Elucidation of a Protease-Sensitive Site Involved in the Binding of Calcium to C-Reactive Protein†

C. M. Kinoshita,‡ S.-C. Ying,‡ T. E. Hugli,§ J. N. Siegel,‡ L. A. Potempa,∥ H. Jiang,‡ R. A. Houghten,⊥ and H. Gewurz*,‡

Department of Immunology/Microbiology, Rush Medical College, Chicago, Illinois 60612, and Departments of Immunology and of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received May 9, 1989; Revised Manuscript Received July 31, 1989

ABSTRACT: C-reactive protein (CRP) is a Ca^{2+}-binding protein composed of five identical 23-kDa subunits arranged as a cyclic pentamer, present in greatly elevated concentration in the blood during the acute phase of processes involving tissue injury and necrosis. In the present work, it was found that treatment of human CRP with Pronase or Nagarse protease produces two major fragments which remain associated in physiological buffers but are separable under denaturing conditions. To localize the cleavage site(s), the fragments were characterized according to molecular mass, amino acid composition, partial amino acid sequence, and reactivity with monoclonal antibodies specific for the fragments and for defined CRP epitopes including residues 147–152 and 199–206. Nagarse protease cleaves the CRP subunit between residues 145 and 146, producing two fragments, 16 and 6.5 kDa (calculated molecular mass). Pronase cleaves the CRP subunit between residues 146 and 147, producing a 16-kDa fragment (A1) and a 6.5-kDa fragment (B); an additional fragment (A2) approximately 1 kDa smaller than fragment A1 is also apparently produced due to a secondary cleavage site in fragment A1. Cleavage appears to be completely inhibited in the presence of 1 mM $CaCl_2$. Ca^{2+} does not protect cleaved CRP from heat-induced aggregation (i.e., precipitation) as it does the intact protein. Protease-cleaved CRP loses the ability to bind to the Ca^{2+}-dependent ligand phosphorylcholine but retains the ability to bind to the Ca^{2+}-independent ligand arginine-rich histone. Equilibrium dialysis indicates that intact CRP binds 2 mol of Ca^{2+}/mol of subunit with a K_d of 6×10^{-5} M. Protease treatment appears to ablate the ability of Ca^{2+} to bind to either Ca^{2+}-binding site. The proteolytically sensitive sites identified in CRP are in a region proposed to bind Ca^{2+} based on similarities to Ca^{2+}-binding sites in calmodulin and related Ca^{2+}-binding proteins. The present work is the first direct evidence that this highly conserved region is involved in binding to Ca^{2+}.

C-reactive protein (CRP)[1] is composed of identical 23-kDa subunits arranged as a cyclic pentamer (Osmand et al., 1977). The ability of CRP to bind to the C-polysaccharide component of *Streptococcus pneumoniae* led to the discovery of CRP (Tillett & Francis, 1930). CRP has been used clinically as an indicator of inflammation and tissue necrosis, because it rises dramatically in concentration during the acute phase of many infectious and inflammatory diseases. CRP has been found to protect against experimental *Streptococcus pneumoniae* infection in mice (Mold et al., 1981; Yother et al., 1982; Nakayama et al., 1983). CRP can opsonize particles and cells for phagocytosis by monocytes and granulocytes and enhance Fc receptor mediated chemiluminescence by these cells (Mortensen et al., 1976; Mold et al., 1982; Kilpatrick & Volanakis, 1985; Zeller et al., 1986), promote platelet activation (Fiedel et al., 1982), and deposit on damaged cell membranes at sites of inflammation (Kushner & Kaplan, 1961; Kushner et al., 1963; Parish, 1976; DuClos et al., 1981). Fragments generated by partial proteolysis of CRP and synthetic CRP peptides can modulate phagocyte (Dougherty et al., 1986; Robey et al., 1987; Shephard et al., 1988) and platelet function (Fiedel & Gewurz, 1986; Fiedel, 1988). Aggregated or ligand-bound CRP can activate the classical pathway of complement (Kaplan & Volanakis, 1974; Siegel et al., 1974; Mold et al., 1982) and can bind to and modify the function of human blood lymphocytes (James et al., 1981; Vetter et al., 1983). In addition, CRP epitopes have been detected on the surface of NK cells and have been shown to be involved in NK cell function (Baum et al., 1983; James et al., 1984; Bray et al., 1988).

The ligands to which CRP binds include phosphorylcholine (Volanakis & Kaplan, 1971), various polycations (DiCamelli et al., 1980), and chromatin (Robey et al., 1984, 1985). Phosphorylcholine is the component in C-polysaccharide to which CRP binds directly, and the binding of CRP to phosphorylcholine has been extensively characterized. This binding depends on Ca^{2+}, which appears to act as an allosteric effector (Volanakis & Kearney, 1981), and the ability of CRP to bind phosphorylcholine in a Ca^{2+}-dependent manner is conserved in most members of the CRP family (Liu et al., 1982; Pepys

† Presented in part at the Annual Meetings of the American Association of Immunologists in 1987 and 1988 (Kinoshita & Gewurz, 1987; Kinoshita et al., 1988).

* Author to whom correspondence should be addressed. H.G. is the holder of the Thomas J. Coogan, Sr., Chair in Immunology established by Marjorie Lindheimer Everett.

‡ Department of Immunology/Microbiology, Rush Medical College.

§ Department of Immunology, Scripps Clinic and Research Foundation.

∥ Present address: Immtech International, Inc., 906 University Place, Evanston, IL 60201.

⊥ Department of Molecular Biology, Scripps Clinic and Research Foundation.

[1] Abbreviations: CRP, C-reactive protein; FPLC, fast protein liquid chromatography; kDa, kilodalton(s); KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris/guanidine, 50 mM Tris-HCl, pH 8.5, containing 6 M guanidine hydrochloride; 10 mM Tris/NaCl, 10 mM Tris-HCl, pH 7.3, containing 0.15 M NaCl; 50 mM Tris/NaCl, 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl.

0006-2960/89/0428-9840$01.50/0

et al., 1985). In contrast, binding of CRP to protamine and other polycations occurs in the absence of Ca^{2+} and can be inhibited by divalent cations (Potempa et al., 1981).

There are two highly conserved regions in the amino acid sequence of proteins in the CRP family (Nguyen et al., 1986a,b). One of these conserved regions (residues 51–66 of human CRP) has been proposed to be the phosphorylcholine-binding site, and the other (residues 133–147) is thought to bind Ca^{2+} on the basis of a sequence similarity to the Ca^{2+}-binding sites in calmodulin and related proteins. In the present work, Ca^{2+}-binding characteristics of human CRP are examined, and direct evidence for localizing the site involved in Ca^{2+} binding is presented.

Experimental Procedures

CRP Preparation. CRP was prepared from human ascites and pleural fluids as described by Potempa et al. (1987) using affinity chromatography on phosphorylcholine-substituted Biogel, ion-exchange chromatography on DE-52, and chromatography on Biogel A 0.5m.

Limited Proteolysis. CRP at 1 mg/mL in 10 mM Tris-HCl, pH 7.3, containing 0.15 M NaCl (10 mM Tris/NaCl) was incubated with 10% (w/w, protease/substrate) Pronase (3 h, 37 °C), Nagarse protease (16 h, 37 °C, unless otherwise indicated), α-chymotrypsin (16 h, 23 °C), or trypsin (16 h, 23 °C). Pronase was purchased from Calbiochem-Behring (110 units/mg), and Nagarse protease (Protease type XXVII), α-chymotrypsin, and trypsin were obtained from Sigma. The digests were chromatographed on either a 1 × 30 cm (analytical) or a 1.6 × 45 cm (preparative) Superose 12 gel filtration FPLC column (Pharmacia) and equilibrated and eluted with 10 mM Tris/NaCl at a flow rate of 0.3 mL/min. Absorbance at 280 nm (A_{280}) of the eluate was continuously monitored, and the material in the peaks was collected. Bio-Rad gel filtration standard, containing thyroglobulin (670 kDa), immunoglobulin G (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa), was used for molecular mass calibration of the FPLC column.

