondary antibody. Color was developed and the $OD_{405}$ read in a microtiter plate reader (Molecular Devices).

To investigate whether CRP binds to factor H in fluid phase, equal amounts of CRP and factor H were incubated at $37^{o}$C for 30 min. Interactions between the 2 proteins was assessed by

solid phase ELISA. Microtiter wells were coated with 10µg/ml rabbit polyclonal anti-CRP Ab overnight at 4°C. Unreacted sites were blocked using 0.5 % gelatin at room temperature for 45 min. The CRP-factor H mixture diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates for 2 h at 37 ºC, the wells were washed with TBS-Ca buffer. Mouse anti-CRP mAb HD2.4, diluted in TBS-Ca buffer, was used (1 h at 37 ºC) to detect captured CRP from the mixture in 1 plate. HRP-conjugated goat anti-mouse IgG diluted in TBS-Ca buffer, were used (1 h at 37 ºC) as secondary antibody. In the other plate, factor H bound to the captured CRP was detected using sheep polyclonal anti-mouse factor H Ab followed by HRP-conjugated rabbit anti-sheep IgG. For both plates, color was developed and $OD_{405}$ read using microtiter plate reader (Molecular Devices).

Pneumococci

   *S. pneumoniae* type 3, strain WU2, were made virulent by sequential i.v. passages in mice, and were stored in aliquots at -80°C in Todd-Hewitt broth containing 0.5% yeast extract and 10% glycerol, as described previously (7, 25). For each experiment, a separate aliquot of frozen pneumococci was thawed. Pneumococci were then grown in Todd-Hewitt broth containing 0.5% yeast extract and collected from mid-log phase cultures. Pneumococci were washed and resuspended in normal saline ($OD_{600} = 0.35 = 2.5$ x $10^8$ CFU/ml). The concentration, purity, and viability of pneumococci were confirmed by plating on blood agar plates. This is broth-grown pneumococci stock.

## Preparation of factor H-coated pneumococci

Broth-grown pneumococci were obtained as described above. Pneumoccocci were centrifuged at 13,000 rpm for 5 min to obtain a pellet. The pellet was resuspended in 300 μl TBS buffer and incubated with 10 μg of factor H at $37^{o}$C for 30 min. Excess factor H was washed by centrifugation using TBS buffer. The bacterial pellet was resuspended in TBS buffer to obtained desired concentration and used to coat microtiter wells for binding assays. Alternatively, broth-grown pneumococci were used to coat microtiter wells overnight at $4^{o}$C. Unoccupied sites in the wells were blocked using 0.5 % gelatin for 45 min at room temperature.

## Isolation of *in vivo* pneumococci from mice

Mice were infected with 5 x $10^{7}$ CFU broth-grown pneumococci by intravenous route. After 40 h blood was collected by cardiac puncture of $CO_2$-utinized mice. Tubes containing EDTA were used for blood collection to prevent clotting and entrapment of the bacteria. Blood was mixed with an equal volume of saline and centrifuged at 2,200 rpm for 2 min and the supernatant recovered. The supernatant was washed 4 times using the same conditions and collecting the bacterial pellet each time. Finally, the pellet was spun at 13,000 rpm for 5 min and the pellet resuspended in saline. The suspension was plated on blood agar at $37^{o}$C for 16 h and enumerated to determine the concentration of pneumococci.

## Pneumococcus-binding assay

Microtiter wells were coated with broth-grown pneumococci, factor H-coated broth-grown pneumococci or mouse-isolated pneumococci in TBS ($10^{7}$ CFU/100μl/well) overnight at

4 ℃. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature.

For binding of CRP to pneumococci, CRP diluted in TBS-Ca buffer was added in duplicate wells. After incubating the plates for 2 h at 37 ℃, the wells were washed with TBS-Ca buffer. Rabbit polyclonal anti-CRP Ab diluted in TBS-Ca buffer, was used (1 h at 37 ℃) to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG diluted in TBS-Ca buffer were used (1 h at 37 ℃) as secondary antibody. Mouse anti-CRP mAb HD2.4, diluted in TBS-Ca buffer, was also used (1 h at 37 ℃) to detect bound CRP, followed by HRP-conjugated goat anti-mouse IgG as secondary antibody. Color was developed and the $OD_{405}$ read in a microtiter plate reader (Molecular Devices).

For binding of factor H to pneumococci, factor H diluted in TBS-Ca buffer was added in duplicate wells and incubated at 37 ℃ for 2 h. Bound factor H was detected using sheep polyclonal anti-mouse factor H Ab (R & D Systems) and goat polyclonal anti-human factor H Ab dissolved in TBS buffer. HRP-conjugated rabbit anti-sheep IgG (Pierce) and HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology) were then used. Color was developed and $OD_{405}$ read using microtiter plate reader (Molecular Devices).

Clearance of CRP from mouse circulation

Mice were injected i.v. with 100 μl of CRP in TBS-Ca buffer through the tail vein. Blood was collected from the tip of the tail vein after 1, 4, 8, 12, 16, 20, 24 and 28 h. The concentration of CRP in the serum was measured by ELISA.

## Sequestration of CRP from mouse blood

To investigate the possibility that CRP could be sequestered from mouse blood by cells, we repurified CRP from mouse blood that was spiked with purified WT CRP and quadruple mutant CRP. CRP-spiked mouse blood was centrifuged at 8,000 rpm for 5 min and the serum recovered. The concentration of CRP was determined by ELISA using mouse anti-CRP mAb (HD2.4) for detection followed by HRP-conjugated goat anti-mouse IgG as secondary Ab.

## Mice

Male C57BL/6J mice (Jackson ImmunoResearch Laboratories) were brought up and maintained according to protocols approved by the University Committee on animal care. Mice were 8-10 weeks old when used in experiments.

## Mouse protection experiments

Mouse protection experiments were performed as described previously (7, 25) with modifications. Mice were first injected i.v. with either 25 µg of WT CRP in 100 µl TBS-Ca buffer or TBS-Ca buffer only. After 30 min, mice in some groups were injected i.v. with $5 \times 10^7$ CFU of pneumococci in 100 µl saline. After 16 h the mice in other groups which received only TBS-Ca buffer were injected with 25 µg of either WT CRP or quadruple mutant CRP. This treatment regimen was repeated 3 more times at 20 h, 24 h, and 28 h.

Survival of mice was recorded 3 times per day for 6 days. Survival curves were generated using the GraphPad Prism 4 software. To determine $p$ values for the differences in the survival curves among various groups, the survival curves were compared using the software's Logrank test. To determine bacteremia (CFU/ml) in the surviving mice, blood was collected daily for 5

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days from the tip of the tail vein, diluted in normal saline, and plated on blood agar plates for colony counting. Bacteremia values for dead mice was taken as $>10^8$ CFU/ml. The plotting and statistical analyses of the bacteremia data were done using the GraphPad Prism 4 software and Mann-Whitney two-tailed test.

