Intersubunit Communications in *Escherichia coli* Cyclic AMP Receptor Protein: Studies of the Ligand Binding Domain†

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ABSTRACT: *Escherichia coli* cAMP receptor protein (CRP) is a homodimer in which each subunit is composed of two domains. The C-terminal domain is responsible for DNA recognition, whereas the larger N-terminal domain is involved in cAMP binding. Biochemical and genetic evidence suggests that both intersubunit and interdomain interactions play important roles in the regulatory mechanism of this protein. Essentially all intersubunit contacts occur via a long C-helix which is a part of the N-terminal domain. In this work, intersubunit interactions in CRP were studied with the use of two proteolytic fragments of the protein. Subtilisin digestion produces a fragment (S-CRP) which includes residues 1–117 and in which about 85% of the C-helix is removed, whereas chymotrypsin digestion produces a fragment (CH-CRP) consisting of residues 1–136, in which the whole C-helix is preserved. Both fragments were purified and subjected to functional tests which included cAMP binding, subunit assembly, and hydrodynamic properties in the presence and absence of cAMP. S-CRP binds cAMP with a similar affinity to that of native CRP but with reduced cooperativity. CH-CRP exhibits about 1 order of magnitude tighter binding of cAMP than S-CRP or CRP and the highest degree of negative cooperativity. Both fragments are dimeric with dimerization constants around 10^8 M^{-1}. Ligand binding promotes dimerization and induces a small contraction of both S-CRP and CH-CRP. There is no apparent correlation between dimer stability and cooperativity of ligand binding. Although residues 117–136 in the C-helix are apparently not critical for dimer stability, they may be important for intersubunit communication that leads to cooperativity in ligand binding.

Escherichia coli cAMP receptor protein (CRP)[1] is a transcription factor which regulates the expression of more than 20 genes (de Crombrugghe et al., 1984). Its binding to specific and nonspecific DNA sites is regulated by a small allosteric effector, cAMP. In the absence of cAMP, CRP exhibits a low affinity for the specific DNA site. However, the presence of cAMP leads to the formation of CRP–cAMP and CRP–$(cAMP)_2$ complexes which have been shown to have different affinities for the specific DNA site in the lac operon (Heyduk & Lee, 1989, 1990). The CRP–cAMP complex displays a stronger affinity than the CRP–$(cAMP)_2$ complex. Furthermore, the binding of cAMP to CRP is characterized by a negative cooperativity (Takahashi et al., 1980; Heyduk & Lee, 1989). It is evident that both homotropic and heterotropic allosteric effects are observed in this system. Many studies have demonstrated that cAMP binding induces a conformational change in the protein which is responsible for the change in affinity for the specific DNA site (Wu et al., 1976; Eilen & Krakow, 1977; Krakow & Pastan, 1973; Heyduk & Lee, 1989). In order to elucidate the molecular mechanism(s) of regulation exhibited by CRP, it is useful to establish a correlation between functional and structural elements of the protein.

CRP is a dimer composed of two identical subunits. Each of them is composed of two domains. The cAMP binding site is located in the N-terminal domain while the C-terminal domain is involved in specific recognition of DNA. These domains are connected by a long C-helix which consists of residues 114–134. On the basis of X-ray crystallographic data, this helix also forms essentially all of the intersubunit contacts in this protein (McKay & Steitz, 1981; McKay et al., 1982; Weber & Steitz, 1987). The structural information also shows that the binding of each cAMP molecule involves ionic and hydrogen-bond interactions. A specifically interesting feature is that both Thr-127 and Ser-128 of the C-helix, with the latter from the adjacent subunit, are involved in hydrogen bonding with the adenine ring of cAMP (Weber & Steitz, 1987). In view of the homotropic and heterotropic functional features exhibited by CRP, it is conceivable that in CRP both intersubunit and interdomain interactions play important roles in the regulatory functions of the protein. Furthermore, the C-helix seems to be a crucial structural feature of the protein involved in these interactions. Other functional data which also support the notion that the C-helix must be intimately linked to the regulatory mechanism are given in the recent study by Brown and Crothers (1989). These authors reported that CRP dissociates into monomer and the monomer–dimer equilibrium is shifted in favor of dimer in the presence of cAMP. Hence, in any consideration about the mechanism of CRP action, one must include the quantitative linkages among CRP–cAMP, CRP subunits, and CRP–DNA interactions. The helix–helix interactions might be the focal point of all of these reactions for signal transduction between domains and subunits.

One of the long-term goals of this laboratory is to obtain information on the pathways of interdomain and intersubunit communication in cAMP receptor protein. In this report, the intersubunit communication in CRP was probed by using proteolytic fragments of the protein. It has been shown that in the presence of cAMP CRP dimer can be cleaved with a number of proteolytic enzymes, producing a dimer of the

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[1] Abbreviations: CRP, cyclic AMP receptor protein; S-CRP and CH-CRP, N-terminal fragments of CRP resulting from proteolytic digestion by subtilisin and chymotrypsin, respectively; α-CRP, generic term for both S- and CH-CRP; FITC, fluorescein isothiocyanate; PMSF, phenylmethanesulfonyl fluoride; DMF, *N,N'*-dimethylformamide.