Effect of Ca^{2+} on Susceptibility to Proteolysis. To test the effect of Ca^{2+} on susceptibility to digestion by Pronase and Nagarse protease, CRP was preincubated for 1 h at 23 °C in 10 mM Tris/NaCl with 0, 0.1, 1.0, or 10 mM added $CaCl_2$. Pronase or Nagarse protease was added to 10% (w/w), and the mixtures were incubated for 3 h (Pronase) or 16 h (Nagarse protease) at 37 °C. The digests were immediately chromatographed on the Superose 12 column as described above for limited proteolysis, and the material in the peaks was collected.

Protein Assay. Protein concentration was determined by the BCA protein assay (Pierce Chemical Co.; Smith et al., 1985).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE on 13% polyacrylamide gels was carried out on the material in the peaks from the FPLC column according to the method of Laemmli (1970). Dithiothreitol to 0.1 M final concentration was added to some samples before heating to reduce the intrachain disulfide bond located between residues 36 and 97 of each subunit. Bio-Rad low molecular weight SDS–PAGE standard, containing phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa), was used for molecular mass calibrations.

Separation of Fragments by Gel Filtration in Guanidine. CRP at 1 mg/mL was mixed with 10% (w/w) Pronase or Nagarse protease, incubated for 3 h (Pronase) or 16 h (Nagarse protease) at 37 °C, and separated on the Superose 12 FPLC column as described for limited proteolysis. In each case, material eluting in the peak having an apparent molecular mass of 110 kDa was collected and lyophilized. The freeze-dried material was dissolved in 50 mM Tris-HCl, pH 8.6, containing 6 M guanidine hydrochloride (Tris/guanidine), chromatographed on the 1 × 30 cm Superose 12 FPLC column, and equilibrated and eluted with the sample buffer. The flow rate was 0.05 mL/min, and the A_{280} was continuously monitored. Carbonic anhydrase (29 kDa), myoglobin (17 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa) were used for molecular mass calibration of the column. Sample peaks were pooled, and the material was dialyzed in Spectrapor dialysis tubing (3.5-kDa cutoff) on a magnetic stirrer against one change of 4 L of 0.1 M ammonium bicarbonate at pH 8.6 and 4 °C. The dialyzed material was lyophilized and subjected to SDS–PAGE, amino acid compositional analysis, or partial protein sequencing.

Amino Acid Analysis and Sequencing. Amino acid analysis and sequencing were performed by the Protein Sequencing and Amino Acid Analysis Service, Department of Immunology, Scripps Clinic and Research Foundation (La Jolla, CA). Amino acid analysis was performed on a Beckman 6900 autoanalyzer. Protein samples of 5–20 μg were placed in ignition tubes, vacuum-sealed, and hydrolyzed at 110 °C for 24 h in constant-boiling 6 M HCl containing 1% (v/v) phenol. For partial amino acid sequencing, automated Edman degradation of CRP fragments was performed on an Applied Biosystems Model 470A sequenator. The PTH-amino acid derivatives were identified by HPLC analysis using a Waters automatic sample injection and chromatographic system fitted with a Zorbax 5-μm PTH column (4.6 × 220 mm).

Monoclonal Antibodies (mAb), Peptides, and Immunoassays. Murine mAb to human CRP were generated and characterized exactly as described (Ying et al., 1989), and the purified IgG fractions were used at a concentration of 20 μg/mL. Peptide III (identical with residues 137–152 of human CRP) was synthesized as described (Houghten, 1985), while peptide IV (identical with residues 199–206 of human CRP) was synthesized and kindly provided by Dr. Frank A. Robey of the National Institute of Dental Research (Bethesda, MD). Dot blot and Western blot analyses were performed as described by Ying et al. (1989).

Effect of Ca^{2+} on Heat Aggregation of Intact and Cleaved CRP. CRP was proteolyzed with Nagarse protease and Pronase and chromatographed as described for partial proteolysis. The digests and an undigested control were adjusted to 80 μg/mL with 10 mM Tris/NaCl and preincubated for 30 min in either 0, 0.1, 1.0, or 10 mM $CaCl_2$. The samples were heated in microcentrifuge tubes in a 63 °C water bath until controls (CRP alone, no $CaCl_2$) were turbid. The samples were centrifuged for 20 min at 12000*g* in a microfuge, after which supernatants and precipitates were freeze-dried and assayed for protein content.

Biotinylation of CRP. CRP at 1 mg/mL in 10 mM Tris-HCl, pH 7.3, containing 0.15 M NaCl and 2 mM $CaCl_2$, was mixed with $^1/_8$th volume of 1 mg/mL NHS-LC-biotin (Pierce Chemical Co.) and incubated for 4 h at 23 °C. The mixture was then dialyzed on a magnetic stirrer against one change of 4 L of 10 mM Tris/NaCl at 4 °C.

Solid-Phase Ligand-Binding Assays. Wells of microtiter plates (C.A. Greiner and Sohne, West Germany) were coated with 100 μL of 1.25 μg/mL phosphorylcholine, covalently attached to keyhole limpet hemocyanin (KLH) by the method of Chesebro and Metzger (1972), or with 10 μg/mL argi-

nine-rich histone, each in 50 mM sodium carbonate, pH 8.6, for 16 h at 23 °C. The wells were washed 4 times with 50 mM veronal, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20, and either 2 mM $CaCl_2$ or 10 mM EDTA. Bovine serum albumin [200 μL of 1% (w/v) in H_2O] was applied to each well. The plates were incubated at 37 °C for 30 min, and the wells were washed. Samples (100 μL) of biotinylated CRP in 10 mM Tris/NaCl, containing 2 mM $CaCl_2$ or 10 mM EDTA, were applied to the wells and incubated for 30 min at 37 °C. The wells were washed, and 100 μL of avidin–peroxidase (18 000-fold dilution in wash buffer) was applied. The plates were incubated for 30 min at 37 °C and washed. Substrate [100 μL of 1 mM 2,2′-azinobis(ethylbenzothiazolinesulfonic acid) diammonium salt in 0.1 M sodium citrate at pH 4.0] was applied and allowed to develop at 23 °C for 15–60 min. A_{414} was read in a Titertek Multiskan MC plate reader.

Equilibrium Dialysis. Equilibrium dialysis of CRP and protease-treated CRP was performed with a microdialysis cell (Hoefer) equipped with pairs of 0.1-mL chambers, separated by a dialysis membrane with a molecular mass cutoff of 12 kDa. The 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl (50 mM Tris/NaCl) used for this experiment was passed through a column of Chelex 100 prior to use. Membranes and 1-μm glass beads were boiled for 5 min in 5% Na_2CO_3, containing 50 mM EDTA, then boiled in three changes of distilled, deionized water, and rinsed and stored in 50 mM Tris/NaCl at 4 °C. Samples were dialyzed in 2 L of 50 mM Tris/NaCl, followed by 2 L of Tris/NaCl buffer containing 3 mL of a 50% slurry of Chelex 100, and two changes of Tris/NaCl buffer without Chelex 100. One half-cell of each dialysis chamber was filled with 0.1 mL of the protein sample, and the other half-cell was filled with 0.1 mL of various concentrations of $^{45}CaCl_2$ plus carrier $CaCl_2$. The stock solution of carrier $CaCl_2$ was 1.02 mM as determined by atomic absorption spectrophotometry (Schwarzkopf Microanalytical Laboratory, Woodside, NY). A glass bead was added to each half-cell to facilitate mixing, and the chambers were rotated at 23 °C for 16 h. Duplicate 35-μL aliquots from each half-cell were added to 10 mL of scintillation fluid, and the mixture was counted by using a ^{32}P window of an LKB β-scintillation counter. Equilibrium was reached in 16 h as judged by the equal counts in a pair of half-cells in which $^{45}CaCl_2$ was placed into one half-cell and buffer was placed into the other. Laser densitometry of the Coomassie-stained SDS gel of the Nagarse protease digests was performed to quantitate the degree of digestion.

