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# *Escherichia coli* cAMP Receptor Protein: Evidence for Three Protein Conformational States with Different Promoter Binding Affinities†

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ABSTRACT: Cyclic AMP receptor protein (CRP) from *Escherichia coli* is assumed to exist in two states, namely, those represented by the free protein and that of the ligand–protein complex. To establish a *quantitative* structure–function relation between cAMP binding and the cAMP-induced conformational changes in the receptor, protein conformational change was quantitated as a function of cAMP concentration up to 10 mM. The protein conformation was monitored by four different methods at pH 7.8 and 23 °C, namely, rate of proteolytic digestion by subtilisin, rate of chemical modification of Cys-178, tryptophan fluorescence, and fluorescence of the extrinsic fluorescence probe 8-anilino-1-naphthalenesulfonic acid (ANS). Each of these techniques reveals a biphasic dependence of protein conformation on cAMP concentration. At low cAMP concentrations ranging from 0 to 200 $\mu$M, the rates of proteolytic digestion and that of Cys-178 modification increase, whereas the fluorescence intensity of the ANS–protein complex is quenched, and there is no change in the fluorescence intensity of the tryptophan residues in the protein. At higher cAMP concentrations, the rates of proteolytic and chemical modification of the protein *decrease*, while the fluorescence intensity of the ANS–protein complex is further quenched but there is an *increase* in the intensity of tryptophan fluorescence. These results show unequivocally that there are at least three conformational states of the protein. The association constants for the formation of CRP–cAMP and CRP–$(cAMP)_2$ complexes derived from conformational studies are in good agreement with those determined by equilibrium dialysis, nonequilibrium dialysis, and ultrafiltration. Therefore, the simplest explanation would be that the protein exhibits three conformational states, free CRP and two cAMP-dependent states, which correspond to the CRP–cAMP and CRP–$(cAMP)_2$ complexes. The binding properties of CRP–cAMP and CRP–$(cAMP)_2$ to the *lac* promoter were studied by using the gel retardation technique. At a high concentration of cAMP which favors the formation of the CRP–$(cAMP)_2$ complex, binding of the protein to DNA is decreased. This, together with conformational data, strongly suggests that only the CRP–cAMP complex is active in specific DNA binding whereas CRP and CRP–$(cAMP)_2$ are not.

Cyclic AMP receptor protein (CRP)[1] from *Escherichia coli* plays a key role in the regulation of expression of more than 20 genes in bacteria. The basic mechanism involves binding of CRP to the promoter regions of these genes. The formation of these CRP–DNA complexes is allosterically regulated by cAMP. There is ample evidence to indicate that binding of

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[1] Abbreviations: CRP, cyclic AMP receptor protein; ANS, 8-anilino-1-naphthalenesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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cAMP to CRP is accompanied by conformational changes and induces binding of CRP–cAMP complex to specific DNA sequences near promoters, resulting in the enhancement of transcription (de Crombrugghe et al., 1984; Pastan & Adhya, 1976; Adhya & Garges, 1982; Ullman & Danchin, 1983). The crystallographic structure of the cAMP–CRP complex shows that CRP is a dimer composed of two identical subunits, each of which has two well-defined domains (McKay & Steitz, 1981; McKay et al., 1982; Weber & Steitz, 1987). In conjunction with chemical studies, the smaller C-terminal domain is proposed to be responsible for binding of the protein to DNA (McKay & Steitz, 1981; McKay et al., 1982; Weber & Steitz, 1987; Eilen et al., 1978). It possesses a helix–turn–helix motif subsequently shown to be present in many bacterial DNA binding proteins such as Cro (Steitz et al., 1982) or λ cI repressor (Pabo & Lewis, 1982). The N-terminal domain contains a single binding site for cAMP (McKay & Steitz, 1981; McKay et al., 1982; Weber & Steitz, 1987); thus, the dimeric CRP can bind two cAMP molecules (Takahashi et al., 1980).