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N-terminal domain (α-CRP) which retains cAMP binding capability (Krakow & Pastan, 1973; Eilen et al., 1978; Tsugita et al., 1982; Ebright et al., 1985). Furthermore, a proton NMR study of the histidine residues indicates that the structure of the N-terminal domain in α-CRP is not dramatically changed relative to that of the intact protein (Clore & Gronenborn, 1982). Thus, the structure and ligand binding ability of these fragments were used to probe the significance of the C-helix in regulating the function of CRP. In particular, fragments produced by cleavage with subtilisin (S-CRP) and chymotrypsin (CH-CRP) were employed. S-CRP is cleaved at Leu-117 (Tsugita et al., 1982); therefore, in this fragment, 85% of the C-helix is removed. CH-CRP, on the other hand, is cleaved at Phe-136 (Angulo & Krakow, 1985). This Phe residue is located in a hinge region between the C- and N-terminal domains. The C-helix in CH-CRP is intact. Results from this study show that the presence of a complete C-helix is necessary for communication between subunits and Thr-127 and Ser-128 may be involved in this process. However, the helix–helix interaction between subunits does not play a dominant role in maintaining dimer stability.

Materials and Methods

Materials

Subtilisin (protease type XXVII) and Tris were purchased from Sigma. Chymotrypsin A and cAMP were from Boehringer-Mannheim. [^{3}H]cAMP of specific activity around 50 Ci/mol was a product of Amersham. ANS (Kodak Lab), before it was used, was purified on a Sephadex LH-20 column (Pharmacia) as described by York et al. (1978). FITC was purchased from Molecular Probes. Bio-Rex 70 and hydroxyapatite were obtained from Bio-Rad, while DEAE-cellulose was from Whatman. The standard proteins for calibrating the Sephacryl 200 HR column (Pharmacia) employed in analytical gel chromatography were bovine pancreas chymotrypsinogen, ribonuclease A, ovalbumin, and *N*-acetyltryptophanamide (Sigma Chemical Co., Inc.). Blue dextran was from Pharmacia. CRP was purified as described by Heyduk and Lee (1989).

Methods

All experiments were conducted in 50 mM Tris, 100 or 300 mM KCl, and 1 mM EDTA, at pH 7.8 and 23 °C. The following extinction coefficients were used: 14 650 M^{-1} cm^{-1} at 259 nm for cAMP (Merck Index, 1976); 6240 M^{-1} cm^{-1} at 351 nm for ANS (Ferguson et al., 1975); 72 000 M^{-1} cm^{-1} at 494 nm for FITC (Molecular Probe).

For determining the concentration of S-CRP and CH-CRP fragments and intact CRP, the same coefficient of 0.91 mL/(mg·cm) at 278 nm was used (Takahashi et al., 1980), since proteolytically modified proteins retain all Trp residues and all but one Tyr of intact CRP. This coefficient differs by less than 10% from the molar extinction coefficient calculated from the amino acids composition of these fragments using the procedure of Gill and von Hippel (1989).

Before use, CRP, S-CRP, and CH-CRP samples were thawed, dialyzed against experimental buffer, and filtered through microporous 0.45-μm filters (Millipore).

Preparation and Purification of S-CRP and CH-CRP. The procedures of CRP digestion by subtilisin and chymotrypsin and purification of fragments were based essentially on the published procedure of Tsugita et al. (1982).

Solutions of CRP at 0.5 mg/mL in 100 mM KCl, 50 mM Tris, and 1 mM EDTA (pH 7.8) containing 0.100 mM cAMP were digested with subtilisin at 6 μg/mL for 85 min and chymotrypsin at 12 μg/mL for 210 min at room temperature. The reactions were stopped with 1 mM PMSF. The reaction mixtures were dialyzed againt 50 mM NaCl, 15 mM phosphate, 0.5 mM EDTA, and 1 mM PMSF (pH 6.8). After dialysis, the protein solutions were adsorbed onto a column of Bio-Rex 70 which was equilibrated with the same buffer. The fragments were eluted with 1 M and a 0.05–1 M NaCl linear gradient for S-CRP and CH-CRP, respectively. The presence of cAMP in these protein fragments was checked by measuring the absorbance ratio at 278 and 260 nm and measuring the remaining radioactivity in samples where digestion was performed in the presence of [^{3}H]cAMP. cAMP was removed easily for S-CRP but only after very extensive dialysis for CH-CRP. Using these procedures, pure S-CRP and CH-CRP were obtained as indicated by the presence of single bands of approximately 13 000 or 15 000 in molecular weight for S-CRP and CH-CRP, respectively, in SDS–polyacrylamide gel experiments.

Preparation of α-CRP Labeled with a Fluorescence Probe. FITC modification of N-terminal fragments of CRP was carried out in 50 mM Tris, 100 mM KCl, 1 mM EDTA, and 1 mM DTT at pH 8.5. Sufficient FITC dissolved in DMF was added to the protein solution to achieve a FITC:protein molar ratio of about 35:1. The reaction mixture was stirred for 3 h at room temperature, after which the unreacted dye was removed by gel filtration through a column of Sephadex G-25 equilibrated with reaction buffer. Fractions displaying an absorbance at both 278 and 494 nm were combined and dialyzed against 50 mM Tris, 100 mM KCl, and 1 mM EDTA at pH 7.8. The extent of modification was measured spectrophotometrically, and it was in a molar ratio ranging from 0.3 to 0.5 of FITC per protein.