Results

Gel Filtration and SDS–PAGE Analysis of Protease-Treated CRP. Gel filtration FPLC was carried out to determine whether protease treatment affects the molecular mass of native CRP. Treatment of CRP with Pronase, Nagarse protease, α-chymotrypsin, or trypsin does not substantially affect the elution position of CRP. The Superose 12 gel filtration FPLC profiles of Pronase-treated and Nagarse protease treated CRP digested in the absence of Ca^{2+} are shown in Figure 1 (α-chymotrypsin and trypsin profiles are not shown). Material in the peak emerging at or near the elution position of the intact CRP pentamer was collected for further analysis. Superose 12 FPLC under nondenaturing conditions serves to separate CRP from protease since the proteases elute after the pentamer peak, as determined by chromatography of the protease alone (not shown).

To assess whether denaturation in SDS would reveal fragmentation of CRP, an aliquot of protease-treated CRP eluting

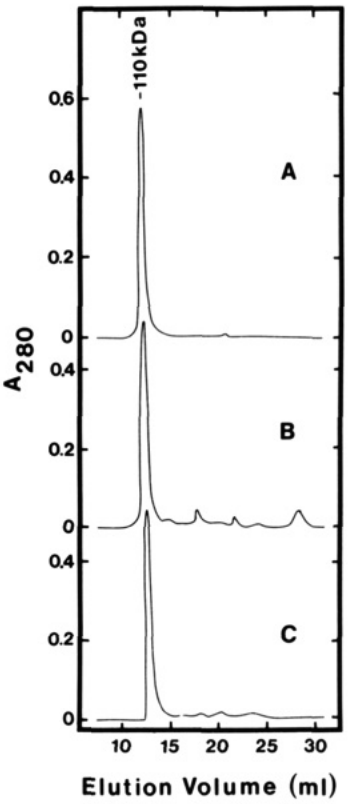

FIGURE 1: Superose 12 gel filtration FPLC of protease-treated CRP under nondenaturing conditions (10 mM Tris/NaCl). Prior to chromatography, CRP was incubated (A) alone for 3 h at 37 °C, (B) with Pronase for 3 h at 37 °C, or (C) with Nagarse protease for 16 h at 37 °C.

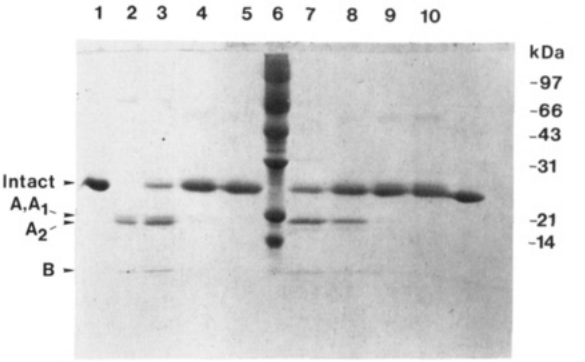

FIGURE 2: SDS–PAGE of CRP treated with Pronase or Nagarse protease in varying concentrations of $CaCl_2$. Gels were stained with Coomassie blue. Prior to electrophoresis, all CRP samples were fractionated on the Superose 12 FPLC column in 10 mM Tris/NaCl to remove protease. As a control, CRP was incubated alone for 16 h at 37 °C (lane 1 and the unlabeled lane on the far right). CRP was treated with Pronase for 3 h at 37 °C in the absence of added $CaCl_2$ (lane 2), in 0.1 mM $CaCl_2$ (lane 3), in 1.0 mM $CaCl_2$ (lane 4), or in 10 mM $CaCl_2$ (lane 5). The Bio-Rad standard is shown in lane 6. CRP was treated with Nagarse protease for 16 h at 37 °C in the absence of added $CaCl_2$ (lane 7), in 0.1 mM $CaCl_2$ (lane 8), in 1.0 mM $CaCl_2$ (lane 9), or in 10 mM $CaCl_2$ (lane 10).

at the position of CRP upon nondenaturing FPLC was subjected to SDS–PAGE (Figure 2). The molecular mass of intact, unreduced CRP on these 13% Laemmli gels is 25 kDa, which is slightly larger than the value of 23 kDa calculated from the amino acid composition. Pronase treatment of CRP in the absence of Ca^{2+} generates three fragments on SDS gels (Figure 2, lane 2). The apparent molecular masses of the two larger fragments, designated A1 and A2, were found to be 18 and 17 kDa, respectively. The third band, designated fragment

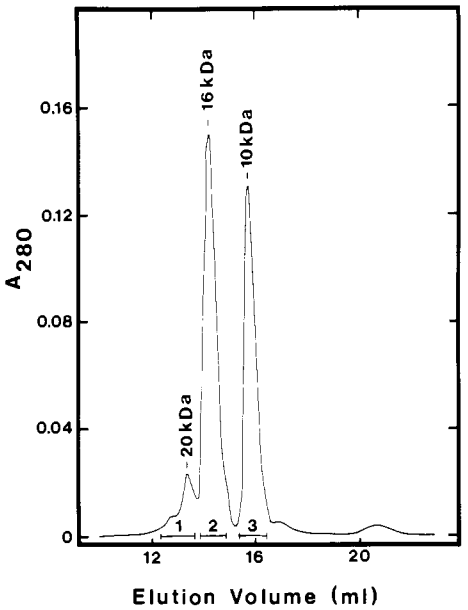

FIGURE 3: Superose 12 gel filtration FPLC of Pronase-treated CRP under denaturing conditions (Tris/guanidine). CRP was digested with Pronase for 3 h at 37 °C and separated from Pronase by gel filtration FPLC in 10 mM Tris/NaCl prior to chromatography in Tris/guanidine.

B, is too small to be sized on the 13% gel (<14 kDa). Nagarse protease digestion in the absence of Ca^{2+} generates just two fragments when analyzed on SDS gels (Figure 2, lane 7). The larger fragment, designated A, has an apparent molecular mass of 18 kDa, and the smaller fragment, designated B, is too small to be sized on this gel (<14 kDa). α-Chymotrypsin treatment (16 h, 23 °C) produces very little fragmentation, and trypsin treatment (16 h, 23 °C) has no effect on the SDS gel pattern of CRP (not shown). The apparent molecular masses of the fragments are not appreciably affected when treated with dithiothreitol (not shown).

Effect of Ca^{2+} on Proteolysis of CRP by Pronase and Nagarse Protease. CRP was incubated with Pronase or Nagarse protease in the presence of various concentrations of $CaCl_2$. Upon gel filtration FPLC in nondenaturing buffer, the elution position of CRP is not affected by protease treatment, regardless of the $CaCl_2$ concentration. SDS–PAGE of aliquots from the peaks which elute at the CRP position demonstrates that $CaCl_2$ at a concentration of 0.1 mM partially protects CRP from proteolysis by Pronase and Nagarse protease as compared to digestion in the absence of $CaCl_2$ (Figure 2, lanes 3 and 8). A concentration of 1.0 mM $CaCl_2$ appears to completely protect CRP from digestion by the two proteases (Figure 2, lanes 4 and 9), as does 10 mM $CaCl_2$ (Figure 2, lanes 5 and 10).