Conformational changes of CRP induced by cAMP binding have been studied in some detail. It was shown that in the presence of a micromolar concentration of cAMP, formation of an intersubunit disulfide bridge between Cys-178 of both subunits can be detected (Eilen & Krakow, 1977a). In addition, free protein is relatively resistant to proteolytic digestion by a number of proteases including subtilisin, trypsin, chymotrypsin, and *Staphylococcus aureus* V8 protease. In the presence of a micromolar concentration of cAMP, CRP is readily digested, producing a protease-resistant core, α-CRP (Eilen et al., 1978; Krakow & Pastan, 1973; Ebright et al., 1985), which was shown to be a dimer of the N-terminal domains of the protein (Eilen et al., 1978; Tsugita et al., 1982). None of these changes could be detected in the presence of cGMP (Eilen & Krakow, 1977a; Krakow & Pastan, 1973), which is known to bind to CRP with an affinity comparable to that of cAMP (Takahashi et al., 1980) but does not induce binding of the protein to DNA (Majors, 1975; Fried & Crothers, 1984). The induced formation of an intersubunit disulfide bridge and the increased rate of proteolytic digestion in the presence of cAMP were, therefore, thought to be related to a functionally important conformational change in the protein, although, as shown by Ebright et al. (1985), there is a class of cAMP analogues which is capable of inducing the same structural changes without an effect on the binding of the protein to DNA. Hence, at present, there is no *quantitative* correlation to link protein conformational changes to cAMP ligation and DNA–protein interaction.

In addition to chemical studies to elucidate the conformational states of CRP, physical techniques were employed to monitor the hydrodynamic properties of CRP. Using neutron scattering, Kumar et al. (1980) demonstrated that CRP undergoes a significant global structural change in the presence of 100 μM cAMP. The radius of gyration is decreased, thus implying that CRP assumes a more symmetric or compact structure in the presence of cAMP. The neutron scattering data are in good agreement with the results by Wu et al. (1974), who showed that cAMP induces a decrease in the rotational correlation time of CRP as detected in their fluorescence depolarization studies.

How is this structural information related to the functional state of CRP? It became customary in recent years to treat CRP as being able to exist in only two states: free protein, which cannot bind to specific DNA sequences, and the cAMP–CRP complex, which binds to specific DNA sequences and induces enhancement of transcription. Since the elucidation of the mechanism of CRP relies on an establishment of linkage between conformational change, cAMP binding to CRP, and the binding of CRP to DNA, a study has been initiated to provide information on this linkage.

## Experimental Procedures

### *Materials*

*Eco*RI, *Bam*HI, polynucleotide kinase, cAMP, and cGMP were purchased from Boehringer-Mannheim while subtilisin (protease type XXVII), Tris, and DTNB were from Sigma. Bio-Rex 70 and hydroxyapatite were obtained from Bio-Rad, and DEAE-cellulose was from Whatman. [$^3$H]cAMP of specific activity around 50 Ci/mol and [$\gamma$-$^{32}$P]ATP were products of Amersham. ANS (Kodak Laboratories) was purified on Sephadex LH-20 (Pharmacia) as described by York et al. (1978) before use. CRP was purified from *E. coli* strain overproducing for CRP containing plasmid pPLcCRP1 (Gronenborn & Clore, 1986) (a kind gift of A. Gronenborn) essentially as described by Boone and Wilcox (1978) with two minor modifications. First, the crude homogenate was subjected to differential ammonium sulfate precipitation. Proteins precipitated by 43–59% ammonium sulfate saturation were desalted on a G-25 column and subsequently applied to a Bio-Rex 70 column, in place of the original phosphocellulose column. Second, the hydroxyapatite column was eluted with a gradient of 0.1–0.7 M KCl in 50 mM phosphate, 1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.5, buffer instead of the phosphate gradient used originally. The KCl gradient gives better resolution as can be expected for a basic protein (Gorbunoff, 1984) [pI of CRP is 9.12 (Anderson et al., 1971)]. The purified protein was at least 98% pure as judged by scannings of SDS–PAGE gels.

### *Methods*

All experiments (with the exception of proteolytic digestion) were conducted in 50 mM Tris, 0.1 M KCl, and 1 mM EDTA at pH 7.8 and 23 °C. For spectrophotometric determination of concentrations, the following absorption 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 (Fergusson et al., 1975), and 20 400 $M^{-1}$ $cm^{-1}$ at 278 nm for CRP monomer (Takahashi et al., 1980).