Titration of Protein–ANS Complexes with cAMP. Binding of cAMP can be monitored by the perturbation of fluorescence intensity of the ANS–protein complex (Heyduk & Lee, 1989). These fluorescence measurements were performed with a Perkin-Elmer 512 double-beam spectrofluorometer. The formation of cAMP–ANS–α-CRP complexes was studied by titrating a solution of 200 μL of α-CRP (2.7–12 μM) containing ANS (20–30 μM) with 1–2 μL of concentrated cAMP solution. The excitation and emission wavelengths were 380 and 480 nm, respectively. The data were corrected for the fluorescence of free ANS by performing analogous titration with cAMP of the samples containing ANS alone without α-CRP.

[^{3}H]cAMP Binding Experiments. Binding of cAMP to α-CRP was measured by equilibrium (Heyduk & Lee, 1989) and nonequilibrium dialysis (Colowick & Womack, 1969; Heyduk & Lee, 1989). In both experiments, Spectra/Por 3 (molecular weight cutoff 3500) was used as a membrane.

Anisotropy of Fluorescence Measurements. The anisotropy measurements were performed with an SLM 500 C spectrofluorometer equipped with a polarization accessory in 50 mM Tris, 300 mM KCl, and 1 mM EDTA, pH 7.8, buffer at 21 °C, in the presence or absence of cAMP. When the fluorescence of protein intrinsic fluorophores was monitored, excitation and emission wavelengths were set at 280 and 340 nm, respectively. For anisotropy measurements of protein labeled with FITC, the excitation and emission wavelengths were 490 and 520 nm, respectively.

Analytical Gel Chromatography. Analytical gel chromatography experiments were performed using a procedure described by Heyduk et al. (1992). In short, the eluant from a thermostated chromatography column (C10/40 with Sephacryl 200 HR from Pharmacia equipped with adaptor) was passed through a flow-cell in a spectrofluorometer. The

Table I: Summary of Fitted Equilibrium Constants[a]

protein	$f_{act.}$	equilibrium dialysis k_1 ($M^{-1} \times 10^{-4}$)	equilibrium dialysis k_2 ($M^{-1} \times 10^{-4}$)	k_1/k_2	fluorescence k_1 ($M^{-1} \times 10^{-4}$)	fluorescence k_2 ($M^{-1} \times 10^{-4}$)
CRP[b]	1.0[c]	4.6 (3.3, 6.8)	0.17 (0.07, 0.3)	27	2.3 (1.9, 2.9)	0.05 (0.01, 2.8)
	1.19 (1.09, 1.27)	3.1 (2.2, 4.4)	0.01 (0, 0.1)	258		
S-CRP	1.0[c]	10.7 (7.4, 15.5)	1.6 (1.1, 2.2)	6.7	7.4[e] (5.9, 9.3)	
	0.72 (0.66, 0.77)	7.6 (3.9, 12.7)	14.3 (8.6, 22.4)	0.5		
CH-CRP	1.0[c]	49.9 (31.1, 80.9)	0.06 (0, 0.26)	832	84.5[e] (63.0, 120.1)	
	1.04 (0.99, 1.10)	42.3 (26.3, 66.9)	nd[d]	high		

[a] Microscopic constants. The numbers in parentheses correspond to 75% confidence intervals calculated as described by Johnson and Frasier (1985). [b] Data from Heyduk and Lee (1989). [c] Data were fitted with $f_{act.} = 1.0$. [d] Too weak to be determined. [e] Only a single equilibrium constant could be resolved.

fluorescence signal was digitized and transferred to an IBM PC computer for data analysis.

The flow rate was controlled with a Gilson peristaltic pump and was determined for each individual run gravimetrically. All solutions used for column experiments were filtered and degassed before use. The column was calibrated with ribonuclease A, chymotrypsinogen A, and ovalbumin. Blue dextran was used to determine the void volume of the column (V_o) and the total volume (V_t) was estimated by using *N*-acetyltryptophanamide. The partition coefficient was calculated as

$$K_{av} = (V_e - V_o)/(V_t - V_o) \quad (1)$$

where V_e is the elution volume of the protein. Elution volumes were calculated from the centroid of the leading boundary in large zone experiments (Ackers, 1970). Linear relationships between $(-\log K_{av})^{1/2}$ and Stokes' radius (Sigel & Monty, 1966) were used to calculate the Stokes' radii of α-CRP fragments.

Results

Since the proteolytic digestion hydrolyzes the C-terminal domain, leaving the N-terminal domain intact, the only functions that can be tested are ligand binding, monomer–dimer association, and changes in hydrodynamic properties of these fragments induced by ligand.

cAMP Binding. The binding of cAMP to S-CRP and CH-CRP was measured by equilibrium dialysis, and the results are shown in Figure 1. The most apparent feature of these results is that the cAMP binding isotherm for CH-CRP lies significantly to the left of that for S-CRP. This indicates that cAMP binds more tightly to CH-CRP than to S-CRP. Since CRP has two binding sites for cAMP (Takahashi et al., 1980), the binding data were fitted to the equation:

$$N_S = f_{act.}(K_1[\text{cAMP}] + 2K_2[\text{cAMP}]^2)/(1 + K_1[\text{cAMP}] + K_2[\text{cAMP}]^2) \quad (2)$$

where N_s is the number of cAMP bound per mole of protein and K_1 and K_2 are Adair constants for cAMP binding. The Adair constants for two equivalent ligand binding sites are related to the microscopic constants by $K_1 = 2k_1$ and $K_2 = k_1k_2$. The fraction of active protein, $f_{act.}$, is equal to the ratio of the concentration of the protein which is competent to bind cAMP to the total concentration of the protein. Including $f_{act.}$ in eq 2 makes allowance for a possibility of some protein being inactive in binding of cAMP or for a systematic error in protein concentration determination.