<div align="center">Results</div>

The E42Q/F66A/T76Y/E81A CRP quadruple mutant binds PEt more avidly than WT CRP does

Since the substitution of Thr[76] to Tyr was based on the structure of SAP, and SAP also binds to PEt (1, 8, 10) as did triple mutant CRP (46), we assessed the expression of the quadruple mutant CRP using culture media supernatant in PEt solid phase ELISA. We observed that both WT CRP and quadruple mutant CRP bound to PEt in a CRP concentration-dependent manner (Fig. 3.1). These data demonstrated that the E42Q mutation had no negative effect on the binding of CRP to PEt. On the contrary, quadruple mutant CRP was much more efficient than WT CRP in binding to PEt since we determined that only 3% of the mutant CRP was required to achieve an equivalent binding of WT CRP to PEt.

The binding of CRP to PEt was further evaluated using purified WT CRP and quadruple mutant CRP. The results were consistent in showing that the quadruple mutant CRP bound more avidly to PEt than WT CRP (Fig. 3.2).

<div align="center">72</div>
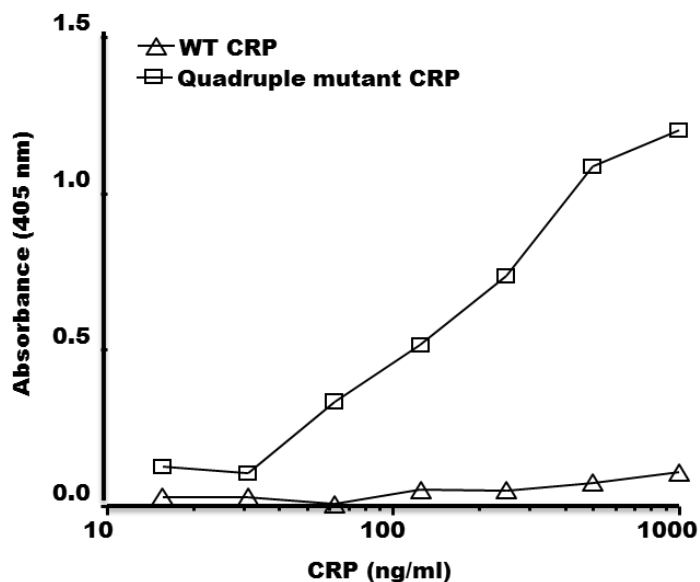
Figure 3.1. E42Q/F66A/T76Y/E81A CRP quadruple mutant in culture media supernatant bound to PEt, enabling its purification by PEt affinity chromatography. Microtiter wells were coated with avidin in TBS. The unreacted sites in the wells were blocked with gelatin. Biotinylated PEt diluted in TBS was then added to the wells. After washing the wells with TBS, purified CRP diluted in TBS-Ca buffer was added to the wells. Bound CRP was detected by using anti-CRP polyclonal rabbit Ab and HRP-conjugated donkey anti-rabbit IgG. Color was developed and the OD was read at 405 nm.
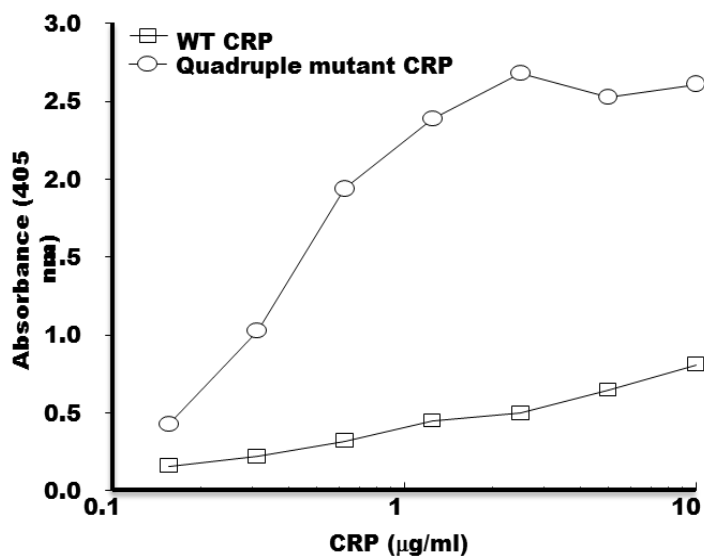


Figure 3.2. The CRP quadruple mutant binds more efficiently to PEt than WT CRP. Microtiter wells were first coated with avidin in TBS. The unreacted sites in the wells were blocked with gelatin. Biotinylated PEt diluted in TBS was then added to the wells. After washing the wells with TBS, purified CRP diluted in TBS-Ca buffer was added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was

<u>The CRP quadruple mutant is expressed as a pentamer</u>

The mutant CRP cDNA was successfully expressed in COS 7 and CHO cells and could be purified by PEt-affinity chromatography followed by gel filtration chromatography. The elution profiles of WT CRP and mutant CRP from the gel filtration column were almost identical. There was a shift of only 250 µl in the elution volume of the mutant CRP compared to that of WT CRP (Fig. 3.3*A*). Denaturing SDS-PAGE analysis of purified WT CRP and mutant CRP showed single bands (Fig. 3.3*B*) and the molecular weight of the subunits of mutant CRP was same as that of WT CRP. The data showed that the mutant CRP was pentameric and that substitution of Glu[42], Phe[66], Thr[76] and Glu[81] with Gln, Ala, Tyr and Ala, respectively, did not affect the overall structure of CRP.
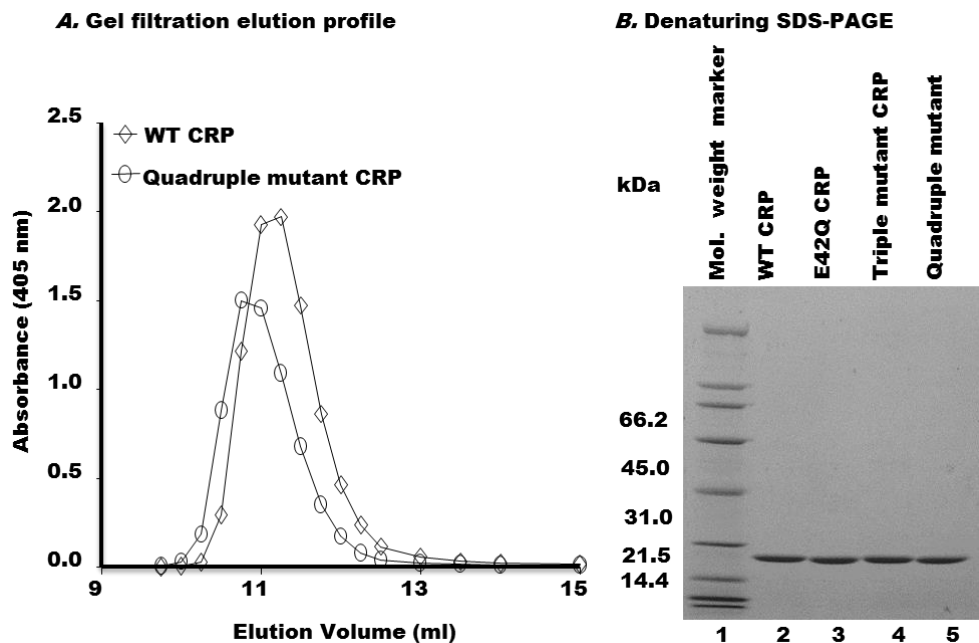


