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Isolation and Characterization of the Amino and Carboxyl Proximal Fragments of the Adenosine Cyclic 3′,5′-Phosphate Receptor Protein of *Escherichia coli*†

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ABSTRACT: The cyclic AMP receptor protein (CRP) is a positive and negative regulatory protein for gene expression in *Escherichia coli*. The protein has been cleaved proteolytically to determine the relation between CRP structure and function. In the presence of sodium dodecyl sulfate ($NaDodSO_4$), chymotrypsin dissects CRP into two stable fragments of molecular weight 9500 (9.5K) and 13 000 (13K). After removal of $NaDodSO_4$, the two fragments are resolved by Bio-Rex 70 chromatography in 6 M urea. Analyses of the terminal amino acids released from each fragment and cyanogen bromide cleavage products indicate that the 9.5K fragment is amino proximal in CRP while the 13K fragment is carboxyl proximal. Notable features of amino acid composition are the relatively high amount of arginine and methionine in the 13K fragment and the retention in the 9.5K fragment of the two tryptophans present in the CRP subunit. Following isoelectric focusing in 8 M urea, the 9.5K fragment, 22.5K CRP, and 13K fragment migrate to pH 5.5, 8.3, and 10.3, respectively. While CRP is a cAMP-stimulated DNA binding protein, the 13K fragment binds to DNA in the presence and absence of cAMP. The 9.5K fragment associates to form dimers and decamers. These data are consonant with a model in which the DNA binding domain is present in the carboxyl proximal region of CRP while the amino proximal region contains the subunit–subunit interaction sites and much of the cAMP binding domain.

The cyclic AMP receptor protein (CRP)[1] is a positive regulatory factor for gene expression in *Escherichia coli*. In the presence of cAMP, CRP binds to sites within the promoter loci of catabolite-repressible operons, enabling RNA polymerase to form the open promoter complex prerequisite to initiation of transcription (deCrombrugghe & Pastan, 1978). CRP is also known to act as a negative effector, acting to repress transcription from one of the two promoters of the *gal* operon (Musso et al., 1977).

The native CRP promoter has a molecular weight of 45 000 and consists of two apparently identical subunits of molecular

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[1] Abbreviations used: CRP, cyclic AMP receptor protein; C core, chymotryptic core of CRP; SAP, *Staphylococcus aureus* V8 protease; $NaDodSO_4$, sodium dodecyl sulfate; $PhCH_2SO_4F$, phenylmethanesulfonyl fluoride; cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

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weight 22 500. CRP is an allosteric protein in which binding of cAMP elicits a conformational transition resulting in an enhanced affinity for DNA and presumably expression of site specificity (Krakow & Pastan, 1973; Wu et al., 1974; Saxe & Revzin, 1979). Digestion of CRP with proteolytic enzymes in the presence of cAMP produces dimeric CRP cores which bind cAMP but no longer bind DNA (Krakow & Pastan, 1973; Eilen et al., 1978). Two regions of the native CRP have been defined: the amino proximal region containing the subunit–subunit interaction sites and the cAMP binding domain; and the carboxyl proximal region which contains the DNA binding domain.

The functional differentiation of CRP suggests that it should be possible to isolate each of the domain-region polypeptides. The analogous experiments have been carried out with the *lac* and λ repressors by proteolytic cleavage of the native protein (Geisler & Weber, 1977; Pabo et al., 1979). A variety of incubation conditions and proteases, including those used for cleaving the native *lac* and λ repressors, proved ineffective in producing a stable carboxyl proximal polypeptide fragment of CRP. CRP in the presence of $NaDodSO_4$ is cleaved into two stable fragments by chymotrypsin (Pampeno & Krakow, 1979). In this paper, we present a procedure for the preparation of the amino proximal and carboxyl fragments of CRP and a characterization of their properties.

Materials and Methods

Materials. All biochemicals were of reagent grade. Trypsin, α-chymotrypsin, α-chymotrypsinogen, lysozyme, sperm whale myoglobin, horse heart cytochrome *c*, ribonuclease A, bovine serum albumin, phenylmethanesulfonyl fluoride (PMSF), dITP, dATP, dCTP, dTTP, cAMP, and Sephadex G-25 were obtained from Sigma Chemical Co. Carboxypeptidase Y, trifluoroacetic acid, phenylisothiocyanate, *N*-methylmorpholine, dansyl chloride, and micropolyamide F 1700 sheets were purchased from Pierce Chemical Co. The dansyl amino acids were obtained as a kit from Mann Research Laboratories, and Ampholine was from LKB. Bio-Rex 70, $NaDodSO_4$, acrylamide, and bis(acrylamide) were products of Bio-Rad Laboratories. Ultrapure urea was obtained from Schwarz/Mann, Sephacryl S-200 was from Pharmacia, and DEAE-cellulose was from Whatman. Nitrocellulose filters (0.45-μm pore size) were obtained from Matheson Higgins. (^{3}H)TTP, (^{3}H)dCTP, (^{3}H)cAMP, and Liquifluor were purchased from New England Nuclear. (^{3}H)d(A-T)$_n$ and (^{3}H)d(I-C)$_n$ were prepared with *E. coli* DNA polymerase I (Jovin et al., 1969).