The data were fitted directly for the microscopic constants k_1 and k_2 with $f_{act.}$ either fixed at $f_{act.} = 1$ (i.e., assuming that all of the protein is capable of binding cAMP) or acting as a fitting parameter. Table I is a summary of the fitted results. When comparing these results, one has to remember that by including $f_{act.}$ as a fitted parameter the correlation between k_1 and k_2 generally increases as judged by correlation matrix

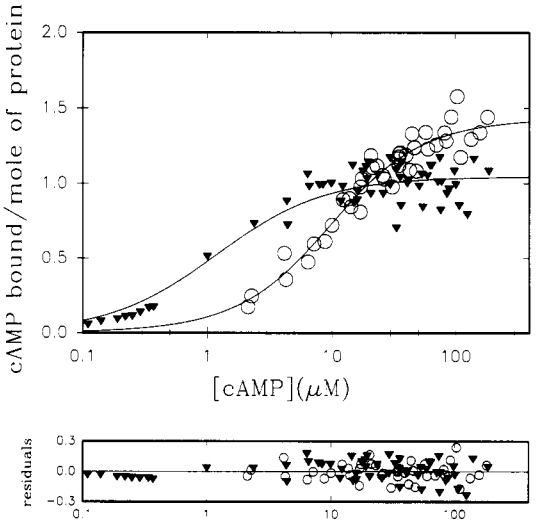

FIGURE 1: [^{3}H]cAMP binding to S-CRP (○) and CH-CRP (▼). Solid lines represent the best fit as described in the text. The ranges of protein concentration employed were 6–20 and 6–12 μM for CH-CRP and S-CRP, respectively.

analysis (Johnson & Frasier, 1985). For example, in the case of S-CRP, the correlation coefficients between k_1 and k_2 are −0.55 and −0.81 when the fitting was performed with $f_{act.}$ fixed at 1 or as a fitting variable, respectively. Furthermore, the correlation coefficient between $f_{act.}$ and k_1 is 0.55, whereas it assumes a value of −0.88 between $f_{act.}$ and k_2. Thus, a floating $f_{act.}$ yields less reliable parameters, and it is worse for k_2 than k_1.

There are two interesting trends which one can identify in the data. First, regardless of the fitting procedure, the values of k_1 are reasonably well defined. Within the experimental uncertainty, the values of k_1 for CRP and S-CRP are similar, whereas this value is about an order of magnitude higher for CH-CRP. Therefore, the order of affinity of cAMP for the first site is as follows: CRP ≤ S-CRP < CH-CRP. The precision of determination of k_2 is quite low, and it is not surprising that its particular values are dependent on the fitting procedure used. However, more revealing is the value of the ratio of k_1 to k_2 which is a measure of cooperativity in cAMP binding. This ratio is quite variable and is dependent on the particular fitting procedure. However, it still shows the following trend: S-CRP < CRP < CH-CRP; i.e., the binding of cAMP to CH-CRP seems to exhibit a very high degree of negative cooperativity which is higher than that of native CRP, whereas the two binding sites in S-CRP exhibit much more similar affinity. An alternative interpretation of the CH-CRP data, namely, no detectable binding beyond a 1:1 stoichiometry, would be a noncooperative binding of 2 cAMP molecules/CRP dimer. The apparent stoichiometry of 1:1 could be a consequence of partial inactivation of the protein toward cAMP binding in the process of preparation of the CH-CRP

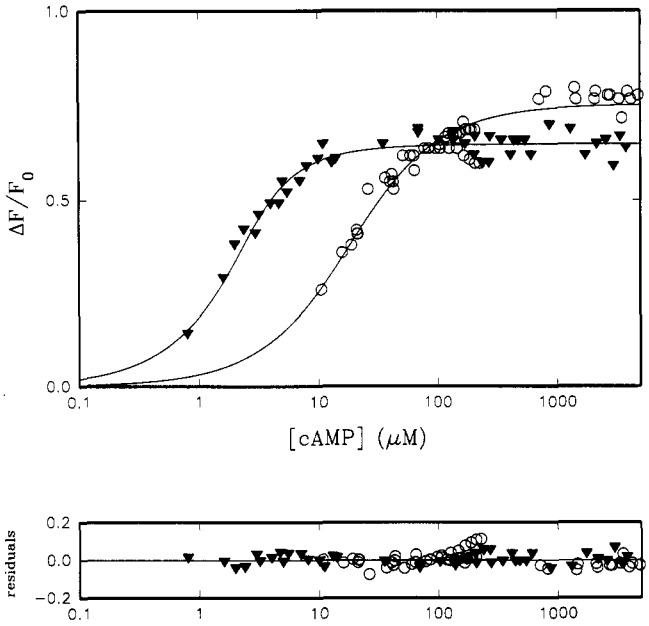

FIGURE 2: Binding of cAMP monitored by the quenching of fluorescence of the α-CRP–ANS complex. The data are expressed as $\Delta F/F_0$ where $\Delta F = F - F_0$ is the difference in the fluorescence intensity between the ANS–protein complex in the absence and presence of cAMP, respectively. The ranges of protein concentration employed were 2.7–3.6 and 4.8–5.8 μM for CH-CRP and S-CRP, respectively. The symbols and identity of CRP fragments are (O) S-CRP and (▼) CH-CRP.