Figure 3.3. Overall pentameric structure of CRP quadruple mutant. *A*. Elution profiles of WT and mutant CRP from the Superose12 gel filtration column are shown. WT CRP in TBS containing 2 mM $CaCl_2$ was applied to the equilibrated column and eluted with the same buffer. Mutant CRP in TBS containing 5 mM EDTA was applied to the equilibrated column and eluted. Sixty fractions (0.25 ml) were collected and protein measured ($OD_{280}$) to determine the elution volume of CRP from the column. A representative of 3 experiments is shown. *B*. Denaturing SDS-PAGE (4%-20% gel) of CRP (5 µg). A representative gel, stained with Coomassie brilliant blue, is shown.

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<u>The CRP quadruple mutant does not bind to PCh or broth-grown pneumococci but binds readily to factor H-coated pneumococci</u>

Using 2 different PCh-containing ligands, PCh-BSA and PnC, we assessed the PCh-binding activity of the mutant CRP. We found that WT CRP bound to both ligands in a CRP concentration-dependent manner, but mutant CRP did not bind to PCh-BSA and PnC (Fig. 3.4*A* and *B*) or to pneumococci (Fig. 3.4*C*).
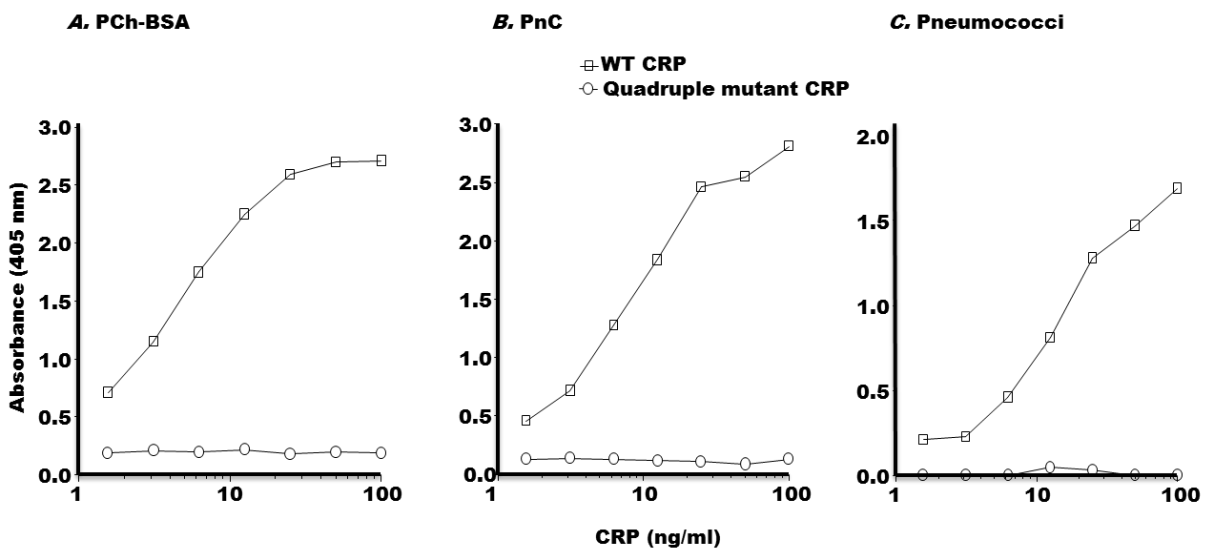


Figure 3.4. The CRP quadruple mutant does not bind to PCh (*A*), PnC (*B*), and pneumococci (*C*). Microtiter wells were coated with PCh-BSA, PnC, and pneumococci. The unreacted sites in the wells were blocked with gelatin. Purified CRP diluted in TBS-Ca buffer was then added to the wells. Bound CRP was detected by using anti-CRP mAb HD2.4 and HRP-conjugated goat anti-mouse IgG. Color was developed and and the OD was read at 405 nm. A representative of 4 experiments is shown. Similar results were obtained when polyclonal anti-CRP Ab was used to detect ligand-bound CRP.

We also determined the binding of mutant CRP to whole pneumococci which we used in the mouse protection experiments. These were broth-grown pneumococci, broth-grown pneumococci coated with factor H or pneumococci isolated from mouse blood. We determined that unlike WT CRP that bound to all types of pneumococci, mutant CRP did not bind to broth-

grown pneumococci (Fig. 3.4*C*).

As hypothesized, unlike WT CRP, quadruple mutant CRP bound readily to factor H-coated broth-grown pneumococci at physiological pH (Fig. 3.5). These results suggested that binding of the mutant CRP to pneumococci may be occurring through the recruited factor H. When incubated with mouse-isolated pneumococci, we found that quadruple mutant CRP bound to the bacteria with even greater avidity (Fig. 3.6).
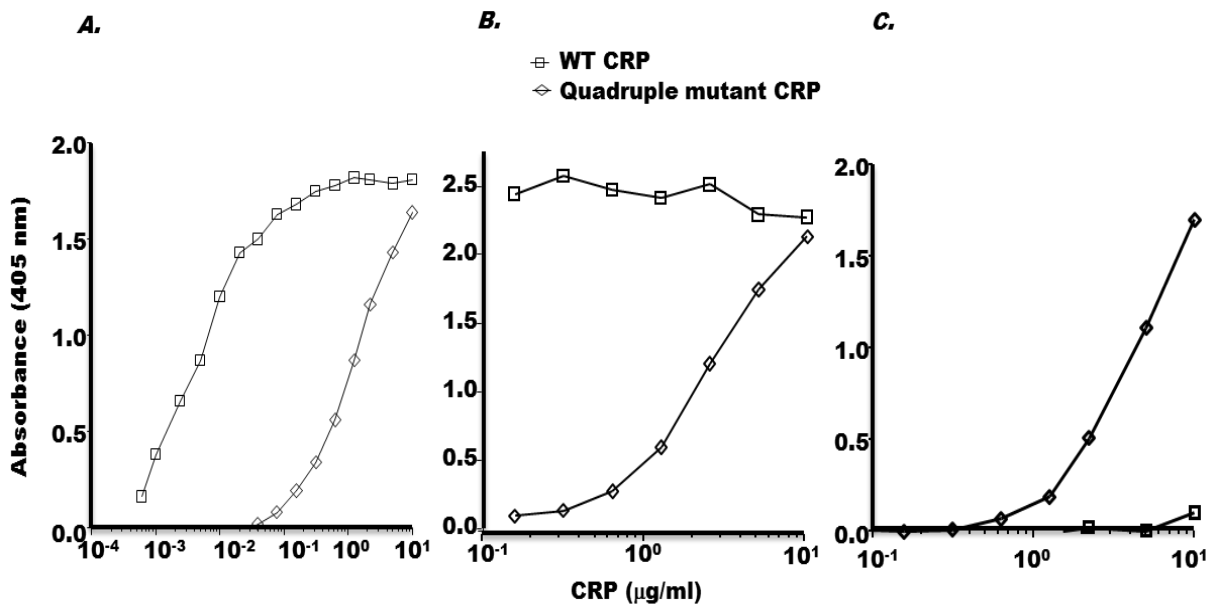