CRP was purified from *E. coli* KLF 41/JC1553 diploid in the CRP structural gene as previously reported (Eilen et al., 1978). The CRP cores were prepared by digestion of CRP in the presence of cAMP with α-chymotrypsin or the *Staphylococcus aureus* V8 protease (SAP) followed by chromatography on Bio-Rex 70 (Eilen et al., 1978).

Protein Determination. Protein was determined by the method of Schaffner & Weissmann (1973).

Binding Assays. Assays for binding of (^{3}H)cAMP, (^{3}H)d(A-T)$_n$, and (^{3}H)d(I-C)$_n$ were carried out as previously described (Aiba & Krakow, 1980).

Buffers. Buffer A: 10 mM sodium phosphate (pH 6.8), 1 mM DTT, 6 M urea, and 0.1 mM $PhCH_2SO_2F$. Buffer B: 10 mM acetic acid (pH 4.8), 0.1 mM DTT, and 0.1 mM $PhCH_2SO_2F$. Buffer C: 10 mM sodium phosphate (pH 8.5), 1 mM DTT, 6 M urea, and 0.1 mM $PhCH_2SO_2F$. Buffer D: 10 mM sodium phosphate (pH 8.5), 0.25 M NaCl, 0.1 mM DTT, and 0.1 mM $PhCH_2SO_2F$. All pH values were determined at room temperature.

Chymotrypsin Cleavage of CRP in $NaDodSO_4$. A mixture containing 1 mg/mL CRP in 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 50% glycerol, and 0.5% $NaDodSO_4$ was heated for 5 min in a boiling water bath. After the mixture cooled, α-chymotrypsin (1 mg/mL in 1 mM HCl) was added to give a concentration 1% of that of CRP (w/w). Cleavage of CRP was carried out at 37 °C for the times indicated, and the reaction was terminated by addition of $PhCH_2SO_2F$ to a concentration of 0.1 mM.

Removal of $NaDodSO_4$. Following cleavage of CRP, removal of $NaDodSO_4$ was accomplished by using procedures based either on binding to an anion-exchange resin (Weber & Kuter, 1971) or on ion-pair extraction (Henderson et al., 1979).

For the anion-exchange resin method, solid urea was added to 5 mL of the digestion mixture to a concentration of 6 M and NaCl to 1 M. Following addition of 0.25 g of Bio-Rad AGl-X8 (200–400 mesh), the mixture was stirred for 30 min at room temperature. The resin was removed by filtration through Whatman 3 paper.

For ion-pair extraction, 5 mL of the digestion mixture was applied to a column (2 × 40 cm) of Sephadex G-25 equilibrated and resolved with 0.2% $NaDodSO_4$. Fractions containing the CRP fragments were pooled, lyophilized, and then dissolved in 1.5 mL of a mixture of acetic acid, triethylamine, and H_2O (1:1:1 v/v). Following addition of 3.5 mL of acetone, the protein precipitate was collected by centrifugation. The extraction procedure was repeated 2 times to complete the removal off $NaDodSO_4$. Finally, the protein precipitate was washed with acetone. Residual acetone was removed under vacuum, and the protein was dissolved in buffer A.

Isolation of the 13K and 9.5K Fragments. The reaction mixture which had been treated with the anion-exchange resin to remove $NaDodSO_4$ was applied to a column of Sephadex G-25 (2 × 40 cm) equilibrated with buffer A. Following elution with buffer A, the protein peak which appeared in the void volume was pooled and applied to a 0.7 × 10 cm column of Bio-Rex 70 equilibrated with buffer A. The column was washed with 20 mL of buffer A and eluted with a linear gradient (100 mL total volume) from 0 to 0.5 M NaCl in buffer A. The fractions were analyzed by $NaDodSO_4$ gel electrophoresis on 15% polyacrylamide slab gels. the M_r 9500 (9.5K) fragment appeared in the flow through while the M_r 13 000 (13K) fragment eluted between 0.1 and 0.3 M NaCl. Fractions containing the 13K fragment were diluted with buffer A to give a conductivity of 1 $m\Omega^{-1}$ and loaded onto a 0.7 × 4 cm Bio-Rex 70 column for concentration. The 13K fragment was eluted with 0.5 M NaCl in buffer A and was dialyzed against 1 L of buffer B.

Fractions which contained the 9.5K fragment were adjusted to pH 8.5 with 0.1 M NaOH and applied to a 0.7 × 4 cm column of DEAE-cellulose equilibrated with buffer C. The 9.5K fragment eluted with 0.5 M NaCl in buffer B and was dialyzed against 1 L of buffer D.

Amino-Terminal Analysis. Manual sequencing was performed by using the dansyl–Edman procedure according to the method of Weiner et al. (1972).