Before use, CRP samples were thawed, dialyzed against 50 mM Tris, 0.1 M KCl, and 1 mM EDTA, pH 7.8, and filtered through microporous filters (0.45 mm, Millipore) to remove any insoluble material.

*Subtilisin Digestion.* Proteolytic digestion of CRP was carried out in the Tris buffer with 0.1 M NaCl in place of KCl so as to avoid SDS precipitation by KCl during SDS–PAGE experiments. Control experiments showed that substitution of KCl by NaCl does not change the rate or pattern of proteolytic modification of CRP by subtilisin. The reaction mixtures (500 μL) contained 5.6 μM CRP and the specified concentration of cAMP. The reaction was started by the addition of 4 μL of a 0.19 mg/mL subtilisin solution. At various time points, 30-μL samples were withdrawn. To those samples was added 5 μL of 20 mM PMSF in 50% DMSO to stop the reaction. After 2 min, 15 μL of sample buffer (0.062 M Tris, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) was added. The samples were boiled for 5 min and then were kept frozen until further analysis on a 15% SDS–PAGE slab gel. The gels were run for 1 h at 175 V in a Mini Protean II electrophoresis system (Bio-Rad) and were stained with Coomassie Blue G-200. To quantitate the amount of proteolysis products, the gels were scanned with

an LKB laser densitometer. The apparent rates of proteolysis were calculated by plotting log (100% − percent of protein remaining) vs time, and slopes of these plots were used to represent the apparent rate of proteolysis.

*Kinetics of Cys-178 Modification.* The changes in reactivity of Cys-178 in response to cAMP binding were studied by monitoring the rate of modification of the protein by DTNB. The reactions were carried out in a Durrum D-110 stopped-flow spectrophotometer at 25 °C. To start the reaction, equal volumes of DTNB (3 mM) and CRP (1.8–2.0 μM) solutions were mixed, and the changes in absorbance at 412 nm were monitored as a function of time. The signal from the stopped-flow spectrophotometer was recorded with a Bascom-Turner recorder and later transferred to an IBM PC microcomputer for data analysis. Typically, for each curve, 500 data points were collected, and for each cAMP concentration, 5–10 reaction curves were recorded. The rate constants for the reaction were obtained from nonlinear fitting of the experimental data to the equation for the pseudo-first-order reaction.

*ANS–CRP Fluorescence.* All fluorescence measurements were performed with a Perkin-Elmer 512 double-beam spectrofluorometer. The dissociation constant of the ANS–CRP complex and the number of binding sites for ANS were obtained by using the procedure outlined by Horowitz and Criscimagna (1985). Briefly, when CRP was titrated with an excess amount of ANS, the relation between various parameters is

$$1/F = 1/n\psi[\mathrm{CRP}]_0 + (K/n[\mathrm{CRP}]_0\psi)(1/[\mathrm{ANS}]_0) \quad (1)$$

where $F$ is the observed fluorescence intensity; [ANS] and $[\mathrm{ANS}]_0$ are the free and total concentrations of ANS, respectively, and since the concentrations of ANS were in much excess, $[\mathrm{ANS}] \simeq [\mathrm{ANS}]_0$; $\psi$ is the proportionality constant relating the fluorescence intensity to the concentration of the ANS–CRP complex; $n$ is the number of binding sites of ANS; $K$ is the dissociation constant of the complex; and $[\mathrm{CRP}]_0$ is the total concentration of CRP. The plots of $1/F$ vs $1/[\mathrm{ANS}]$ for different protein concentrations should be linear and should have a common abscissa intercept of $-1/K$.

When solutions of fixed concentrations of ANS were titrated with an excess amount of CRP, then

$$1/F = 1/\psi[\mathrm{ANS}]_0 + (K/\psi n[\mathrm{ANS}]_0)(1/[\mathrm{CRP}]_0) \quad (2)$$

In this case, the plots of $1/F$ vs $1/[\mathrm{CRP}]_0$ for different ANS concentrations should have a common abscissa intercept of $-n/K$. By the combination of eq 1 and 2, it is possible to extract values for both $n$ and $K$. In all cases, the signal was corrected for the fluorescence of free ANS in buffer.