Figure 3.5. The CRP quadruple mutant binds to factor H-coated broth-grown bacteria. *A*. E42A-F66A-T76Y-E81A mutant CRP binds to bacteria in TBS containing 2mM $CaCl_2$.  Microtiter wells were coated with broth-grown pneumococci, factor H-coated broth-grown pneumococci or mouse-isolated pneumococci in TBS. Unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca buffer was added in duplicate wells. Bound CRP was detected using rabbit polyclonal anti-CRP Ab diluted in TBS-Ca buffer, followed by HRP-conjugated donkey anti-rabbit IgG diluted in TBS-Ca buffer as secondary antibody. Color was developed and the OD read at 405 nm. *B*. The experiment was carried out as
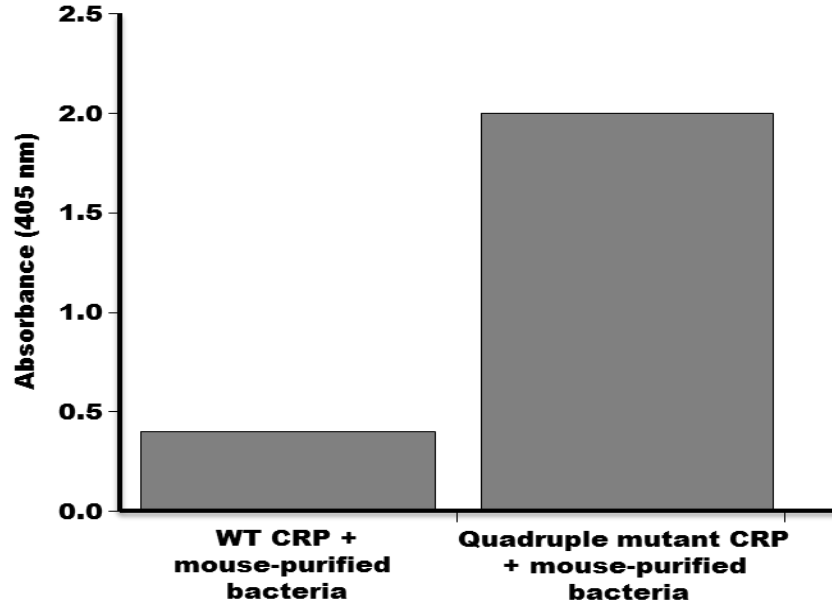
Figure 3.6. The CRP quadruple mutant binds to pneumococci isolated from mouse blood. Mice were infected with 5 x $10^7$ CFU pneumococci. After 40 h, blood was collected by cardiac puncture and used to isolate the pneumococci. Microtiter wells were coated with the pneumococci at $4^0$C overnight. CRP diluted in TBS-EDTA (5 mM) was added to duplicate wells and incubated at $37^0$C for 2 h. Bound CRP was detected using Bound CRP was detected using rabbit polyclonal anti-CRP Ab diluted in TBS-EDTA, followed by HRP-conjugated donkey anti-rabbit IgG diluted in TBS-Ca buffer as secondary antibody. Color was developed and the OD read at 405 nm.

We have verified that both broth-grown pneumococci and mouse-isolated pneumococci actually recruited factor H onto their surface. We found that factor H was detected on the surface of broth-grown pneumococci and on mouse-isolated pneumococci (Fig. 3.7).
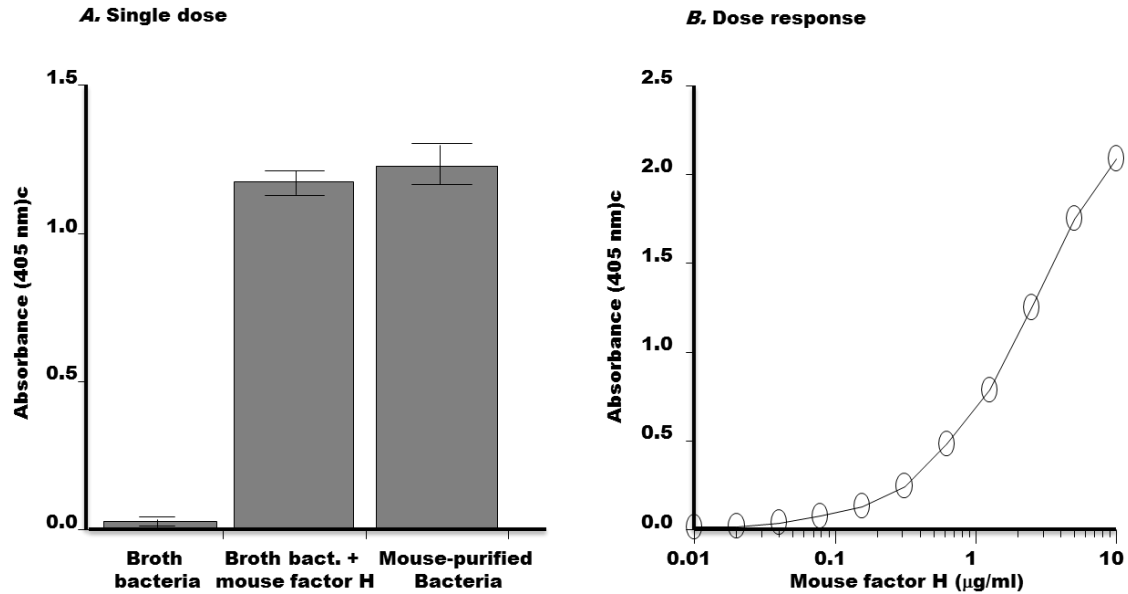
Figure 3.7. Pneumococci recruit factor H *in vivo. A.* Factor H binds to bacteria *in vitro* and *in vivo.* Pneumococci were cultured in broth. Some of the pneumococci were incubated with factor H and excess factor H removed by centrifugation. Some were used to infect mice and isolated from mouse blood after 40 h. Microtiter wells were coated with broth-grown pneumococci, factor H-coated pneumococci and mouse-purified pneumococci. Unoccupied sites were blocked using gelatin. Factor recruited onto pneumococci was detected using anti-factor H Ab followed by the appropriate HRP-tagged secondary Ab. Color was developed and read at 405 nm. *B.* Dose response of factor H binding to broth-grown pneumococci. Microtiter wells were coated with broth-grown pneumococci and unreacted sites blocked. Factor H diluted in TBS was added and incubated. Bound factor H was detected as described for *A.*

The CRP quadruple mutant binds readily to immobilized factor H

We have shown that the quadruple mutant CRP binds to factor H-coated pneumococci. We then attempted to confirm the hypothesis that the mutant CRP was binding to the pneumococci through recruited factor H by determining the binding of the mutant CRP to purified factor H. As expected, we confirmed that the mutant CRP in fact bound to factor H (Fig. 3.8).