Gel Filtration FPLC of CRP Digests under Denaturing Conditions. After removal of protease by gel filtration under nondenaturing conditions, CRP treated with Pronase or Nagarse protease in the absence of $CaCl_2$ was subjected to gel filtration under denaturing conditions (6 M guanidine) to obtain purified fragments for further analysis. Gel filtration in guanidine also provides an estimate of the molecular mass of the fragments obtained from the digests. The Superose 12 FPLC profiles of Pronase-treated and Nagarse protease treated CRP, chromatographed in Tris/guanidine, are shown in Figure 3. For Pronase-treated CRP, the apparent molecular masses of the material in peaks 1, 2, and 3 were found to be 20.2 ± 0.65, 16.4 ± 0.60, and 10.0 ± 0.38 kDa (mean ± standard error of the mean for two determinations), respectively (Figure 3). For Nagarse protease treated CRP, the apparent molecular masses of the material in peaks 1, 2, and 3 were determined to be 21.2 ± 1.7, 16.3 ± 1.1, and 10.1 ± 0.36 kDa (mean ± standard error of the mean for two determinations), respectively (not shown). SDS–PAGE of the dialyzed peaks from Pronase and Nagarse protease digests demonstrates that peak 1 contains intact CRP, peak 2 contains fragments A1 and A2 (Pronase digest) or fragment A (Nagarse protease digest), and peak 3 contains fragment B from the Pronase or Nagarse protease digests (not shown).

Table I: Amino Acid Composition of Fragment B from Pronase-Treated CRP

amino acid	theoretical	experimental[a]
Asx	8	6.9 ± 1.1
Thr	2	1.8 ± 0.35
Ser	4	3.4 ± 0.25
Glx	7	8.0 ± 0.15
Pro	5	4.8 ± 0.05
Gly	6	7.1 ± 0.10
Ala	1	1.7 ± 0.10
Val[b]	6	6.0
Ile	3	2.9 ± 0.10
Leu	6	5.8 ± 0.55
Tyr	2	2.1 ± 0.00
Phe	3	3.4 ± 0.05
His	0	0.04 ± 0.04
Lys	2	2.2 ± 0.15
Arg	1	0.6 ± 0.55

[a] Mean ± standard error of the mean for two experiments. [b] Normalized to Val.

Characterization of CRP Fragments from the Protease Digests. To localize the position of CRP cleavage by Pronase, the sequence of the first eight residues from the amino-terminal end of fragment B (Pronase digest) was determined and found to coincide with residues 147–154 of CRP (Figure 4). The apparent molecular mass of fragment B from the Pronase digest by gel filtration FPLC in Tris/guanidine is 10 kDa, somewhat larger than the theoretical value of 6.5 kDa for a polypeptide composed of residues 147–206 (residue 206 is the carboxy-terminal amino acid of CRP). The amino acid composition of fragment B corresponds well to the theoretical composition of residues 147–206 (Table I), and further supports the conclusion that cleavage by Pronase occurs between residues 146 and 147. As mentioned above, fragments A1 and A2 emerge in one peak by gel filtration FPLC in Tris/guanidine with an apparent molecular mass of about 16 kDa. This agrees with the theoretical molecular mass of residues 1–146 of 16 kDa. Fragment A2 is about 1 kDa smaller than fragment A1 by SDS–PAGE. It is not known whether the additional cleavage occurs on the amino- or carboxy-terminal end of fragment A1, or both.

Fragment B from the Nagarse protease digest also was partially sequenced to determine the position of the cleavage. The first nine residues from the amino-terminal end of fragment B were identified and correspond to residues 146–154 of CRP, which indicates that cleavage occurs between residues 145 and 146 (Figure 4). This is one residue away from the identified Pronase cleavage site, which is between residues 146 and 147. The molecular mass of fragment B of CRP from Nagarse protease treatment is 10 kDa by gel filtration in Tris/guanidine, which is somewhat larger than the expected value of 6.5 kDa for a polypeptide composed of residues 146–206. The molecular mass of fragment A is 16 kDa by gel filtration in Tris/guanidine, which is consistent with a polypeptide composed of residues 1–145 of CRP.

Further Characterization of CRP Fragments from the Pronase Digest Using mAb. Fragments from Pronase-treated CRP were further studied by using mAb which react selec-

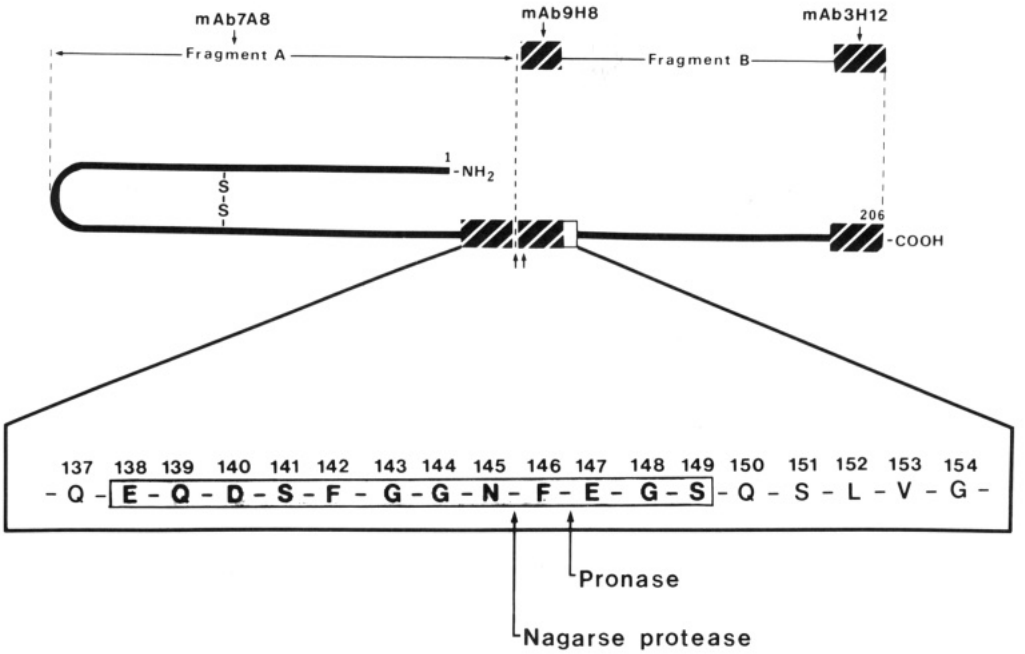

FIGURE 4: Schematic representation of the CRP subunit, with a detailed view of amino acid residues 137–154, indicating the protease-sensitive cleavage sites (upward arrows) and the resultant fragments A and B, the residues defined upon partial amino-terminal amino acid sequenation of fragment B (residues 146–154 and 147–154 for cleavage by Nagarse protease and Pronase, respectively), the synthetic CRP peptides III and IV used in these investigations (residues 137–152 and 199–206, respectively; darkened hatches on the subunit), the sites of reactivity of mAb 7A8 (large fragment) and of mAb 9H8 (residues 147–152) and mAb 3H12 (residues 199–206) with the latter two indicated by the darkened hatches at the termini of fragment B, and the proposed Ca^{2+}-binding region (residues 138–149; shaded box) as discussed in the text.

Table II: Reactivity of mAb with CRP Cleavage Fragments and Peptides[a]

	Western blots			dot blots	
antibody	intact subunit	fragment A1,A2	fragment B	peptide III, 137–152	peptide IV, 199–206
26-7A8	+	+	–	–	–
32-9H8	+	–	+	+	–
13-3H12	+	–	+	–	+

[a] mAb raised to CRP were reacted with Pronase-treated CRP in Western blot assays and with synthetic peptides identical with residues 137–152 (peptide III) and 199–206 (peptide IV) of human CRP in dot blot assays. Antibodies 26-7A8 and 13-3H12 were described previously (Ying et al., 1989).