fragment. This would require inactivation of about 50% of the protein. There is no experimental evidence to indicate the presence of such heterogeneity in the various preparations of CH-CRP. On the contrary, CH-CRP preparations were generally better than that of S-CRP in which occasionally a faint band of lower molecular weight could be detected in SDS–PAGE gels.

cAMP binding was also followed by the quenching of fluorescence of the ANS–protein complex. It has been shown previously (Heyduk & Lee, 1989) that CRP binds 2 mol of ANS/mol of protein and that the binding of cAMP quenches the ANS fluorescence signal by displacing it from a CRP–ANS complex. ANS also binds to the proteolytic fragments of CRP although more weakly (data not shown). As in the case of CRP, cAMP quenches the fluorescence signal of the ANS–α-CRP complex, which permits the determination of equilibrium constants. Results from fluorescence titrations of S-CRP and CH-CRP are shown in Figure 2. It is evident that the titration curve for cAMP binding to CH-CRP has shifted to the left of that for S-CRP. This implies that the affinity of CH-CRP for cAMP is higher than that of S-CRP. An attempt was made to fit the titration data to eq 3, which

$$\Delta P_{obs} = \frac{\Delta P_1 K_1[\text{cAMP}] + \Delta P_2 K_2[\text{cAMP}]^2}{1 + K_1[\text{cAMP}] + K_2[\text{cAMP}]^2} \quad (3)$$

was used to fit the fluorescence data for CRP (Heyduk & Lee, 1989). ΔP_{obs} is the measured value of fluorescence intensity, and ΔP_1 and ΔP_2 are normalized specific values of the change in fluorescence intensity corresponding to the formation of CRP–cAMP and CRP–$(cAMP)_2$ complexes, respectively. The data for both S-CRP and CH-CRP could not be fitted to eq 3, but they could adequately be analyzed in terms of a simple binding isotherm with a single equilibrium constant and an overall ΔP of 0.76 and 0.65 for S-CRP and CH-CRP, respectively. This could mean either that the affinities for the binding of the first and second cAMP moleucles are quite similar or that binding of the second cAMP molecule is very

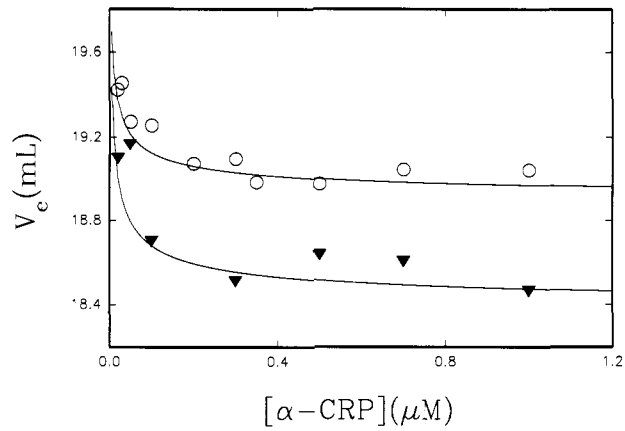

FIGURE 3: Elution volume of α-CRP as a function of protein concentration. The lines represent the best fit to eq 4 and 5 describing dissociation of α-CRP dimers to monomers. The symbols and identity of CRP fragments are the same as in Figure 2: (O) S-CRP; (▼) CH-CRP.

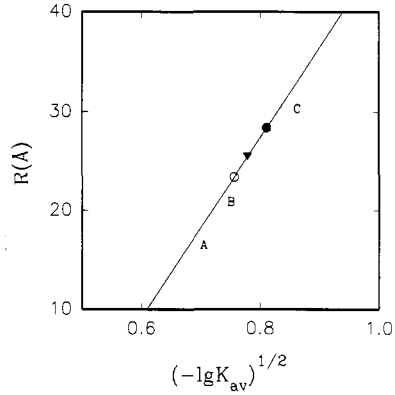

FIGURE 4: Stokes' radius as a function of partition coefficient for the column used in analytical gel chromatography experiments. The symbols and identity of protein are (A) ribonuclease, (B) chymotrypsinogen, and (C) ovalbumin. (O) CRP; (▼) CH-CRP; (●) S-CRP.

weak and essentially unmeasurable under the experimental conditions. Comparing fluorescence data with the equilibrium dialysis data suggests that the first explanation would apply to the S-CRP data. The fluorescence data indicate that $k_1 = k_2 = 7.4 \times 10^4$ M^{-1} (Table I). The second explanation would be applicable to the CH-CRP fluorescence data. The derived equilibrium constant of 84.5×10^4 M^{-1} should be assigned to k_1, and k_2 is very small. These results are in good agreement with those determined by equilibrium dialysis.

Monomer–Dimer Equilibrium. As the intersubunit contact is predominantly associated with the C-helix, it is useful to monitor the subunit assembly process of the proteolytic fragments of S-CRP and CH-CRP by large-zone analytical gel chromatography. The elution volumes of S-CRP and CH-CRP were monitored as a functon of protein concentration, and the results are shown in Figure 3. It is evident that the elution volume varies with protein concentration for both fragments. The elution volume increases with decreasing protein concentration; thus, these results imply the presence of a subunit assembly–disassembly process. Since the elution volume seems to reach a constant value at high protein concentration, it implies that a final stable species is formed. In order to identify the molecular weight of the stable species, the elution volumes of S-CRP and CH-CRP at high concentrations were expressed as partition coefficients, K_{av}, by eq 1. The Stokes' radii of S-CRP and CH-CRP were determined to be 23.4 and 25.5 Å, respectively, by the procedure of Sigel and Monty (1966), as shown in Figure 4. Such Stokes' radii