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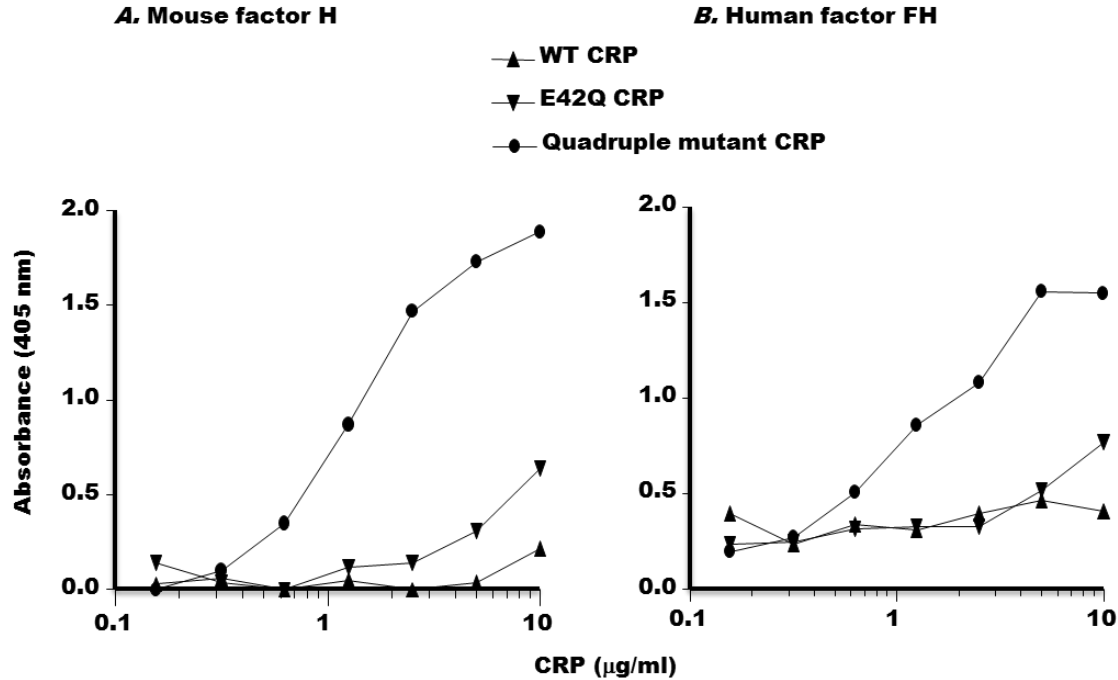
Figure 3.8. CRP quadruple mutant binds to purified factor H. Microtiter wells were coated with 10 µg/ml of mouse factor H (*A*) and human factor H (*B*) in TBS, overnight at 4 ℃. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca buffer was added in duplicate wells. and incubated for 2 h at 37 ℃. The wells were washed with TBS-Ca buffer. Bound CRP was detected using rabbit polyclonal anti-CRP and HRP-conjugated donkey anti-rabbit IgG diluted in TBS-Ca buffer. Color was developed and the OD read in a microtiter plate reader at 405 nm.

This observation suggested that combining the E42Q and T76Y mutations resulted in a synergistic enhancement of the factor H binding ability of E42Q in the quadruple mutant CRP. In order to rule out the possibility that the mutant CRP could bind to circulating factor H, we assessed its binding to factor H in fluid phase. We found that the mutant CRP did not bind to factor H in fluid phase. This was consistent with our understanding that CRP was binding only to immobilized factor H (Fig. 3.9).
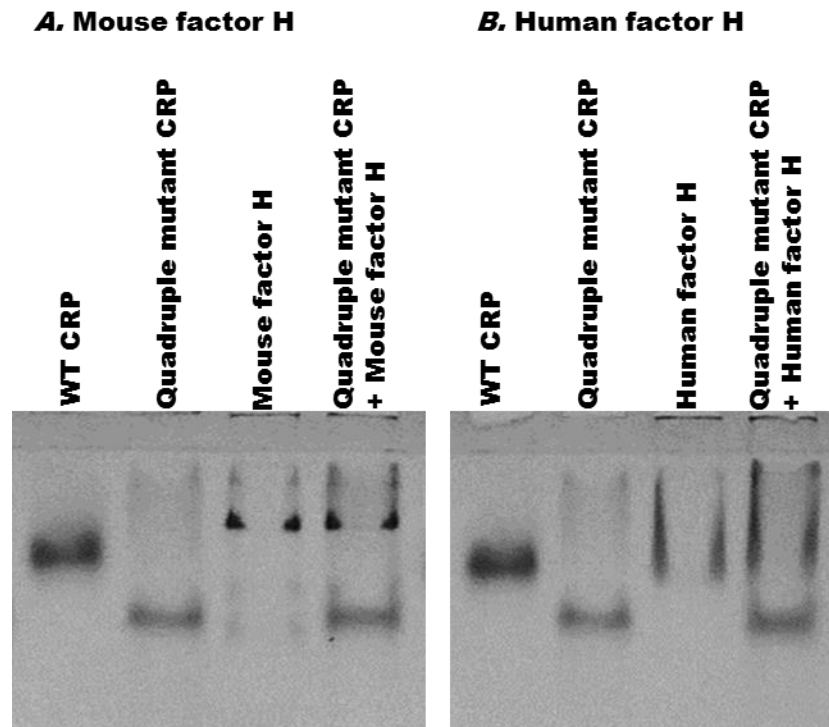
Figure 3.9. CRP quadruple mutant does not bind to factor H in fluid phase. Equal amounts (28.6 μg) of CRP and mouse factor H (*A*) and human factor H (*B*) were mixed and incubated at $37^0$C for 30 min. After incubation, 10 μg of the proteins were subjected to native PAGE. The same amounts of the individual proteins were used as loading controls.