Binding of cAMP to the ANS–CRP complex was studied by sequential addition of 1–2 μL of a concentrated cAMP solution to 220 μL of CRP solution (4.4–12 μM) which contained ANS (10–20 μM). The excitation and emission wavelengths were 350 and 480 nm, respectively. The data were corrected for the fluorescence of free ANS by performing analogous titrations with cAMP of the samples containing ANS alone without CRP.

*Tryptophan Fluorescence.* Binding of cAMP to CRP was also monitored by the tryptophan fluorescence. The changes in the intensity of the tryptophan fluorescence of CRP were monitored by titrating 220 μL of CRP solution (2.2–13.3 μM) with 1–2-μL aliquots of a concentrated cAMP solution. Fluorescence emission was monitored at 343 nm. To diminish the inner filter effect caused by cAMP, the excitation wavelength was set at 300 nm. The data were corrected for protein dilution. To correct for the inner filter effect, ovalbumin which does not bind cAMP was titrated with cAMP under identical conditions. The decrease in fluorescence intensity of this protein (after correction for dilution) was used to correct the data for CRP. This correction amounted to about 10% of the fluorescence intensity at the highest cAMP concentration employed in this study.

*Data Analysis for the Structural Studies.* CRP dimer possesses two cAMP binding sites (Takahashi et al., 1980). Hence, in the presence of cAMP in solution, there should be an equilibrium mixture of three species: free CRP, CRP–cAMP, and CRP–$(\mathrm{cAMP})_2$. In this study, each of the examined properties displays a biphasic dependence on cAMP concentration. Therefore, the data were analyzed by assuming that, for each of the protein properties tested, each of those three species exhibits its own specific value of that property (i.e., specific rates of Cys-178 modification, proteolysis, and fluorescence intensities of the complex with ANS and that of tryptophan). Such an assumption results in the expression for the observed property:

$$\Delta P_{\mathrm{obs}} = \Delta P_1 V_1 + \Delta P_2 V_2 \quad (3)$$

where $\Delta P_{\mathrm{obs}}$ is the measured value of property, $\Delta P_1$ and $\Delta P_2$ are normalized specific values of the change in property $P$ on going from free protein to CRP–cAMP and CRP–$(\mathrm{cAMP})_2$, respectively, and $V_1$ and $V_2$ are molar fractions of corresponding CRP–cAMP complexes. Equation 3 can be rewritten as

$$\Delta P_{\mathrm{obs}} = \frac{\Delta P_1 K_1[\mathrm{cAMP}] + \Delta P_2 K_2[\mathrm{cAMP}]^2}{1 + K_1[\mathrm{cAMP}] + K_2[\mathrm{cAMP}]^2} \quad (4)$$

where $K_1$ and $K_2$ are Adair constants of formation of CRP–cAMP and CRP–$(\mathrm{cAMP})_2$, respectively, in accordance with

$$\begin{aligned} \mathrm{CRP} + \mathrm{cAMP} &\overset{K_1}{\rightleftharpoons} \mathrm{CRP\text{–}cAMP} \\ \mathrm{CRP} + 2\mathrm{cAMP} &\overset{K_2}{\rightleftharpoons} \mathrm{CRP\text{–}(cAMP)_2} \end{aligned} \quad (5)$$

where

$$K_1 = [\mathrm{cAMP}][\mathrm{CRP}]/[\mathrm{CRP\text{–}cAMP}]$$

$$K_2 = [\mathrm{cAMP}]^2[\mathrm{CRP}]/[\mathrm{CRP\text{–}cAMP_2}]$$

All the data were then fitted to eq 4 by using the nonlinear regression procedure based on the Marquardt algorithm. In eq 4, [cAMP] corresponds to the free ligand concentration. For fittings, only data at sufficiently high cAMP concentrations were used so that the assumption of $[\mathrm{cAMP}]_{\mathrm{free}} = [\mathrm{cAMP}]_{\mathrm{total}}$ is valid.