Table III: Effect of $CaCl_2$ on Heat Aggregation of Intact and Pronase-Treated and Nagarse Protease Treated CRP

		μg of protein[a]	
CRP	mM $CaCl_2$	precipitate	supernatant
no protease	0	178 ± 6.0	26 ± 8.5
	0.1	169 ± 17	43 ± 18.5
	1.0	74 ± 21.5	143 ± 23
	10.0	42 ± 10.5	167 ± 27
Pronase-treated	0	153 ± 2.9	57 ± 11
	0.1	169 ± 6.5	56 ± 7.3
	1.0	148 ± 2.0	71 ± 11
	10.0	138 ± 8.0	59 ± 7.0
Nagarse protease treated	0	163 ± 3.0	45 ± 9.0
	0.1	171 ± 5.0	43 ± 5.0
	1.0	159 ± 20	57 ± 17
	10.0	117 ± 34	86 ± 28

[a] Mean ± standard error of the mean for two determinations.

tively with neodeterminants of human CRP (Table II). Western blot analysis demonstrates that mAb 7A8 reacts with fragments A1 and A2 (Ying et al., 1989) while mAb 3H12 (Ying et al., 1989) and newly generated mAb 9H8 (IgG1, κ) react with fragment B. By dot blot analysis, mAb 3H12 reacts with a synthetic peptide (peptide IV) identical with the carboxy-terminal octapeptide of CRP (residues 199–206) (Ying et al., 1989), while mAb 9H8 reacts with a synthetic hexadecapeptide (peptide III) identical with residues 137–152 of CRP. These results indicate that mAb 9H8 reacts with a hexapeptide identical with residues 147–152 of CRP and support the contention that fragment B from the Pronase digest consists of residues 147–206 (Figure 4).

In addition, multiple anti-CRP mAb selectively reactive with native CRP were tested for the ability to bind CRP digested with Pronase. Ten mAb and a polyclonal antibody, each known to react with native CRP both in the presence and in the absence of Ca^{2+}, were found to bind to Pronase-treated as well as untreated CRP by dot blot analysis. In contrast, two mAb (4H2 and JEV-EA4-1) previously shown to react with CRP only in the presence of Ca^{2+} (Kilpatrick et al., 1982; Ying et al., 1989), as well as a third newly developed mAb (1B1) with comparable properties, did not react with digested CRP, even though optimal amounts of Ca^{2+} were present. These results are consistent with the hypothesis that protease-modified CRP loses its ability to bind Ca^{2+} and hence to express Ca^{2+}-dependent determinants.

Effect of Ca^{2+} on Heat Aggregation of Intact and Cleaved CRP. Intact CRP precipitates when heated in the absence of added $CaCl_2$ or in the presence of only 0.1 mM $CaCl_2$ but is protected from precipitating when heated in the presence of 1 or 10 mM $CaCl_2$, as shown in Table III. $CaCl_2$ at a concentration of 1 or 10 mM does not prevent the heat-induced precipitation of protease-treated CRP, even though these levels of $CaCl_2$ protect unproteolyzed CRP. About 40% of the CRP remains intact in the Nagarse protease digest as determined by laser densitometry, which may explain the partial protection afforded by $CaCl_2$, especially at the 10 mM level.

Effect of Cleavage on Binding of CRP to Phosphorylcholine and Arginine-Rich Histone. The ability of CRP to bind to phosphorylcholine in a Ca^{2+}-dependent manner may be important physiologically since this characteristic is conserved

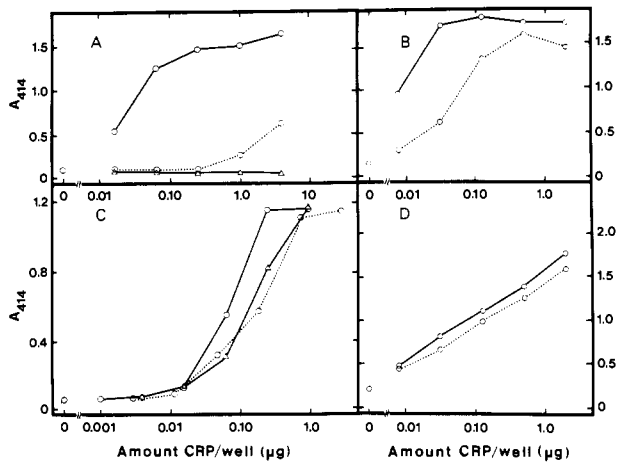

FIGURE 5: Binding of intact and protease-treated biotinylated CRP to immobilized phosphorylcholine–KLH and arginine-rich histone. An enzyme-linked immunosorbent assay was used to compare the binding of (A) intact and Pronase-treated CRP to phosphorylcholine–KLH, (B) intact and Nagarse protease treated CRP to phosphorylcholine–KLH, (C) intact and Pronase-treated CRP to arginine-rich histone, and (D) intact and Nagarse protease treated CRP to arginine-rich histone. The symbols used are intact CRP in $CaCl_2$ (O—O), intact CRP in EDTA (Δ—Δ), and protease-treated CRP in $CaCl_2$ (O- -O).

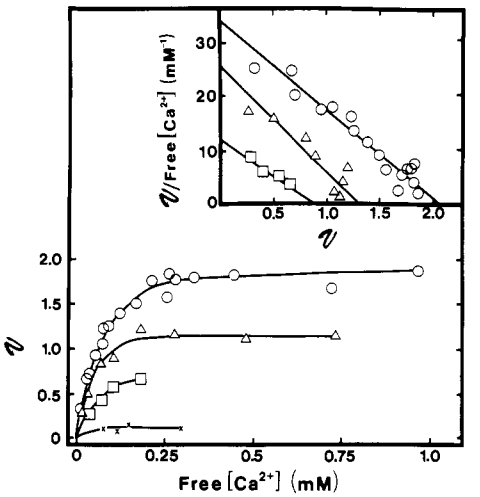

FIGURE 6: Characteristics of Ca^{2+} binding to CRP and protease-treated CRP. Equilibrium dialysis was used to study the binding of Ca^{2+} to CRP and Pronase-treated and Nagarse protease treated CRP. The inset shows the Scatchard plot of the data with linear regression used to fit the lines to the points. ν represents moles of Ca^{2+} bound per mole of CRP subunit. The symbols used are intact CRP (O), CRP treated with Nagarse protease for 14 h at 23 °C followed by 3 h at 37 °C (Δ) or for 16 h at 37 °C (□), and CRP treated with Pronase for 3 h at 37 °C (×).

in most members of the CRP family of proteins (Nguyen et al., 1986a,b). Ca^{2+}-dependent binding of CRP to phosphorylcholine is markedly depressed with Pronase treatment of CRP (Figure 5A), and partially depressed with Nagarse protease treatment (16 h, 37 °C, Figure 5B). The depression in binding is in approximate proportion to the amount of cleaved CRP in the preparation, as Pronase-treated CRP contains no detectable intact subunit on SDS gels and the Nagarse protease treated CRP contains about 40% intact subunit as determined by laser densitometry. Ca^{2+}-independent binding to arginine-rich histone is retained by Pronase-treated and Nagarse protease treated CRP (Figure 5C,D).