The CRP quadruple mutant can be repurified from mouse serum

We demonstrated that the mutant CRP did not bind to fluid phase factor H. However, we eliminated any worries about the possibility of binding to serum components by repurifying it from mouse serum spiked with quadruple mutant CRP. We also evaluated recovery from mouse blood spiked with the protein. We showed by SDS-PAGE that the mutant CRP could be repurified successfully from mouse serum (Fig. 3.10). Similarly, we demonstrated very good recovery of the mutant CRP from mouse blood by ELISA. These, put together, mean that the mutant CRP is not sequestered in mouse blood.
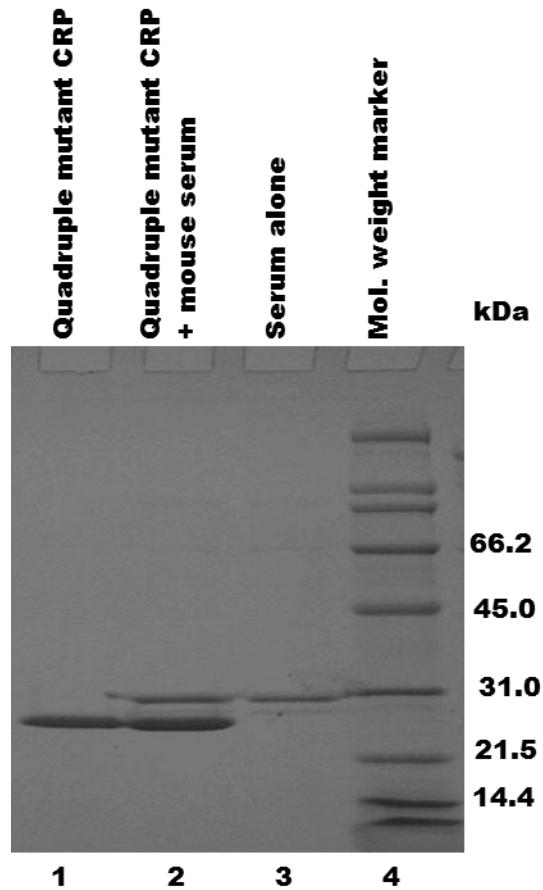
Figure 3.10. The CRP quadruple mutant can be repurified from mouse serum. CRP quadruple mutant was repurified from mouse blood that was spiked with purified WT CRP and mutant CRP. CRP-spiked mouse blood was centrifuged at 8,000 rpm for 5 min and the serum recovered. The concentration of CRP was determined by ELISA using mouse anti-CRP mAb (HD2.4) for detection followed by HRP-conjugated goat anti-mouse IgG as secondary Ab.

The clearance rate of CRP quadruple mutant from mouse circulation is similar to that of WT

CRP

We evaluated the clearance of mutant CRP from mouse circulation relative to that of

WT CRP. We observed a wide variation in the time to detect the peak concentration of CRP in

mouse circulation, ranging from 1 h to 12 h after injection of CRP. The data were normalized

before plotting. The time at which the CRP concentration in serum was at its peak was taken as

zero time taking the CRP concentration at this time taken as 100%. Clearance of CRP was then

followed at various time intervals. The results indicated that the clearance of mutant CRP in mice was similar to that of WT CRP (Fig. 3.11). These data suggested that the mutations did not affect the stability of CRP *in vivo* and that the same amount of both WT and mutant CRP could be used in mouse protection experiments for comparing the protective effects of the 2 CRP species.
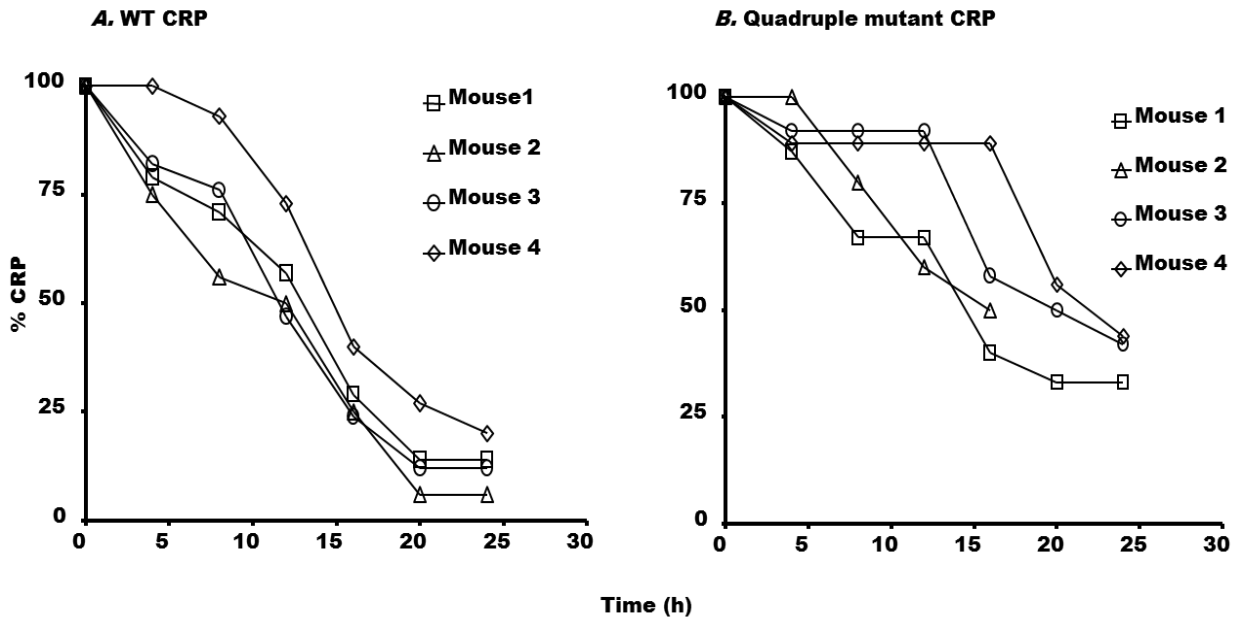


Figure 3.11. The CRP quadruple mutant is not cleared faster than WT CRP from mouse circulation. To evaluate the clearance of WT CRP (*A*) and quadruple mutant CRP (*B*), mice were injected with 100 μg of CRP in TBS buffer containing 2 mM CaCl$_2$. Blood was collected at various time points, sera separated, and the concentration of CRP measured. The time at which the CRP concentration in serum was at its peak was considered as zero time and the CRP concentration at this time was taken as 100% injected CRP; clearance of CRP was then followed at various time intervals.

CRP quadruple mutant which binds to factor H does not protect mice against pneumococcal infection when administered during late stages of infection

We have demonstrated that the quadruple mutant CRP binds to factor H and could be protective against pneumococcal infection in mice if administered later during infection. This treatment regimen was based on the observation that WT CRP does not offer protection if not

administered within 6 h before to 2 h after infection, and the assumption that the quadruple

mutant CRP will bind to factor H recruited by pneumococcus to facilitate complement attack. To

our dismay, such administration did not offer any protection against pneumococcus in mice.

There was no difference in mortality between mice that received either WT or mutant CRP in 4

25 µg doses starting at 16 h, with mean survival time (time taken for 50% of mice to die) of 40 h

(Fig. 3.12). Mice in which WT CRP was administered at time of infection as a control had a

mean survival time of 64 h ($p$ value = 0.04). Unlike WT CRP, mutant CRP administered at time

of infection did not have any protective effect either.