*Error Analysis.* The estimation of errors of fitted parameters was performed essentially as described by Brodersen et al. (1987). The parameters representing the best fit were modified in random manner to generate 30–200 sets of parameters. These sets of parameters yield acceptable fits with a larger sum of squared deviations than the best fit. However, all of these parameters are acceptable within a probability limit of 0.90, as judged by the $F$ test (Sprague et al., 1980). Minimum and maximum values of parameters in these acceptable sets were taken to represent the error of a given parameter.

*[$^3H$]cAMP Binding Experiments.* Binding of cAMP to CRP was measured by three methods in 50 mM Tris, 0.1 M KCl, and 1 mM EDTA at pH 7.8 and 23 °C. The methods include the following:

(*A*) *Equilibrium Dialysis.* The experiments were performed by using simple dialysis cells constructed in our laboratory. A Spectrapor 1 membrane with a molecular weight cutoff of 6000–8000 was used. The membrane was boiled in EDTA

solution, rinsed with deionized water, and soaked for at least 2 h in buffer before use; 90–100 μL of CRP solution at 11.1–45.0 μM was placed in one compartment of the cell, and 100–400 μL of [$^3$H]cAMP solution was placed in the other compartment. Dialysis was conducted for 14–18 h at room temperature with continuous shaking. Control experiments were performed to assure that the time of dialysis was sufficient for the system to reach equilibrium. Fifty-microliter samples of dialysates from both compartments were withdrawn, then mixed with 10 mL of Aquasol-2 (NEN), and counted in a Beckman LS 1701 scintillation counter. Concentrations of cAMP in the protein and ligand compartments were calculated by using the values of specific activities determined for each of the cAMP solutions used.

(*B*) *Nonequilibrium Dialysis.* The basic procedure and the dialysis cell used were essentially the same as described by Colowick and Womack (1969). Spectrapor 3 (molecular weight cutoff of 3500) or Spectrapor 1 (molecular weight cutoff of 6000–8000) membranes were used. These membranes were treated before being used. The upper chamber of the cell was filled with 1.2–1.5 mL of protein solution at 0.6–2 mg/mL and radioactive cAMP at a specific concentration. The lower chamber was filled with buffer which flowed through the chamber at a constant rate of 2–4 mL/min. Fractions of effluent were collected. The actual number of tubes which has to be collected to reach a steady state is dependent on the membrane used and the flow rate of buffer through the lower chamber. Generally, about 7–10 fractions were collected. Having established that a steady state was reached, 2-μL aliquots of concentrated cold cAMP were added to the upper chamber to establish a new protein–ligand equilibrium. In each experiment, only six to nine additions of cold ligand were made so that the amount of radioactive ligand removed from the upper chamber during the experiment was less than 4% of the starting concentration. In the last addition of ligand, the concentration of cold cAMP was about 200 times the concentration of CRP. Under those conditions, essentially all ligand in the upper chamber was free. A 1-mL aliquot from each fraction was mixed with 10 mL of Aquasol-2 and counted. The data were corrected for dilution of protein in the upper chamber due to the addition of cold cAMP. The calculation of free and bound ligand concentrations was performed as in Colowick and Womack (1969), i.e.:

$$L_{free} = L_{total}(cpm/cpm_{max}) \quad (6)$$

$$L_{bound} = L_{total} - L_{free} \quad (7)$$

where $L_{free}$, $L_{bound}$, and $L_{total}$ correspond to the concentration of free, bound, and total ligand, respectively, cpm is the counts in fractions after establishing steady state at a certain ligand concentration, and $cpm_{max}$ is the counts in fractions when the concentration of cAMP is large as compared to CRP (i.e., $L_{total} = L_{free}$).

(*C*) *Ultrafiltration.* The procedure of Paulus (1969) was adopted. In this work, an Amicon MPS-1 micropartition system and YMT membranes were used. Five hundred microliters of CRP solution (6.7–8.9 μM) containing different concentrations of [$^3$H]cAMP of known specific activity was filtered by centrifugation of MPS-1 in a clinical centrifuge until the whole protein solution has passed through. Filters were then soaked in 200 μL of 0.1 M NaOH for about 15 min. Subsequently, the filters were rinsed consecutively with 100 μL of acetic acid and 700 μL of water. After 15 min, 10 mL of Aquasol-2 was added, and the mixture was counted. Counts of the filters treated in the same way but without CRP were used as background.