Carboxyl-Terminal Analysis. The carboxyl-terminal amino acid residues of CRP and the 9.5K and 13K fragments were analyzed by digestion with carboxypeptidase Y. Digestion mixtures contained (final volume 0.5 mL) 10 nmol of CRP or fragment (225 μg of CRP, 130 μg of 13K fragment, or 95 μg of 9.5K fragment) in 0.1 M *N*-methylmorpholine acetate (pH 4.0 for CRP or the 13K fragment; pH 7.0 for the 9.5K fragment), 0.15 M NaCl, and 0.1 nmol of carboxypeptidase

Y. The mixtures were incubated at 25 °C. At appropriate times, aliquots of 10 and 50 μL were removed, and carboxypeptidase Y was inactivated by heating for 2 min at 100 °C. The 10-μL samples were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis to monitor for possible endoproteolytic cleavage of CRP or the fragments. Acetone (0.5 mL) was added to the 50-μL sample, and the precipitate (protein plus salt) was removed by centrifugation at 3000 rpm for 10 min. The acetone supernatant containing the released amino acids was dried. After addition of 10 μL of 0.2 M $NaHCO_3$, dansylation was carried out with the addition of 10 μL of a solution of dansyl chloride in acetone (0.25% w/v, obtained from Pierce Chemical Co.) followed by incubation at 37 °C for 1 h. The sample was dried, dissolved in 20 μL of 0.1 M HCl, and twice extracted with 0.5 mL of water-saturated ethyl acetate. The ethyl acetate extract containing the dansylated amino acids was dried and the residue dissolved in 20 μL of 95% ethanol. A 2-μL aliquot was spotted on a micropolyamide plate (5 × 5 cm), and two-dimensional chromatography was performed according to the method of Hartley (1970).

Cyanogen Bromide Cleavage. Cleavage of CRP and fragments was carried out by using the method of Chen-Kiang et al. (1979). To the protein (5 nmol) in 100 μL of 70% formic acid was added 10 μL of freshly prepared cyanogen bromide solution (100 mg of cyanogen bromide in 100 μL of 70% formic acid). The mixture was incubated for 20 h at 37 °C. The sample was then diluted to 1 mL with water and lyophilized. The sample was then analyzed by $NaDodSO_4$ gel electrophoresis on a 15% polyacrylamide slab gel.

Isoelectric Focusing Gel Electrophoresis. Electrophoresis was carried out in glass tubes (120 × 5 mm i.d.). The gel mixture contained 5% acrylamide, 0.2% bis(acrylamide), 2% Ampholine (pH 3.5–10) or an Ampholine mixture (pH 7–9, pH 9–10, and pH 3.5–10, 4:4:2), 6 M urea, 0.025% Temed, and 0.025% ammonium persulfate. The electrode reservoirs contained 1 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). Electrophoretic focusing was carried out at 100 V for 15 h at room temperature. The standard gel was sliced into 5-mm segments which were shaken with 2 mL of deionized water for 2 h in order to determine to pH gradient. Sample gels were stained with 0.05% Coomassie brilliant blue R-250 in 25% 2-propanol–10% acetic acid.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis on slab gels was carried out by the method of Laemmli (1970). The determination of the molecular weight of the fragments was carried out in cylindrical gels according to the method of Weber & Osborn (1969).

Results

Chymotryptic Cleavage of CRP in $NaDodSO_4$. It was previously reported that two fragments were generated following incubation of CRP with chymotrypsin in the presence of $NaDodSO_4$ (Pampeno & Krakow, 1979). In order to effect a complete conversion of CRP to fragments, a high level of chymotrypsin had been used relative to CRP (1:5 chymotrypsin:CRP w/w). Initial attempts to isolate the fragments were unsuccessful due to extensive proteolysis following removal of $NaDodSO_4$.

A variety of digestion conditions were surveyed in order to find one which allowed for the recovery of intact fragments following removal of the detergent. It was found that the amount of chymotrypsin necessary for cleavage of CRP could be markedly reduced when the reaction mixture contained 50% glycerol. Figure 1 shows the time course for the cleavage of 100 μg of CRP by 1 μg of chymotrypsin in 50 mM Tris-HCl

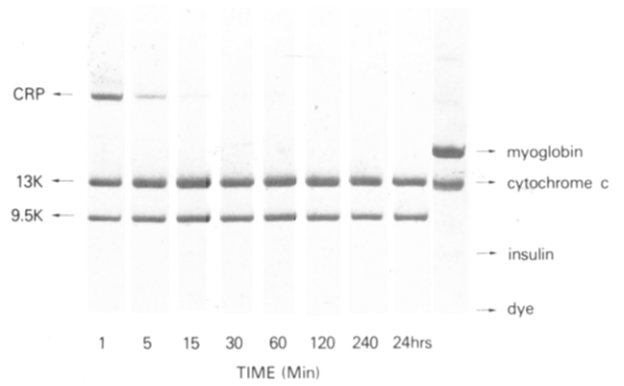

FIGURE 1: Time course of digestion of CRP in $NaDodSO_4$ by chymotrypsin. CRP (100 μg) in 0.1 mL of 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 0.5% $NaDodSO_4$, 50% glycerol, and 1 μg of α-chymotrypsin was incubated at 37 °C. At the times indicated, 10-μL aliquots were removed and heated for 2 min at 100 °C. The samples were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis (15% slab gel). Protein markers are shown in the last lane: sperm whale myoglobin, M_r 17 800; Horse heart cytochrome *c*, M_r 12 600; insulin, M_r 5700.