Figure 3.13 shows the bacteremia values in mice from the experiment presented in

Fig. 3.12. By the first day following infection mice that were injected with bacteria alone had

median bacteremia value of approximately 7.0 x $10^5$ CFU/ml of blood. For the treatment groups

the median bacteremia values were 4.4 x $10^5$ CFU/ml with WT CRP administered at 16 h, 2.2 x

$10^5$ CFU/ml and 8.1 x $10^4$ CFU/ml respectively for mutant CRP administered at time of infection

and at 16 h. The median bacteremia value for WT CRP administered at time of infection,

however, was 2.3 x $10^3$ CFU/ml. These data indicated that the observed deaths in Fig. 3.12 were

due to overwhelming bacterial loads as the median bacteremia values increased exponentially by

day 2 post infection when most of the mice were dying.

Group A: Bacteria alone (*n* = 8)
Group B: Bacteria + 25 µg WT CRP (*n* = 8)
Group C: Bacteria + 25 µg WT CRP 16, 20, 24, 28 h (*n* = 8)
Group D: Bacteria + 25 µg Quadruple mutant CRP (*n* = 8)
Group E: Bacteria + 25 µg Quadruple mutant CRP, 16, 20, 24, 28 h (*n* = 8)
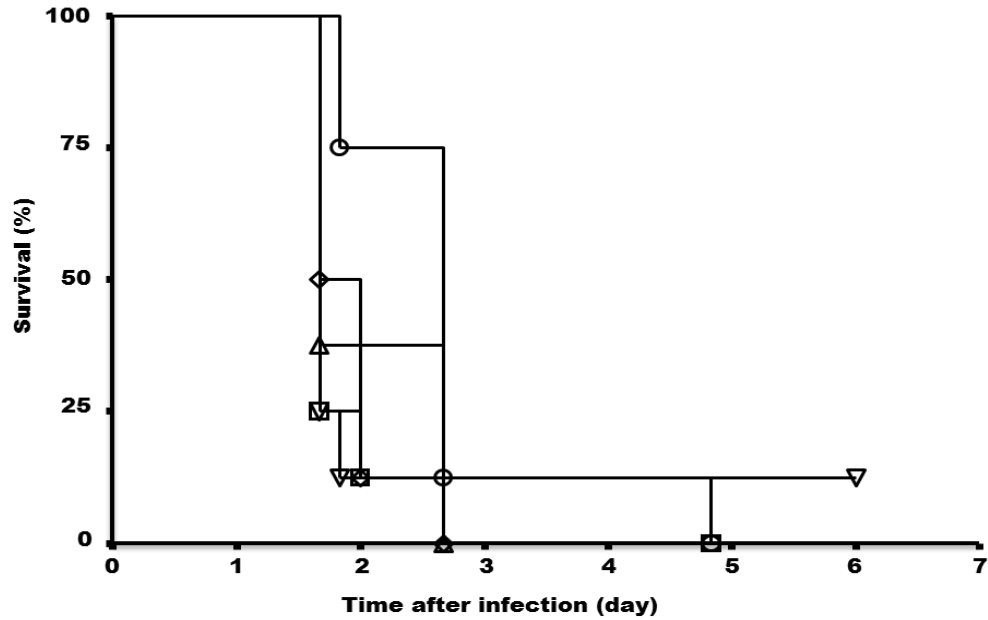
Figure 3.12. Survival curves of mice infected with *S. pneumoniae* with CRP. Mice were injected with 5 x $10^7$ CFU pneumococci, with or without 25 µg single dose of either WT or quadruple mutant CRP at time of infection or 4 doses starting at 16 h. CRP was injected first; bacteria were injected 30 min later. Deaths were recorded 3 times a day for 6 days.

A. Bacteria alone (*n* = 8)
B. Bacteria + 25 µg WT CRP (*n* = 8)
C. Bacteria + 25 µg WT CRP, 16 h (*n* = 8)
D. Bacteria + 25 µg Quadruple mutant CRP (*n* = 8)
E. Bacteria + 25 µg Quadruple mutant CRP, 16 h (*n* = 8)
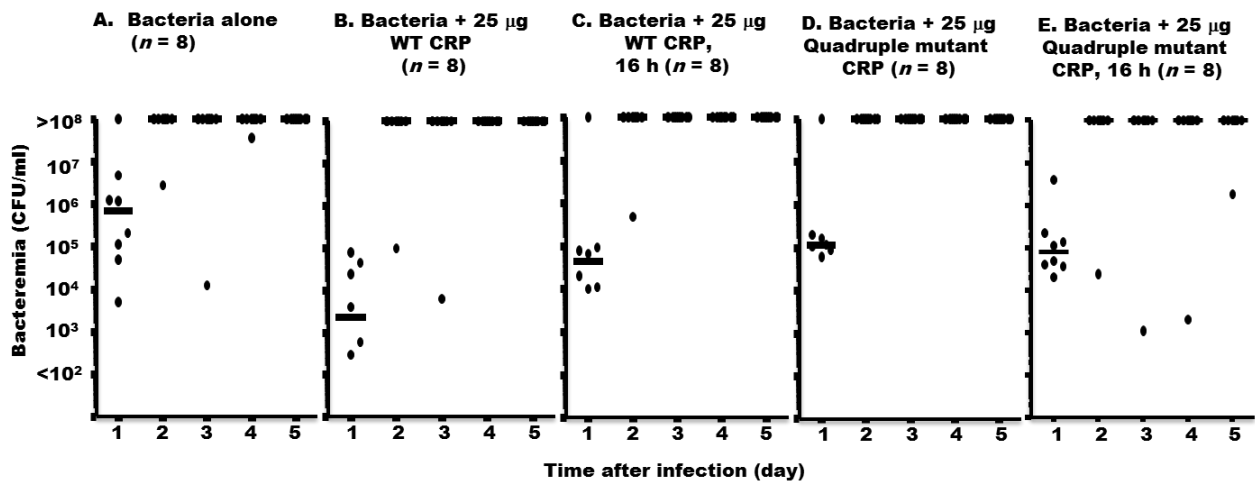
Figure 3.13. Bacteremia in mice treated with or without 25 µg of either WT or CRP quadruple mutant. Blood samples were collected from each surviving mouse for the first 5 days after infection. Bacteremia was determined by plating. Each dot represents 1 mouse. The horizontal line in each group of mice represents the median bacteremia value in that group. A bacteremia value of $>10^8$ CFU/ml indicates a dead mouse. Groups B and D received 25 µg CRP at the time of infection. Groups C and E received 4 25 µg doses of CRP starting at 16 h post infection.

In this study, we hypothesized that bacteria recruit factor H *in vivo* and use the complement regulatory ability of the protein to prevent complement activation. We held that a mutant CRP that does not bind to PCh but binds to factor H will allow us to investigate the involvement of factor H in serum resistance. Using site-directed mutagenesis we generated the quadruple mutant CRP that combined the property of not binding to PCh but with binding to factor H. This strategy was based on previous findings that E42Q mutant CRP binds moderately to factor at physiological pH (45) and the inability of triple mutant CRP to bind to PCh (46). We found that: 1) Combination of the E42Q mutant CRP with the triple mutant CRP did not affect the inability of the triple mutant CRP to bind to PCh. Thus the quadruple mutant CRP did not bind to PCh, PnC or whole pneumococci as predicted (Fig. 3.4). 2) Combining the 2 mutations resulted in a synergistic improvement in the binding ability of quadruple mutant CRP to factor H (Fig. 3.8). We observed a 4 fold increase of binding of quadruple mutant CRP to factor H over the binding of E42Q to factor H.