Analysis of Ca^{2+} Binding Using Equilibrium Dialysis. The effect of protease treatment on the ability of CRP to bind to Ca^{2+} was assessed by equilibrium dialysis (Figure 6). Scatchard analysis of the data demonstrates that intact CRP has two binding sites of equal affinity for Ca^{2+}, with a K_d of 6.0×10^{-5} M (Figure 6, inset). Laser densitometry of the bands from SDS–PAGE was used to quantitate the proportion of CRP which was cleaved in preparations of protease-treated CRP in order to correlate the proportion of CRP subunits cleaved with the proportion of Ca^{2+}-binding sites lost. The relative amounts of intact subunit and fragment A were estimated by densitometry; fragment B was not used in the calculation since it is too small to quantitatively remain in the gel. The area of the fragment A band was corrected by a factor of 23/16 to determine the relative number of moles of fragment A and intact subunit in the preparation. CRP treated with Nagarse protease at 37 °C for 16 h consists of 38% intact and 62% fragmented CRP, and equilibrium dialysis demonstrates that Nagarse protease-treated CRP binds 44% as many Ca^{2+} ions as does the same amount of intact CRP (Figure 6). CRP treated with Nagarse protease at 23 °C for 14 h followed by 3 h at 37 °C consists of 55% intact and 45% fragmented CRP and binds about 64% as many Ca^{2+} ions as intact CRP. The preparations of CRP treated with Nagarse protease for 16 h at 23 °C and for 14 h at 23 °C plus 3 h at 37 °C have dissociation constants of 7.6×10^{-5} and 5.0×10^{-5} M, respectively (calculated from Figure 6, inset). Two preparations of intact CRP incubated alone for 16 h at 37 °C behaved the same as unincubated intact CRP upon equilibrium dialysis (not shown).

Discussion

These investigations were carried out to determine whether protease treatment of CRP could be used as a tool to explore structure–function relationships in CRP. Both gel filtration and SDS–PAGE were utilized to monitor the effects of protease treatment on CRP. Partial proteolysis by Pronase or Nagarse protease fails to substantially change the elution position of the digested CRP upon nondenaturing gel filtration while fragments are evident upon PAGE in SDS, indicating that strong noncovalent forces hold the fragments together in physiological buffers. SDS–PAGE of protease-treated CRP was performed in the presence and absence of dithiothreitol. In the absence of reducing agent, fragments with apparent molecular masses of 17–18 kDa (fragment A or A1 and A2) and <14 kDa (fragment B) were identified. SDS–PAGE of protease-treated CRP in the presence of dithiothreitol demonstrates that no additional fragments are generated with reduction of the single intrachain disulfide bond, indicating that proteolytic cleavage occurs only outside of the disulfide loop which is located between residues 36 and 97.

Amino acid sequence and compositional analysis, as well as apparent molecular weight estimates by gel filtration FPLC in Tris/guanidine, indicate that fragments B from the Nagarse protease and Pronase digests contain the carboxy-terminal end of the CRP subunit, i.e., residues 146(147)–206. Further evidence that fragment B from the Pronase digest consists of the carboxy-terminal end of the CRP subunit is provided by experiments with the mAb to human CRP. Several anti-CRP mAb were found to react on dot blots with a synthetic peptide corresponding to the carboxy-terminal octapeptide of CRP. Binding of these mAb to CRP is inhibited by the synthetic peptide as determined by the enzyme-linked immunosorbent assay (Ying et al., 1989). These mAb also react on Western blots with fragment B of CRP from the Pronase digest (Ying et al., 1989), indicating that the carboxy-terminal octapeptide is included in fragment B. Similarly, another mAb to CRP was generated which reacts specifically with a synthetic peptide

Table IV: Amino Acid Sequence Comparison of Known Ca^{2+}-Binding Loops[a] with the Proposed Ca^{2+}-Binding Region of CRP

protein	first residue	position 1	2	3	4	5	6	7	8	9	10	11	12	last residue
calmodulin														
loop I	20	D	K	D	G	N	G	T	I	T	T	K	E	31
loop II	56	D	A	D	G	N	G	T	I	D	F	P	E	67
loop III	93	D	K	D	G	N	G	Y	I	S	A	A	E	104
loop IV	129	N	I	D	G	D	G	E	V	N	Y	E	E	140
parvalbumin														
loop CD	51	D	Q	D	K	S	G	F	I	E	E	D	E	62
loop EF	90	D	S	D	G	D	G	K	I	G	V	D	E	101
galactose-binding protein	134	D	L	N	K	D	G	Q	I	Q	–	I[b]	E	205
troponin C														
loop I	30	D	A	D	G	G	G	D	I	S	T	K	E	41
loop II	66	D	E	D	G	S	G	T	I	D	F	E	E	77
loop III	106	D	K	N	A	D	G	F	I	D	I	E	E	117
loop IV	142	D	K	N	N	D	G	R	I	D	F	D	E	153
human CRP	138	E	Q	D	S	F	G	G	N[c]	F[d]	E	G	S	149

[a] Includes loops in calmodulin (Watterson et al., 1980), parvalbumin (Coffee & Bradshaw, 1973), galactose-binding protein (Mahoney et al., 1981), and troponin C (Wilkinson, 1976). [b] Residue 204 (I) of galactose-binding protein. [c] Position of cleavage by Nagarse protease. [d] Position of cleavage by Pronase.

identical with residues 137–152 of human CRP; this mAb also reacts preferentially with fragment B from the Pronase digest. These mAb results, together with the partial protein sequence and amino acid composition of fragment B, are consistent with the conclusion that fragment B from the Pronase digest consists of the carboxy-terminal end of the CRP subunit beginning at residue 147.

Ca^{2+} apparently stabilizes CRP so that it is not as readily precipitated by heat (Potempa et al., 1983). The present work shows that $CaCl_2$ protects intact CRP from heat precipitation at a concentration between 0.1 and 1.0 mM. Pronase-treated or Nagarse protease treated CRP is not substantially protected from heat-induced precipitation even with 10 mM $CaCl_2$. A $CaCl_2$ concentration between 0.1 and 1.0 mM also protects the CRP molecule from partial proteolysis by either Pronase or Nagarse protease. These results are consistent with equilibrium dialysis results which show that the high-affinity Ca^{2+}-binding sites are largely saturated with Ca^{2+} at a $CaCl_2$ concentration of 0.3 mM and that Ca^{2+} is unable to bind to cleaved CRP. The binding of Ca^{2+} to CRP may serve to protect CRP from proteolytic degradation or denaturation in the blood during the acute phase of infection.

Shephard et al. (1988) studied lysosomal degradation of human CRP at pH 4.5 and 7.4. They found that treatment of CRP with lysosomal enzymes at pH 7.4 generates a 16-kDa fragment as seen on SDS–PAGE (intact subunit apparent molecular mass of 24 kDa), similar in size to the approximately 16-kDa fragments seen with Pronase and Nagarse protease in the present work. $CaCl_2$ at 1.5 mM slows the rate of digestion. The same general effect of $CaCl_2$ is observed in the present work. Shephard et al. further report that smaller (<14 kDa) peptides of CRP generated by lysosomal degradation at either pH 4.5 or 7 are active in decreasing lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence of opsonized zymosan-activated neutrophils. The <14-kDa peptides generated by lysosomal degradation were not further characterized but may be similar to fragment B from Pronase or Nagarse protease treatment of CRP.

Equilibrium dialysis indicates that human CRP has two Ca^{2+}-binding sites per subunit with a K_d of 6×10^{-5} M (Figure 6). In earlier studies, Gotschlich and Edelman (1967) obtained a K_d of 1.3×10^{-4} M by equilibrium gel filtration. They also found that CRP binds 1.5 Ca^{2+} per CRP subunit but suggested that in view of the experimental conditions the actual value might be two Ca^{2+}-binding sites per subunit. The reason for the difference in K_d values is not known.