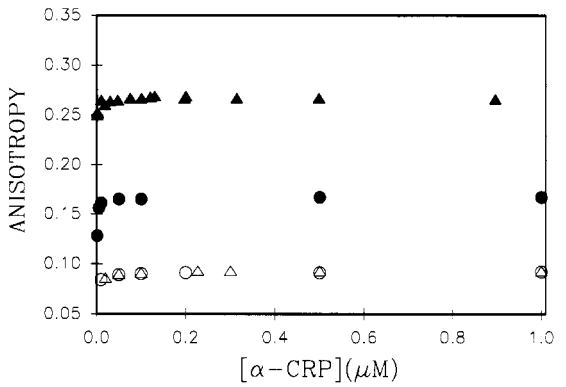

FIGURE 5: Fluorescence anisotropy as a function of protein concentration. Open symbols, tryptophan fluorescence; closed symbols, fluorescence of FITC-labeled proteins. The symbols and CRP fragments are the same as in Figure 2.

would correspond to globular proteins of molecular weights of 26 900 and 32 000, respectively. Since the subunit molecular weights of S-CRP and CH-CRP are 13 300 and 16 000, respectively, it can be concluded that the stable species at high concentrations are dimers of S-CRP and CH-CRP. This conclusion is in total agreement with the results from sedimentation equilibrium experiments for S-CRP [this laboratory and Anderson et al. (1971)].

The stabilities of S-CRP and CH-CRP can be estimated from the data of Figure 3. The data were fitted to the equation:

$$V_e = V_m + (V_d - V_m)[D]/P_{tot} \quad (4)$$

where V_e is the elution volume at a given protein concentration, V_m and V_d are the elution volumes of monomer and dimer, respectively, [D] is the concentration of dimer, and P_{tot} is the total protein concentration expressed in dimers. [D] can be determined by solving the equation:

$$P_{tot} = 1/2[M] + [D] = 1/2\sqrt{[D]/K_a} + [D] \quad (5)$$

where $K_a = [D]/[M]^2$.

The data were fitted for K_a and V_d with the values for V_m fixed since there are no data at sufficiently low protein concentration to allow fitting for V_m as well. Within a large range of V_m values, e.g., 20.5–23 mL, the values for the best fit for V_d are essentially the same (18.89 mL for S-CRP and 18.37 mL for CH-CRP), and K_a values vary only 3–4-fold. For both S-CRP and CH-CRP, the best-fit values for K_a are in the range of (1–4) × 10^8 M^{-1}, leading to a ΔG of about −11.3 kcal/mol.

The dissociation of CRP fragments can also be detected by fluorescence polarization measurements by monitoring the fluorescent intensity of natural fluorophores (Trp) or of the extrinsic probe FITC. Figure 5 shows that for both S-CRP and CH-CRP the anisotropy of polarization decreases with a decrease in the concentration. Interestingly, the values of the anisotropy of S-CRP and CH-CRP labeled with FITC are very different (see Figure 5), although the sizes of these two proteins are not very different. The simplest explanation would be that in CH-CRP there is an additional Lys residue (Lys-130) which may become a target for FITC modification, giving rise to the observed difference in polarization.

The effect of cAMP on subunit assembly–disassembly was monitored by column chromatography. Figure 6 shows the difference between the elution volumes of S-CRP and CH-CRP in the presence and absence of saturating cAMP concentrations as a function of protein concentration. For both fragments of CRP, the elution volume decreases in the presence of cAMP, indicating that cAMP induces an increase in the apparent molecular weight of CRP. By combining the observations that this difference in elution volumes is dependent on protein concentration, i.e., the difference is larger at low protein concentrations and that in the absence of cAMP both fragments dissociate to monomers at low protein concentrations, one may conclude that cAMP stabilizes the dimeric structure of both S-CRP and CH-CRP.

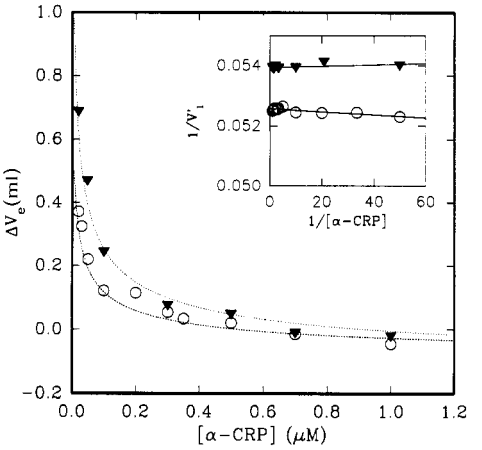

FIGURE 6: Difference in elution volume for α-CRP in the absence and presence of 200 μM cAMP (ΔV_e) as a function of protein concentration. The symbols and CRP fragments are the same as in Figure 2. Analytical gel chromatography experiments were performed as described in the text. The lines in the plot correspond to simulated data generated according to Scheme I as described in the text. The following parameters were used: for S-CRP, $K_a = 1 \times 10^8$ M^{-1}, $K_{DL} = 7 \times 10^4$ M^{-1}, $V_m = 20.5$ mL, $V_d = 18.89$ mL, and $V_{dl} = 19.00$ mL; for CH-CRP, $K_a = 1 \times 10^8$ M^{-1}, $K_{DL} = 1 \times 10^6$ M^{-1}, $V_m = 20.5$ mL, $V_d = 18.37$ mL, and $V_{dl} = 18.51$ mL. Inset: Extrapolation of V_l' to infinite protein concentration.