To assess our assertion that the resulting mutant CRP will be able to bind to factor H recruited onto pneumococci and terminate its complement inhibiting ability, we went on to confirm that pneumococci actually recruited factor H. We detected factor H on the surface of broth-grown pneumococci previously exposed to factor H as well as on pneumococci isolated from mouse but not on unexposed broth-grown pneumococci (Fig. 3.7A). We also found that factor H bound to broth-grown pneumococci in a concentration-dependent manner (Fig. 3.7B). When we used factor H-coated pneumococci, the results showed that the quadruple mutant CRP bound readily to the bacteria (Fig. 3.5A), which binding we did not see with unexposed pneumococci (Fig. 4C). Because detection of bound CRP was done using polyclonal anti-CRP

Ab, we also detected using the pentameric-specific monoclonal Ab, HD2.4, to rule out the possibility of false positive result due to monomerized CRP (Fig. 3.5B). WT CRP also equally bound to factor H-exposed pneumococci since it still retains binding to PCh. To eliminate this confounding factor, we performed the binding assay in TBS containing EDTA. The data clearly suggested that the quadruple mutant CRP was specifically binding to factor H on the surface of factor H-exposed pneumococci and mouse-isolated pneumococci (Fig. 3.5C and fig. 3.6). To ascertain the quadruple mutant CRP only binds to immobilized and not circulating factor H, we incubated CRP with human and mouse factor H in fluid phase and subjected the mixture to native PAGE. The resulted showed no interaction between the 2 proteins in fluid phase (Fig. 3.9).

Due to concerns that the quadruple mutant CRP could be sequestered in mouse blood, we repurified quadruple mutant CRP from quadruple mutant CRP-spiked mouse blood. We determined that there was no sequestration since we could not detect any additional CRP band(s) when the sample was subjected to denaturing SDS-PAGE (Fig. 3.10). The next concern was whether the quadruple mutant CRP could be metabolized faster than WT CRP when injected. We injected C57BL/6J mice with CRP and collected blood samples at defined time points for analysis. Detection of CRP in recovered serum was achieved using ELISA. We determined that the rate of clearance of the quadruple mutant CRP was not faster than that of WT CRP (Fig. 3.11) similar to our previous observations with the Triple mutant CRP (46). Taken together, the quadruple mutant CRP we have generated is a potential antibacterial therapeutic molecule with unique properties that may allow it to bind to factor H-protected bacteria and render them susceptible.

We administered the quadruple mutant CRP to mice that were infected with pneumococcus at 25 µg starting at 16 h for a total of 4 doses. Analysis of the survival data

indicated that there was no difference in the mean survival time between the mice that received

the protein and those which did not. This was contrary to the mean survival time for mice that

received WT CRP at time of infection Fig. 3.12). When the bacteremia values were examined we

determined that the median bacteremia values for the mice that received the quadruple mutant

CRP were not as low as in mice that received WT CRP at time of infection. They were close to

those observed in mice that did not receive CRP. These data indicated that the quadruple mutant

CRP was not able to reduce bacteremia and hence mortality. It is not clear why the mutant CRP

could not improve survival when it bound to factor H on pneumococcus as demonstrated *in vitro*.

Although additional treatment regimens will be investigated, there is a likelihood that the

assumption that the quadruple mutant CRP would bind to recruited factor H and facilitate

complement activation and deposition may not be feasible.  Furthermore, additional elements of

the immune system may be necessary for such scenario to materialize.

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CHAPTER 4

SUMMARY

Major Findings of the Project

The key findings of our work were:

1. Both triple mutant CRP and quadruple mutant CRP species possessed improved binding to PEt as a result of the substitution of Tyr for Thr$^{76}$. This enabled the use of PEt-conjugated sepharose beads for purification of the mutant CRP species.

2. The mutant CRP retained HD2.4 epitope and the elution profiles of the mutant CRP species from the gel filtration column were similar. These data confirmed that the substitution of Phe$^{66}$, Thr$^{76}$, and Glu$^{81}$ did not affect the overall pentameric structure of the proteins.

3. Neither triple mutant CRP nor quadruple mutant CRP bound to PCh, PnC, and pneumococci. The EA4.1 epitope was lost in the mutant CRP, indicating that the PCh-binding site was successfully prohibited.

4. Mutant CRP species were not sequestered in mouse blood/serum and could be successfully repurified from CRP-spiked mouse blood/serum.

5. The rate of clearance of mutant CRP was similar to or even slower than that of wild-type CRP, suggesting that there was enough mutant CRPs available in mice during *in vivo* experiments.

6. The triple mutant CRP did not protect mice against pneumococcal infection because the PCh-binding site of CRP was necessary, at least, for the initial stages of infection.

7. The quadruple mutant CRP bound to pneumococci only after the pneumococci were pre incubated with factor H or when pneumococci were isolated from mice. This suggested that the binding of the mutant CRP to pneumococci was mediated by factor H.

8. We showed that the quadruple mutant CRP effectively bound to purified factor H in a dose-dependent manner.

9. Mutant CRP bound to factor H only when immobilized; CRP did not bind to factor H in the fluid phase.

10. We have demonstrated that combination of the E42Q and the triple mutations results in a highly synergistic factor H-binding ability in the quadruple mutant CRP.

11. We confirmed that pneumococci recruit factor H onto their surface both *in vitro* and *in vivo*. This observation was consistent with the hypothesis that pneumococci may recruit factor H to achieve serum resistance.


Pitfalls

The main difficulty we faced was with the expression of the quadruple mutant CRP cDNA. Although we successfully expressed the cDNA, the expression was so low that we invested a lot of time and material wealth in the accumulation of enough protein for the experiments. The problem seemed to be associated with undefined additional properties of the mutant CRP. We observed that the older the expressing CHO cells became and as they die, we started to lose expressed protein The loss ranged from an initial expression level of about 2 mg/l down to about 0.4 mg/ml compared to up to 10 mg/ml expression level with the triple mutant CRP under serum-free medium adapted conditions. This observation suggested that dead CHO cells may be exposing some ligands to which the mutant CRP bound.  It was not clear if CRP

bound to such ligands lost their native structure because attempts to detect CRP on cell pellets immobilized onto ELISA plates were not successful.

We assumed that WT CRP failed to protect mice against pneumococcal infection in the late stages of infection because pneumococci-recruited factor H prevents the activation and deposition of complement on the bacterial surface. It is not clear whether binding of mutated CRP to pneumococci-coated factor H will indeed translate into the elimination of those complement-inhibiting properties of factor H. Is there a scenario where mutated CRP preoccupies factor and allow WT CRP to activate and deposit complement on the bacteria?

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