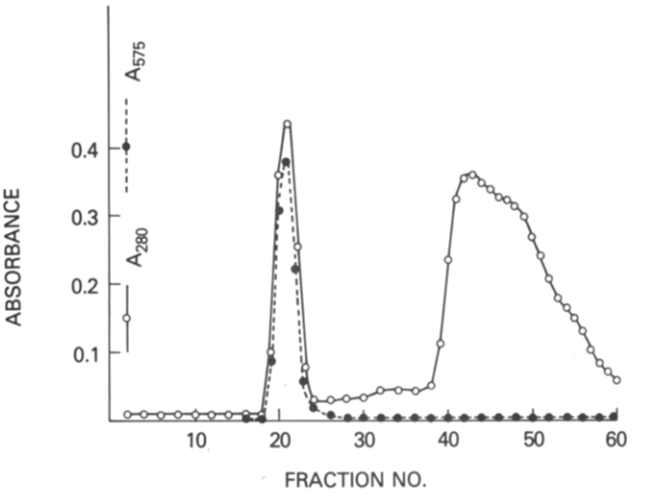

FIGURE 2: Chromatography on Sephadex G-25 of the CRP fragments produced in $NaDodSO_4$ by chymotrypsin. CRP (5 mg) was cleaved with chymotrypsin in 0.5% $NaDodSO_4$ (see Materials and Methods) for 60 min at 37 °C. The digest was resolved by chromatography on a Sephadex G-25 column (2 × 40 cm) equilibrated and developed with 0.2% $NaDodSO_4$ plus 1 mM DTT. The fractions (3 mL) were assayed for absorbance at 280 nm and ninhydrin-reacting material. For the ninhydrin assay, 0.1 mL of a ninhydrin solution (0.25% ninhydrin in butanol) was added to 0.7 mL of each fraction. After the mixture was heated for 10 min at 100 °C, the absorbance at 575 nm was determined.

(pH 8.0), 1 mM DTT, 0.1 mM EDTA, 0.5% $NaDodSO_4$, and 50% glycerol. Within 10 min at 37 °C, most of the CRP has been clipped to yield the two fragments, with complete conversion occurring by 1 h. The two fragments are stable after 24 h of incubation. Addition of another 1 μg of chymotrypsin after 60 min of incubation does not result in further digestion.

Only two fragments produced by chymotrypsin were noted following electrophoresis. The possibility remained that other smaller polypeptides might also have been produced which were not detected. A chymotryptic digest of 5 mg of CRP in $NaDodSO_4$ was resolved by chromatography on Sephadex G-25 using 0.25% $NaDodSO_4$ plus 1 mM DTT. The fractions were analyzed by reaction with ninhydrin (Figure 2). The results showed that ninhydrin-reacting material was present only in the void volume which corresponded to the position of the CRP fragments. No ninhydrin-reacting material was noted in any of the other fractions. The data suggest that

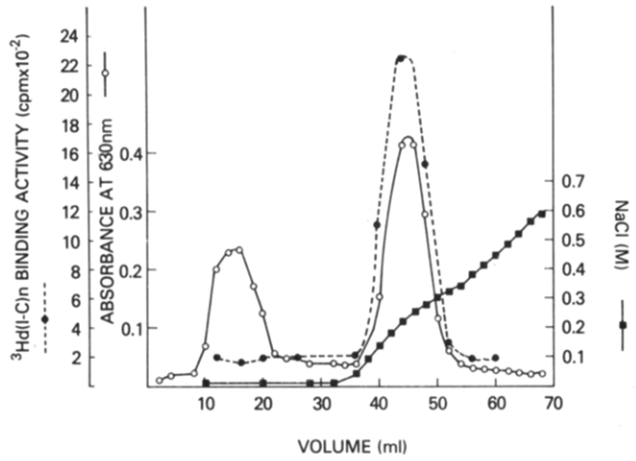

FIGURE 3: Resolution of the CRP fragments by chromatography on Bio-Rex 70. Following removal of $NaDodSO_4$, the fragments were chromatographed on Bio-Rex 70 (see Materials and Methods), and 2-mL fractions were collected. The protein concentration in 50 μL of each fraction (A_{630}) was determined by the method of Schaffner & Weissmann (1973). Binding of $(^3H)d(I\text{-}C)_n$ was assayed with 20 μL of each fraction.

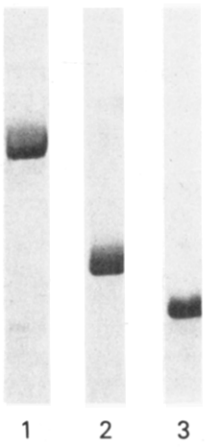

FIGURE 4: Electrophoretic analysis of purified CRP fragments. Electrophoresis of (1) CRP (10 μg), (2) 13K fragment (10 μg), and (3) 9.5K fragment (10 μg) was carried out on a $NaDodSO_4$–polyacrylamide (15%) gel.