Discussion

Stability of α-CRP Dimers. Brown and Crothers (1989) reported that the free energy of association of CRP dimers is around −14.3 kcal/mol. The ΔG of dimerization of S-CRP and CH-CRP estimated in this study is about −11.3 kcal/mol. Therefore, the stability of both S-CRP and CH-CRP is reduced by about 3 kcal/mol as compared to intact CRP. The crystal structure of the CRP–$(cAMP)_2$ complex shows that all intersubunit contacts between CRP subunits are accomplished by residues from the N-terminal domains and most of them involve interaction between the two long C-helices. Altering the number of residues in the C-helix as occurs in α-CRP is, therefore, expected to influence the stability of dimer formation. A majority of the C-helix was removed in S-CRP. On the other hand, CH-CRP possesses essentially the whole C-helix, and, therefore, all the intersubunit interactions observed in intact CRP protein can in principle occur in CH-CRP, as well. On the basis of the difference in the number of residues remaining in the C-helix, one may expect that CH-CRP should exhibit a K_a value similar to that of intact CRP while S-CRP should dissociate into monomers more easily. However, the data show that the stability of CH-CRP is of the same order as that observed for S-CRP. This surprising observation may indicate that the C- and N-terminal domains of CRP are not independent and removal of the C-terminal domain affects the structure of the N-terminal domain. This in turn may affect subunit–subunit interactions. These structural changes must be quite subtle since the NMR study indicates that the structure of the N-terminal domain in α-CRP is not dramatically changed (Clore & Gronenborn, 1982).

Results shown in Figure 6 indicate that cAMP binding enhances the stability of both S-CRP and CH-CRP dimers. The simplest mechanism of this stabilization would be to assume that cAMP can bind exclusively to dimers but not to monomers of α-CRP, as shown in Scheme I.

Scheme I

$$2M \overset{K_a}{\rightleftharpoons} D + L \overset{K_{DL}}{\rightleftharpoons} DL$$

According to this scheme, the elution volume in the presence of cAMP (V_{el}) can be calculated as

$$V_{el} = V_m + (V_m - V_d)[D]/P_{tot} + (V_m - V_{dl})[DL]/P_{tot} \quad (6)$$

where V_{dl} and [DL] are the elution volume and the concentration of a dimer–cAMP complex, respectively. [D] can be calculated by solving the equation:

$$P_{tot} = 1/2[M] + [D] + [DL] = 1/2\sqrt{[D]/K_a} + [D] + K_{DL}[D][cAMP] \quad (7)$$

It is, therefore, possible to calculate the theoretical dependence of $V_e - V_{el}$ on protein concentration (lines in Figure 6) and compare them to experimental results (data points in Figure 6), using the assumption presented in Scheme I and values of K_a, K_{DL}, V_m, V_d, and V_{dl} obtained as discussed above. As can be seen, the theoretical lines correspond quite well in the experimental results. Hence, it can be concluded that Scheme I is sufficient to explain the observed effect of stabilization of α-CRP dimers by cAMP. This conclusion is consistent with the report that cAMP stabilizes dimers of intact CRP (Brown & Crothers, 1989).

cAMP-Induced Conformational Changes in S-CRP and CH-CRP. A number of techniques show that CRP in solution exists in two cAMP-dependent conformational states which correspond to the singly and fully liganded protein (Heyduk & Lee, 1989; Tan et al., 1991). Upon formation of the CRP–cAMP complex, native CRP has been shown by analytical gel chromatography to undergo a very small contraction of the protein, whereas a much more pronounced contraction is associated with the formation of CRP–$(cAMP)_2$ (Heyduk et al., 1992). These results are consistent with the conclusion deduced from a spectroscopic study (Lee et al., 1991).

It is of interest to obtain information regarding the influence of cAMP binding on the hydrodynamic properties of the CRP fragments. An attempt was made to estimate the differences in the elution volume of dimeric S-CRP and CH-CRP in the absence and presence of 200 μM cAMP. Since both CRP fragments dissociate to monomers at low protein concentrations, this comparison has to be performed under conditions where both proteins are 100% dimeric. Therefore, the information on the cAMP-induced conformational change was estimated from elution volumes of α-CRP in the absence and presence of cAMP at infinite protein concentration. The elution volumes of the unliganded S-CRP and CH-CRP, V', were obtained as mentioned above from the data of Figure 3 and using eq 4. The elution volume of the α-CRP–cAMP complexes at a given protein concentration were then tabulated as $V' - \Delta V_e = V_l'$ as a function of protein concentration. The values of ΔV_e were taken from the data shown in Figure 6. The elution volumes of dimeric S-CRP–cAMP and CH-CRP–cAMP complexes were estimated from double-reciprocal plots of $1/V_l'$ vs $1/[\alpha\text{-CRP}]$, as shown in the inset of Figure 6. The values of V_{dl} for S-CRP and CH-CRP are 19.00 and 18.51 mL, respectively. Since the values of V_d for S-CRP and CH-CRP are 18.89 and 18.37 mL, respectively, this analysis showed that for both CRP fragments the values of V_{dl} are greater than V_d. These results imply that S-CRP and CH-CRP undergo contraction upon binding of cAMP, a structural change that is also observed in native CRP. The difference in elution volumes was estimated to be 0.11 and 0.14 mL for S-CRP and CH-CRP, respectively, with an error of about ±0.06 mL. The magnitude of difference corresponds to about a 0.5-Å change in Stokes' radius. Therefore, in addition to inducing stability of a dimeric structure of S-CRP and CH-CRP, cAMP binding induces a conformational change in both proteins such that the molecules become more compact. Similar conclusions were presented by Eilen and Krakow (1977).