Cleavage of CRP with Nagarse protease or Pronase appears to destroy both Ca^{2+}-binding sites in the subunit. Values for the percent of Ca^{2+}-binding sites remaining as determined by equilibrium dialysis are somewhat higher than those expected on the basis of the percent of intact subunits remaining as determined by densitometry (44% obtained versus 38% expected from the 16-h, 37 °C Nagarse protease treated preparation, and 64% obtained versus 55% expected from the 14-h, 23 °C plus 3-h, 37 °C preparation). However, if only one Ca^{2+}-binding site per subunit were destroyed by the cleavage, 69% of the Ca^{2+}-binding sites would remain for the 16-h, 37 °C preparation, which is much higher than the experimentally determined value (44%) of Ca^{2+}-binding sites remaining. The Pronase-treated preparation contains very little intact subunit as determined by SDS–PAGE and binds very little Ca^{2+} compared to intact CRP (Figure 6). Thus, it appears that both Ca^{2+}-binding sites are affected by proteolysis, such that their ability to bind Ca^{2+} is largely lost. The phosphorylcholine-binding ability of CRP also is lost upon cleavage by Pronase or Nagarse protease. This indicates that one or both of the Ca^{2+}-binding sites that are lost upon proteolysis are important for the allosteric effect which allows binding of phosphorylcholine to CRP. Furthermore, the finding that antibodies specific for the Ca^{2+}-bound form of CRP do not react with Pronase-treated CRP even in the presence of Ca^{2+} is consistent with the conclusion that the partial proteolysis destroys the ability of CRP to bind Ca^{2+}.

Nguyen et al. (1986a,b) found two regions in the amino acid sequence of members of the CRP family which are highly conserved. One region (residues 51–66 of human CRP) is thought to contain the phosphorylcholine-binding site, and the other region (residues 133–147) includes a sequence of amino acids (residues 138–147) similar to Ca^{2+}-binding sites in calmodulin and related Ca^{2+}-binding proteins (Dang et al., 1985; Nguyen et al., 1986a,b). The present work provides direct evidence that the highly conserved region identified by Nguyen et al. is involved in the binding of Ca^{2+}.

A careful comparison of this region to the Ca^{2+}-binding sites of several Ca^{2+}-binding proteins is presented below and suggests that the binding of Ca^{2+} may involve residues 138–149 of human CRP. As described by Herzberg and James (1985), the Ca^{2+}-binding sites of calmodulin and related Ca^{2+}-binding

proteins have been assigned to a usually continuous sequence of 12 residues (Table IV). Residues at positions 1, 3, 5, 7, 9, and 12 apparently participate in coordination to Ca^{2+}. The residues at positions 1, 3, and 12 are Asx or Glu and coordinate to Ca^{2+} via their side-chain oxygen atoms, while residues 5, 7, and 9 may coordinate to Ca^{2+} via the amide carbonyl oxygen atom or via a water molecule. Due to structural requirements, position 6 is invariably occupied by Gly, and the residue in position 8 has an aliphatic hydrophobic side chain (Herzberg & James, 1985).

The proposed Ca^{2+}-binding region of CRP is compared with other known Ca^{2+}-binding sites in Table IV. The residue in position 1 of the known Ca^{2+}-binding sites is Asp in all cases except in loop IV of calmodulin. Position 1 of the proposed CRP site is Glu, which is a conservative replacement. Position 3 of the CRP site is Asp, which agrees with the Asx in this position in the known sites. Position 5 in the CRP site is occupied by Phe, whereas it is usually Asx in the other loops. However, there is some variability in this position, as loop CD of parvalbumin and loop II of troponin C are occupied by Ser and loop I of troponin C is filled by Gly. Position 6 of the CRP site is Gly, as it is in all other sites. Thus, the residues in the key positions 1, 3, and 6 of CRP are the same as or a conservative replacement for the corresponding residues in the other Ca^{2+}-binding loops. However, the residues in the key positions 8 and 12 of CRP as labeled in Table III are not the same as or a conservative replacement for corresponding residues in the known loops. Residues 8, 10, and 12 correspond to positions 1, 3, and 5 of the known Ca^{2+}-binding loops. Position 8 (Asn) of the CRP site is the same as the Asn in position 1 of calmodulin loop IV. Position 10 (Glu) of the CRP site is a conservative replacement for the Asp which generally occupies position 3 of the known Ca^{2+}-binding loops. Position 12 (Ser) is the same residue as position 5 of parvalbumin loop CD and troponin C loop II. Thus, it may be that the proposed Ca^{2+}-binding region of CRP contains two partial sites, each corresponding to approximately the first half of calmodulin-like Ca^{2+}-binding sites. The second half of these sites may come from other regions of CRP. In the galactose-binding protein (GBP) site, the last two residues of the loop come from another part of the polypeptide chain, which demonstrates that the entire site need not be sequential (Vyas et al., 1987). If there are parts of two Ca^{2+}-binding sites within the proposed Ca^{2+}-binding region, this could explain the loss of the two Ca^{2+}-binding sites in the CRP subunit when cleavage between residues 145 and 146 (Nagarse protease) or 146 and 147 (Pronase) occurs. Confirmation of this hypothesis must await analysis by X-ray crystallography.

The main findings of this work are that human CRP is cleaved between residues 145 and 146 by Nagarse protease or between residues 146 and 147 by Pronase and that this cleavage results in disruption of the two high-affinity (K_d = 6×10^{-5} M) Ca^{2+}-binding sites identified in the present work. Binding of Ca^{2+} to one or both of these sites is important for (1) the binding of CRP to phosphorylcholine, (2) protection against heat-induced precipitation or denaturation, and (3) protection against proteolysis. The cleavage occurs in a region of CRP that is similar to the Ca^{2+}-binding sites in calmodulin and related Ca^{2+}-binding proteins (Nguyen et al., 1986a,b). The present work is the first direct evidence that this highly conserved region of CRP may be involved in the binding of Ca^{2+}.

Acknowledgments

We thank Frank A. Robey (National Institute of Dental Research) for generously providing CRP peptide IV (residues 199–206). The excellent technical assistance of Putrina Dunlap, Michael Chen, and Michele Halberg is much appreciated. The expert advice of Dr. Eric Bremer is gratefully acknowledged.

Registry No. Ca^{2+}, 7440-70-2; phosphorylcholine, 107-73-3.

References

Baum, L. L., James, K. K., Glaviano, R. R., & Gewurz, H. (1983) *J. Exp. Med. 157*, 301.

Bray, R. A., Samberg, N. L., Gewurz, H., Potempa, L. A., & Landay, A. L. (1988) *J. Immunol. 140*, 4271.

Chesebro, B., & Metzger, H. (1972) *Biochemistry 11*, 766.

Coffee, C. J., & Bradshaw, R. A. (1973) *J. Biol. Chem. 248*, 3305.

Dang, C. V., Ebert, R. F., & Bell, W. R. (1985) *J. Biol. Chem. 260*, 9713.

DiCamelli, R., Potempa, L. A., Siegel, J., Suyehira, L., Petras, K., & Gewurz, H. (1980) *J. Immunol. 125*, 1933.

Dougherty, T. J., Zeller, J. M., Potempa, L. A., Gewurz, H., & Siegel, J. (1986) in *Proceedings of the XXXIV Colloquium, Protides of the Biofluids* (Peeters, H., Ed.) pp 267–271, Pergamon Press, New York.

DuClos, T. W., Mold, C., Paterson, P. Y., Alroy, J., & Gewurz, H. (1981) *Clin. Exp. Immunol. 43*, 565.

Fiedel, B. A. (1988) *Immunology 64*, 487.

Fiedel, B. A., & Gewurz, H. (1986) *J. Immunol. 136*, 2551.

Fiedel, B. A., Potempa, L. A., Frenzke, M. E., Simpson, R. M., & Gewurz, H. (1982) *Immunology 45*, 15.

Gotschlich, E. C., & Edelman, G. M. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 706.

Herzberg, O., & James, M. N. G. (1985) *Biochemistry 24*, 5298.

Houghten, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5131.

James, K., Hansen, B., & Gewurz, H. (1981) *J. Immunol. 127*, 2545.

James, K., Baum, L., Adamowski, C., & Gewurz, H. (1984) *J. Immunol. 131*, 2930.

Kaplan, M. H., & Volanakis, J. E. (1974) *J. Immunol. 112*, 2135.