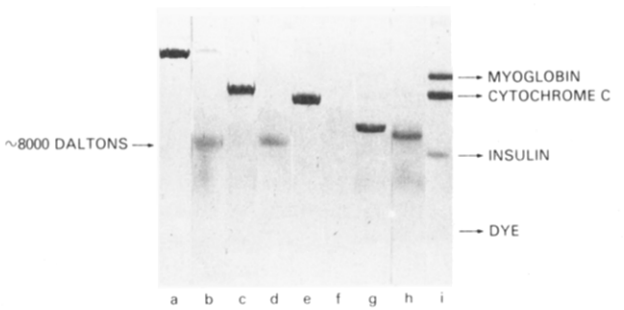

FIGURE 5: Cyanogen bromide cleavage of CRP and fragments. Cyanogen bromide treated (see Materials and Methods) CRP, C core, and 13K and 9.5K fragments (10 μg of each) were lyophilized. The lyophilized preparations were dissolved in 30 μL of sample buffer and resolved by electrophoresis on a $NaDodSO_4$–polyacrylamide (20%) slab gel. (a) CRP; (b) cleaved CRP; (c) C core; (d) cleaved C core; (e) 13K fragment; (f) cleaved 13K fragment; (g) 9.5K fragment; (h) cleaved 9.5K fragment; (i) molecular weight marker proteins: sperm whale myoglobin (17 800), horse heart cytochrome *c* (12 600), and insulin (5700).

under the conditions used chymotrypsin attacks at only one site within the CRP polypeptide to generate the two fragments.

Isolation of the Fragments. In order to resolve the CRP fragments, it was necessary to remove the $NaDodSO_4$ prior to chromatography. The removal of $NaDodSO_4$ was possible by either ion-pair extraction (Henderson et al., 1979) or binding to an anion-exchange resin (Weber & Kuter, 1971), with the latter being more convenient. For optimal recovery of the CRP fragments following removal of $NaDodSO_4$, both 6 M urea and 1 M NaCl must be present prior to addition of the anion-exchange resin (Bio-Rex AG1×8). In the absence of added NaCl, recovery of the CRP fragments was about 10–20% of that expected, while in the presence of 1 M NaCl, the recovery was greater than 90% without a significant effect on adsorption of the $NaDodSO_4$ to the resin. The amount of resin necessary to adsorb $NaDodSO_4$ was determined in trial runs (digestion mixture plus 6 M urea and 1 M NaCl but without CRP) by addition to the resin supernatants of KOH to form the insoluble $KDodSO_4$. A 50-mg sample of resin per mL of sample solution was sufficient for removal of $NaDodSO_4$ while resin at even 500 mg/mL did not affect the recovery of fragments. Routinely, 100 mg/mL Bio-Rex AG1×8 was used. Following removal of $NaDodSO_4$, the concentration of NaCl was lowered by gel filtration on Sephadex G-25.

The fragments were resolved by chromatography on the cation-exchange resin, Bio-Rex 70. Under the conditions used, the 9.5K fragment did not bind to the resin and appeared in the flow through (Figure 3). The 13K fragment was eluted from the Bio-Rex 70 between 0.1 and 0.3 M NaCl. Undigested CRP would have eluted just before the 13K fragment (data not shown). Following chromatography, each fragment is homogeneous (Figure 4). While the 13K fragment was soluble at pH 6.8 in the presence of 6 M urea, it tended to aggregate at neutral pH in the absence of urea. Concentrated solutions (1–2 mg/mL) remain soluble when stored in 10 mM acetic acid (pH 4.8).

Determination of Fragment Molecular Weights. The mobility of the CRP fragments gives molecular weights of 13 000 and 9500 following $NaDodSO_4$–polyacrylamide gel electrophoresis in sodium phosphate buffer (data not shown). These values are higher than those previously estimated (12 000 and 9000; Pampeno & Krakow, 1979) by $NaDodSO_4$–polyacrylamide gel electrophoresis in the Tris-glycine buffer system of Laemmli (1970). The molecular weights of 13 000 and 9500 are consonant with a single cut by chymotrypsin since the sum equals that of the molecular weight of the intact CRP subunit (Anderson et al., 1971). The molecular weights for the CRP cores formed by *Staphylococcus aureus* V8 protease and chymotrypsin attack on CRP in the presence of cAMP are the following: SAP core, 19 500; C core, 15 000.

Origin of Fragments. Our results indicate that CRP has been cleaved into two fragments, one of which comprises the amino proximal region and the other the carboxyl proximal region of the CRP polypeptide. CRP contains six methionine residues (Anderson et al., 1971). Cleavage of CRP by cyanogen bromide yields one large fragment of molecular weight 9000 comprising the amino proximal region (Schlesinger, 1978). Cyanogen bromide cleavage of CRP, the amino proximal C core (produced by chymotrypsin from CRP plus cAMP), or the 9.5K fragment all yield a large fragment of molecular weight ~8000. Cyanogen bromide cleavage of the 13K fragment does not yield any detectable fragments following $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 5). The results indicate that the 9.5K fragment is amino proximal in CRP while the 13K fragment is carboxyl proximal.