Cooperativity of cAMP Binding. S-CRP seems to bind cAMP in an essentially noncooperative mode. The affinity of cAMP to S-CRP is almost the same as that for the binding of the first cAMP molecule to CRP. This is quite surprising since S-CRP lacks several residues (Thr-127, Ser-128) which were indicated by the crystallographic structure of CRP–$(cAMP)_2$ to be a part of the cAMP binding site. One possible explanation of this would be that a part of the energy of cAMP binding to CRP is consumed to change the conformation in the C-terminal domains. Since S-CRP does not have these domains, this unfavorable term in the apparent ΔG for cAMP binding to S-CRP disappears. This could, therefore, offset the loss of binding energy due to the absence of some residues in the cAMP binding site. This would be consistent with the observation that in CH-CRP, the binding of the single cAMP molecule is very tight. The interaction of cAMP with CH-CRP is more favorable by 1.7 kcal/mol than that with intact CRP. CH-CRP contains all of the residues indicated by the crystal structure as part of a cAMP binding site in CRP. Therefore, this tight binding would reflect the fact that in this protein the cAMP binding site is fully functional and no energy is spent for a cAMP-induced conformational change in the C-terminal domains. This interpretation is consistent with the observations that minimal changes in hydrodynamic and spectroscopic properties have been detected (this study; Clore & Gronenborn, 1982).

S-CRP does not show any significant cooperativity in cAMP binding. Again, that points out the importance of the Thr-127 and Ser-128 residues (and possibly other residues that span the region of amino acids 117–134), which are absent in S-CRP, in the intersubunit communication in CRP. Interestingly, residues from one subunit have been proposed to interact with cAMP from another subunit (Weber & Steitz, 1987). This could be the way of accomplishing intersubunit communication. Site-directed mutagenesis experiments indicated that these residues are not essential for cAMP binding (Gronenborn et al., 1988) but they still can play a role in communication between subunits in CRP.

CH-CRP seems to bind only one cAMP molecule per protein dimer. This may be due to a high degree of negative cooperativity or to the fact that, for some reason, only one binding site is functional in this protein. Since binding of cAMP to intact CRP exhibits negative cooperativity, the former explanation is favored. This would be consistent with the suggestion that residues between 117 and 137 in CRP are important for intersubunit communication.

Registry No. cAMP, 60-92-4.

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Reaction of O^6-Alkylguanine-DNA Alkyltransferase with O^6-Methylguanine Analogues: Evidence That the Oxygen of O^6-Methylguanine Is Protonated by the Protein To Effect Methyl Transfer†

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ABSTRACT: The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) repairs the promutagenic O^6-methylguanine lesion by transferring the methyl group to a cysteine residue on the protein. A mechanism in which AGT activates the guanyl moiety as a leaving group by protonation of a heteroatom on guanine was probed by reacting AGT with analogues of O^6-methylguanine in which the heteroatoms were changed. The initial rates of reaction were measured at various substrate concentrations in 50 mM Hepes, 1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.8 at 37 °C. The k_{inact} (h^{-1}) and K_{in} (mM) were determined for O^6-methylguanine (1.66 ± 0.19, 1.51 ± 0.32), 6-methoxypurine (1.07 ± 0.25, 10.6 ± 4.2), S^6-methyl-6-thioguanine (0.63 ± 0.04, 1.17 ± 0.18), 6-methylthiopurine (no reaction), Se^6-methyl-6-selenoguanine (1.76 ± 0.28, 10.6 ± 5.0), 6-methylselenopurine (2.51 ± 0.62, 15.7 ± 6.3), O^6-methyl-1-deazaguanine (1.71 ± 0.34, 14.8 ± 4.4), O^6-methyl-3-deazaguanine (1.90 ± 0.24, 2.54 ± 0.59), and O^6-methyl-7-deazaguanine (1.97 ± 0.26, 2.56 ± 0.72). These results indicate that replacement of the nitrogens does not affect the k_{inact} parameter but the K_{in} is increased upon removal of the exocyclic amino group and the nitrogen at the 1-position. Replacement of the oxygen with sulfur decreases the k_{inact}, and replacement with selenium increases the K_{in}. The results are consistent with a mechanism in which O^6-methylguanine binds to the active site of AGT with hydrogen bonds to the oxygen, the exocyclic amino group, and the nitrogen at the 1-position of the substrate. The methyl group is then displaced from the guanine as a proton is transferred to the oxygen, neutralizing the charge on the leaving group.

O^6-Alkylguanine lesions are generally believed to be important in the initiation of carcinogenesis (Pegg, 1984). Mammals and bacteria have proteins, O^6-alkylguanine-DNA alkyltransferases (EC 2.1.1.63) (AGT),[1] which repair these lesions (Pegg, 1990; Demple, 1990). AGT repairs the O^6-methylguanine lesion by transferring the methyl group from the DNA to a cysteine residue on the protein (Olsson & Lindahl, 1980; Foote et al., 1980; Demple et al., 1985; Ryberg et al., 1990). This action restores the DNA, but since the free

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; S^6mGua, S^6-methyl-6-thioguanine; Se^6mGua, Se^6-methyl-6-selenoguanine.

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