Kilpatrick, J. M., & Volanakis, J. E. (1985) *J. Immunol. 134*, 3364.

Kilpatrick, J. M., Kearney, J. F., & Volanakis, J. E. (1982) *Mol. Immunol. 19*, 1159.

Kinoshita, C. M., & Gewurz, H. (1987) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 46*, 397.

Kinoshita, C. M., Siegel, J. N., Potempa, L. A., & Gewurz, H. (1988) *FASEB J. 2*, A1149.

Kushner, I., & Kaplan, M. H. (1961) *J. Exp. Med. 114*, 961.

Kushner, I., Rakita, L., & Kaplan, M. H. (1963) *J. Clin. Invest. 42*, 286.

Laemmli, U. K. (1970) *Nature 227*, 680.

Liu, T.-Y., Robey, F. A., & Wang, C. M. (1982) *Ann. N.Y. Acad. Sci. 389*, 151.

Mahoney, W. C., Hogg, R. W., & Hermodson, M. A. (1981) *J. Biol. Chem. 256*, 4350.

Mold, C., Nakayama, S., Holzer, T. J., Gewurz, H., & Du Clos, T. W. (1981) *J. Exp. Med. 154*, 1703.

Mold, C., DuClos, T. W., Nakayama, S., Edwards, K. M., & Gewurz, H. (1982) *Ann. N.Y. Acad. Sci. 389*, 251.

Mortensen, R. F., Osmand, A. P., Lint, T. F., & Gewurz, H. (1976) *J. Immunol. 117*, 774.

Nakayama, S., Gewurz, H., Holzer, T., Du Clos, T. W., & Mold, C. (1983) *Immunology 54*, 319.

Nguyen, N. Y., Suzuki, A., Cheng, S.-M., Zon, G., & Liu, T.-Y. (1986a) *J. Biol. Chem. 261*, 10450.

Nguyen, N. Y., Suzuki, A., Boykins, R. A., & Liu, T.-Y. (1986b) *J. Biol. Chem. 261*, 10456.
Osmand, A. P., Friedenson, B., Gewurz, H., Painter, R. H., Hofmann, T., & Shelton, E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 739.
Parish, W. E. (1976) *Clin. Allergy 6*, 543.
Pepys, M. B., Rowe, I. F., & Baltz, M. L. (1985) *Int. Rev. Exp. Pathol. 27*, 83.
Potempa, L. A., Siegel, J. N., & Gewurz, H. (1981) *J. Immunol. 127*, 1509.
Potempa, L. A., Maldonado, B. A., Laurent, P., Zemel, E. S., & Gewurz, H. (1983) *Mol. Immunol. 20*, 1165.
Potempa, L. A., Siegel, J. N., Fiedel, B. A., Potempa, R. T., & Gewurz, H. (1987) *Mol. Immunol. 24*, 531.
Robey, F. A., Jones, K. D., Tanaka, T., & Liu, T.-Y. (1984) *J. Biol. Chem. 259*, 7311.
Robey, F. A., Jones, K. D., & Steinberg, A. D. (1985) *J. Exp. Med. 161*, 1344.
Robey, F. A., Ohura, K., Futaki, S., Fujii, N., Yajima, H., Goldman, N., Jones, K. D., & Wahl, S. (1987) *J. Biol. Chem. 262*, 7053.
Shephard, E. G., Anderson, R., Beer, S. M., Van Rensburg, C. E. J., & DeBeer, F. C. (1988) *Clin. Exp. Immunol. 73*, 139.
Siegel, J., Rent, R., & Gewurz, H. (1974) *J. Exp. Med. 140*, 631.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goede, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76.
Tillett, W. S., & Francis, T. (1930) *J. Exp. Med. 52*, 561.
Vetter, M. L., Gewurz, H., Hansen, B., James, K., & Baum, L. L. (1983) *J. Immunol. 130*, 2121.
Volanakis, J. E., & Kaplan, M. H. (1971) *Proc. Soc. Exp. Biol. Med. 136*, 612.
Volanakis, J. E., & Kearney, J. F. (1981) *J. Exp. Med. 153*, 1604.
Vyas, N. K., Vyas, M. N., & Quiocho, F. A. (1987) *Nature 327*, 635.
Watterson, D. M., Sharief, F. S., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962.
Wilkinson, J. M. (1976) *FEBS Lett. 70*, 254.
Ying, S.-C., Gewurz, H., Kinoshita, C. M., Potempa, L. A., & Siegel, J. N. (1989) *J. Immunol. 143*, 221.
Yother, J., Volanakis, J. E., & Briles, D. E. (1982) *J. Immunol. 128*, 2374.
Zeller, J. M., Landay, A. L., Lint, T. L., & Gewurz, H. (1986) *J. Leukocyte Biol. 40*, 769.

Intramolecular Electron Transfer in Proteins. Radiolysis Study of the Reductive Activation of Daunorubicin Complexed in Egg White Apo-Riboflavin Binding Protein†

Chantal Houée-Levin,*,‡ Monique Gardès-Albert,‡ Kouider Benzineb,‡ Christiane Ferradini,‡ and Bernard Hickel§
Laboratoire de Chimie Physique, UA 400, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France, and CEN Saclay, IRDI, DESICP, DPC/SCM, UA 331, 91191 Gif-sur-Yvette, France
Received February 6, 1989; Revised Manuscript Received July 28, 1989

ABSTRACT: Daunorubicin, an anthracycline antitumor antibiotic, can be complexed in egg white apo-riboflavin binding protein. The reduction of this complex was studied by γ-radiolysis and pulse radiolysis using $COO^{\bullet -}$ free radicals as reductants. The final products are 7-deoxydaunomycinone intercalated in the protein and thiol groups coming from the reduction of disulfide bonds of the protein, in the respective proportions of 90% and 10%. One-electron reduction of the complex gives daunorubicin semiquinone radical and a disulfide anion. The rate constants of the reactions of $COO^{\bullet -}$ ions with the complex and with the disulfide bond in the protein alone are respectively equal to 2.4×10^8 $mol^{-1} \cdot L \cdot s^{-1}$ and 6.4×10^7 $mol^{-1} \cdot L \cdot s^{-1}$. Daunorubicin semiquinone decays by a first-order process, the rate constant of which is independent of the initial protein and radical concentrations. Without protein, daunorubicin semiquinone undergoes a disproportionation–comproportionation equilibrium [Houée-Levin, C., Gardès-Albert, M., Ferradini, C., Faraggi, M., & Klapper, M. (1985) *FEBS Lett. 179*, 46–50]. We propose that a protein residue reduces the semiquinone by an intramolecular path. This creates an electron hole in the protein which may alter its function. This reduction process is very different from the reduction mechanism of riboflavin binding protein by the same reductant [Faraggi, M., Steiner, J. P., & Klapper, M. H. (1985) *Biochemistry 24*, 3273–3279]. These results suggest a new deleterious pathway to explain the antitumor and/or cytotoxic effect of this drug.

Flavoproteins are a very important class of proteins which carry out a wide variety of different biochemical processes including electron transfer. Numerous studies have been devoted to a better understanding of how electrons are transmitted at flavoenzyme active sites through the protein matrix (Moore & Williams, 1976; Simondsen et al., 1982; Merrill et al., 1981; Klapper & Faraggi, 1983; Faraggi & Klapper, 1984; Tegoni et al., 1984; Tollin et al., 1984; Faraggi et al., 1985; Ghisla & Massey, 1986; Anderson et al., 1986). One of the most extensively studied flavoproteins is the chicken egg white riboflavin binding protein (RBP),[1] because of its

† The financial aid of Ligue Nationale contre le cancer is acknowledged.
* To whom correspondence should be addressed.
‡ Laboratoire de Chimie Physique.
§ CEN Saclay.

0006-2960/89/0428-9848$01.50/0