Amino-Terminal Sequence. If the 9.5K fragment is amino proximal in CRP it should contain an amino-terminal sequence identical with that of CRP. As a demonstration, this limited sequencing was carried out by the manual dansyl–Edman procedure (Table I). . The amino-terminal sequence found

Table I: Properties of CRP and Fragments

	CRP	C core	9.5K	13K
M_r	22 500	15 000	9500	13 000
p*I*	8.3	7.0	5.6	10.3
NH_2-terminal sequence	Val-Leu-Gly	Val-Leu-Gly	Val-Leu-Gly	Val-Lys-Ala
COOH-terminal amino acids	Ile, Leu, Val, and Tyr		Ile, Leu, Ala, Tyr, and Trp	Ile, Leu, Val, and Tyr
cAMP binding	+	+	−	−
DNA binding				
−cAMP	−	−	−	+
+cAMP	+	−	−	+

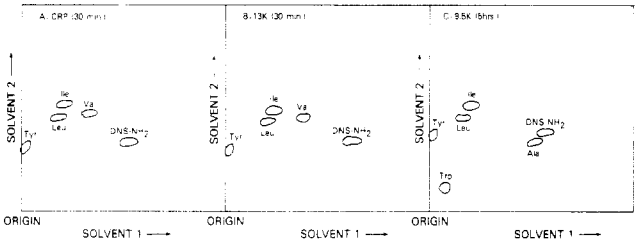

FIGURE 6: Release of amino acids from CRP and fragments following carboxypeptidase Y digestion. Digestion with carboxypeptidase Y and chromatography were carried out as described under Materials and Methods: (A) CRP (30-min incubation); (B) 13K fragment (30-min incubation); (C) 9.5K fragment (5-h incubation). Chromatography solvent 1, 3% formic acid; solvent 2, benzene:acetic acid (9:1 v/v).

for CRP and the 9.5K fragment, Val-Leu-Gly, is in agreement with the more extensive sequence presented by Schlesinger (1978). The sequence determined for the 13K fragment, Val-Lys-Ala, indicates that the fragment was not derived from the amino proximal region of CRP.

Carboxyl-Terminal Analysis. The generation of the 9.5K and 13K fragments appears to be a result of a scission within the CRP polypeptide. Hydrolysis by carboxypeptidase Y was carried out to determine whether the carboxyl proximal 13K fragment retained the amino acid residues present at the carboxyl terminus of CRP. The data presented in Figure 6 indicate that the amino acids released from the 13K fragment and CRP were identical: Ile, Leu, Val, and Tyr. The amino acids present at the carboxyl terminus of the 9.5K fragment are Ile, Leu, Ala, Tyr, and Trp.

Amino Acid Composition. The amino acid compositions of CRP and the 9.5K and 13K fragments are presented in Table II. Notable features of the composition are the relatively high amount of arginine and methionine in the 13K fragment and the retention in the 9.5K fragment of the two tryptophans present in the CRP subunit. The markedly basic behavior of the 13K fragment in binding to the cation-exchange resin, Bio-Rex 70, and its high isoelectric point (see below) suggest that most of the Asx and Glx are probably in the amide form.

Spectrophotometric Properties. The spectrophotometric properties of the fragments are presented in Table III. The extinction coefficients determined for each of the fragments are quite different; the $E^{1\%}_{280nm}$ of the 9.5K fragment is 17.9 while the $E^{1\%}_{280nm}$ of the 13K fragment is only 2.8. The $E^{1\%}_{280nm}$ of CRP is 8.8, which reflects the presence of both regions in the intact polypeptide.

The major contributor to the absorption at 280 nm is tryptophan. For determination of the number of tryptophans (N_{Trp}) in each fragment, the absorptions at 280 and 288 nm were determined in 6.5 M guanidine hydrochloride. N_{Trp} was determined with the equation $N_{Trp} = E'_{288nm}/3103 - E'_{280nm}/10\,318$ (Edelhoch, 1967) where E' is the molar extinction coefficient. The two tryptophans present in the intact CRP polypeptide are contained in the 9.5K fragment.

Table II: Amino Acid Composition of CRP and Fragments

	CRP	9.5K	13K
Arg	9	1	8
Lys	14	6	8
His	5	3	2
Cys	2	1	1
Asx	15	5	10
Glx	31	15	17
Thr	12	4	8
Ser	10	5	5
Pro	6	3	3
Gly	16	9	7
Ala	14	4	10
Val	13	4	10
Met	5	1	4
Ile	15	5	10
Leu	22	10	12
Tyr	5	3	1
Phe	5	3	2
Trp	2	2	0
total	201	84	118

Table III: Spectrophotometric Properties of CRP and Fragments

	absorption max[a] (nm)	$E^{1\%}_{280nm}$[a]	no. of Trp[b]	no. of Cys[c]
CRP	277.5	8.8	2.32	2
C core	277.5	14.2	2.54	1
SAP core	277.5	9.9	2.32	1
13K fragment	275	2.8	0.26	1
9.5K fragment	279	17.9	2.35	1

[a] The following buffers were used: 10 mM sodium phsophate (pH 6.8), 0.5 M NaCl, and 0.1 mM DTT for CRP, C core, and SAP core; 10 mM acetic acid, 0.1 mM DTT, and 0.1 mM PMSF for the 13K fragment; 10 mM sodium phosphate (pH 8.5), 0.25 M NaCl, 0.1 mM DTT, and 0.1 mM PMSF for the 9.5K fragment. [b] The number of tryptophan residues was calculated according to the equation $N_{Trp} = E^{1\%}_{288nm}/3103 - E^{1\%}_{280nm}/10\,318$ (Edelhoch, 1967). The absorption spectra were measured in 6.5M guanidine hydrochloride (pH 6.5). [c] Based on the amino acid composition (Table II) and previous reports (Eilen & Krakow, 1977; Pampeno & Krakow, 1978, 1979).

Isoelectric Focusing. Isoelectric focusing of the fragments and CRP in the presence of 8 M urea showed distinctive differences in the pH to which each migrated (Figure 7). The 9.5K fragment, 22.5K CRP polypeptide, and 13K fragment migrated to pH 5.5, 8.3, and 10.3, respectively. It is evident that the 9.5K fragment is acidic while the 13K fragment is basic relative to the parental CRP polypeptide. The content of basic amino acids in the 13K fragment (Table III) is not sufficient to account for its high isoelectric point; it is probable that many of the Glx and Asx residues will be present as glutamine and asparagine.

9.5K Oligmers. The amino proximal portion of CRP contains the cAMP binding domain and the region involved in subunit–subunit interactions (Eilen et al., 1978). Attempts to reconstitute cAMP binding activity after renaturation of

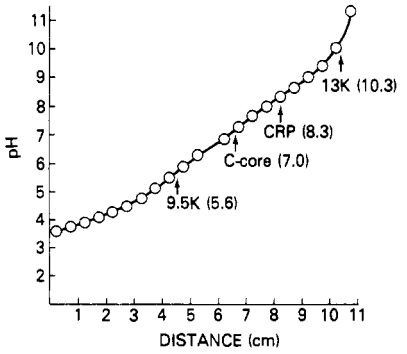

FIGURE 7: Isoelectric focusing of CRP and fragments. Electrophoresis was carried out as indicated under Materials and Methods, using 10 μg of each of the indicated proteins. The arrows indicate the position at which the protein focused. The values given in parentheses are the pHs determined at which focusing occurred.

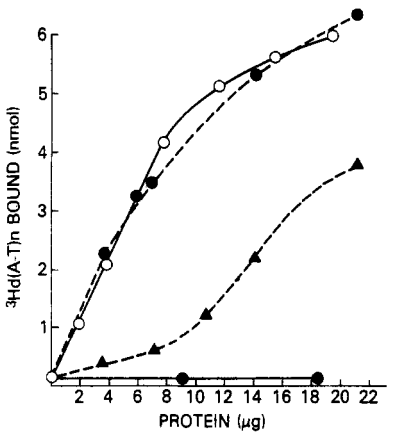

FIGURE 8: Binding of CRP and fragments to $(^3H)d(A\text{-}T)_n$. The assay mixture contained (final volume 0.5 mL) 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 nmol of $(^3H)d(A\text{-}T)_n$ (1360 cpm/nmol), 0.1 mM cAMP (where indicated), and the indicated amount of CRP, 13K fragment, or 9.5K fragment. After 5 min at 37 °C, the mixture was passed onto a nitrocellulose membrane filter. (●---●) CRP + cAMP; (▲) CRP; (○) 13K fragment; (●—●) 9.5K fragment.

the 9.5K fragment under a variety of conditions have been unsuccessful.

Following removal of urea, the 9.5K fragment was chromatographed on Sephacryl S-200 (data not shown). Less than 10% of the protein applied was recovered in a position corresponding to the monomeric 9.5K fragment. The protein eluted predominantly with molecular weights of 95 000 and 18 000, corresponding to decameric and dimeric forms of the 9.5K fragment. The results show that the 9.5K fragment can self-associate and suggest that this segment of the CRP polypeptide contains the site(s) involved in subunit–subunit interactions.

Recovery of DNA Binding in the 13K Fragment. We have previously shown that the DNA binding domain is located predominantly in the carboxyl proximal third of CRP; neither the M_r 15 000 chymotrypsin CRP core nor the M_r 19 500 SAP core (Pampeno & Krakow, 1978) retains DNA binding activity. Thus, the loss of a segment from the carboxyl-terminal end resulted in loss of DNA binding. Assays for binding of $(^3H)d(A\text{-}T)_n$ by the 9.5K and 13K fragments were carried out (figure 8). As expected, the 9.5K fragment showed no binding of $(^3H)d(A\text{-}T)_n$. (Binding of the 9.5K fragment to the nitrocellulose membrane could be demonstrated by staining the filter with amido black.) Binding as a function of protein concentration showed that CRP in the absence of cAMP binds cooperatively to $(^3H)d(A\text{-}T)_n$ while this property is not noted in the presence of cAMP. The 13K fragment bound $(^3H)d(A\text{-}T)_n$, and this was unaffected by the presence or absence of cAMP. The binding curves for the 13K fragment and CRP plus cAMP were similar although on a molar basis the affinity of the 13K fragment for $d(A\text{-}T)_n$ would be less than that of CRP plus cAMP.

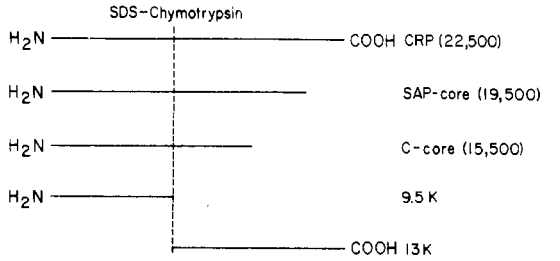

FIGURE 9: Location of CRP fragments.

Discussion

CRP is comprised of two functionally distinct regions: the amino proximal region containing the cAMP binding domain and the region involved in dimerization; the carboxyl proximal region which contains the DNA binding domain. In the absence of cAMP, CRP is resistant to attack by chymotrypsin and other proteases (Eilen et al., 1978). Binding of cAMP alters the conformation of CRP, allowing for proteolytic attack on the carboxyl proximal region. The resulting cores remain dimeric and bind cAMP but no longer bind DNA (Eilen & Krakow, 1977; Eilen et al., 1978). The stability of native CRP (in the absence of added cAMP) to chymotrypsin indicates that there is a stable interaction between regions of the amino proximal and carboxyl proximal polypeptides. When CRP is denatured by $NaDodSO_4$, one peptide bond, inaccessible in native CRP, is hydrolyzed by chymotrypsin, resulting in the formation of the amino proximal 9.5K fragment and the 13K carboxyl proximal fragment (Figure 9). The 9.5K fragment can also be excised from the 15K chymotryptic core by digestion with chymotrypsin in $NaDodSO_4$. Thus, a region of CRP normally inaccessible to chymotrypsin is rendered available in the presence of $NaDodSO_4$ while chymotrypsin-sensitive bonds present in the carboxyl proximal region of CRP plus cAMP are blocked in the presence of the detergent. Removal of $NaDodSO_4$ from the fragments results in a conformation in which the 9.5K and the 13K fragments are sensitive to digestion by chymotrypsin. It is apparent that the interaction within and between the domain structure of CRP is an important factor in determining the susceptibility to attack by chymotrypsin and other proteolytic enzymes. The most serious problem encountered in the isolation of the 9.5K and 13K fragments was their breakdown following removal of $NaDodSO_4$. This was due to reactivation of the $PhCH_2SO_2F$-inhibited chymotrypsin in the presence of urea (James, 1978). This was resolved by the reduced amount of chymotrypsin necessary for cleavage of CRP in 50% glycerol and by the readdition of $PhCH_2SO_2F$ during the course of the isolation of the fragments.

The data presented are consistent with the 9.5K fragment being amino terminal in CRP while the 13K fragment comprises the carboxyl-terminal region. The 9.5K fragment can be excised by chymotryptic cleavage in $NaDodSO_4$ of CRP or the amino proximal CRP cores. Schlesinger (1978) has shown that cleavage of CRP with cyanogen bromide yields an amino-terminal fragment of M_r 9000 with smaller cyanogen bromide polypeptides derived from the carboxyl proximal region. Cyanogen bromide cleavage of CRP, the amino proximal cores, and the 9.5K fragment yields a M_r ~8000 fragment; no large polypeptides are noted following cleavage of the 13K fragment with cyanogen bromide. Finally, the

amino-terminal tripeptide sequence in CRP and the 9.5K fragment is identical.

CRP is comprised of a relatively basic carboxyl proximal region and a relatively acidic amino proximal region. CRP is a DNA binding protein, and the isolated 13K fragment is able to bind DNA. Of the nine arginine residues in CRP, eight are present in the 13K fragment; lysine and histidine are distributed proportionately between the two regions in CRP. Since in the absence of cAMP native CRP is resistant to trypsin, it is probable that electrostatic interactions between basic amino acid residues in the carboxyl proximal region and acidic amino acid residues in the amino proximal region play a role in establishing the tertiary conformation of CRP.

The functional differentiation of the CRP domains is also a property of other DNA binding regulatory proteins. In both the λ and *lac* repressors, the DNA binding domain is amino proximal while the subunit–subunit interaction sites are carboxyl proximal (Platt et al., 1973; Geisler & Weber, 1977, 1978; Pabo et al., 1979). In CRP, the DNA binding domain is carboxyl proximal while the subunit–subunit interaction sites and the cAMP binding domain are amino proximal. The inducer binding site of the *lac* repressor is present in the carboxyl proximal domain. Incubation of the *lac* repressor with trypsin or chymotrypsin yields a resistant tetrameric core which retains inducer binding activity while having lost DNA binding activity (Platt et al., 1973). Under more restrictive incubation conditions, the *lac* repressor can be dissected by trypsin into a homogeneous tetrameric core and monomeric amino-terminal polypeptide fragments termed headpieces. The headpieces bind DNA (Giesler & Weber, 1977; Jovin et al., 1977; Ogata & Gilbert, 1978). Similar dissection into two functional domains is obtained for the λ repressor by papain cleavage (Pabo et al., 1979). The DNA binding activity of the λ repressor is recovered in the amino proximal fragment (Sauer et al., 1979). The DNA binding domain of CRP, *lac* repressor, and λ repressor contains a relatively high amount of basic amino acids presumably involved in electrostatic interaction with DNA. This region also contains potential hydrogen-bonding amino acids such as tyrosine, threonine, and serine (Weber et al., 1972; Fanning, 1975; Alexander et al., 1977). It is also noteworthy that all the DNA binding domains lack tryptophan. In *lac* and λ repressors, the isolated DNA binding fragments have been shown to bind specifically to the operator DNA by the chemical methylation experiments (Ogata & Gilbert, 1978; Sauer et al., 1979). Moreover, the amino-terminal DNA fragment of λ repressor can mediate positive and negative control of transcription in vitro and in vivo (Sauer et al., 1979). The present study shows that the DNA binding property of the 13K fragment is similar to that of the cAMP–CRP complex.

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