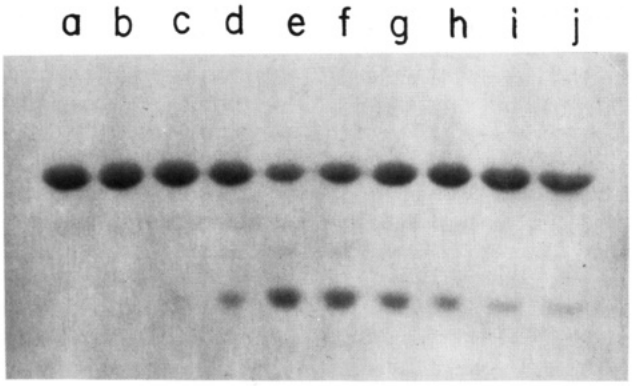



FIGURE 1: Proteolytic digestion of CRP by subtilisin at different cAMP concentrations. 20 μg of CRP was digested with 0.3 μg of subtilisin in a 30-μL volume for 100 min at 25 °C at different cAMP concentrations. Lane a and lanes b–j contained no protease and the specific amount of subtilisin, respectively. cAMP concentration: b, 0; c, 5 μM; d, 20 μM; e, 200 μM; f, 2 mM; g, 5 mM; h, 10 mM; i, 27 mM; j, 48 mM.

*DNA Fragment Isolation and Labeling.* Plasmid pRZ4006 (a kind gift from W. Reznikoff) with a 203 bp fragment of wild-type *lac* promoter region (bp −140 to +63) was purified by CsCl banding (Maniatis et al., 1982). Purified plasmid was linearized with *Eco*RI, and the fragment was released with *Bam*HI. The final purification step involved elution of the fragment from a 5% polyacrylamide gel. The fragment was labeled by the polynucleotide kinase exchange reaction (Berkner & Folk, 1977) using [$\gamma$-$^{32}$P]ATP as described in Maniatis et al. (1982).

*Binding of CRP to DNA.* Binding of CRP to DNA was examined by using a gel retardation assay (Garner & Revzin, 1981; Fried & Crothers, 1984). Reaction mixtures (10 μL) contained $2 \times 10^{-8}$ M DNA fragment and various amounts of CRP, cAMP, and, in some experiments, poly[d(A-T)] in 50 mM Tris, 0.1 M KCl, and 1 mM EDTA, pH 7.8 (25 °C). After gentle mixing of all components, the samples were allowed to equilibrate for 30–45 min, and then 1.5 μL of 15% Ficoll with tracking dye was added. The samples were immediately loaded onto 7.5% polyacrylamide gels (7 × 8 cm). For each experiment, duplicate gels were run in a Mini Protean II electrophoresis system (Bio-Rad) at 150 V for about 35 min. Electrophoresis was carried out in TBE buffer (0.09 M Tris, 0.09 M borate, and 0.0025 M EDTA) supplemented with the same cAMP concentration as was used in the binding reaction. Before application of the samples, the gels were prerun for 20 min at 150 V. Following electrophoresis, gels were dried and subjected to autoradiography using Kodak X-OMAT AR5 film.

## RESULTS

*Sensitivity to Proteolytic Digestion.* Figure 1 shows the results of subtilisin digestion of CRP under different cAMP concentrations. Under the experimental conditions employed, there is no digestion of the protein in the absence of cAMP (Figure 1, lane b). When the cAMP concentration is raised from 0 to about 200 μM, the formation of α-CRP can be detected (Figure 1, lanes c–e) as was reported before (Eilen et al., 1978; Krakow & Pastan, 1973; Ebright et al., 1985). However, when the concentration of cAMP is further increased, a decreasing amount of α-CRP was produced (Figure 1, lanes f–j). The effect of cAMP on the activity of subtilisin was monitored by using the chromophoric substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. At 10 mM cAMP, the activity of subtilisin was 98 ± 7% of that in the absence of cAMP. Therefore, the changes in the rate of CRP digestion

Table I: Summary of Fitted Parameters

| technique | $K_1$[a] ($M^{-1}$) | $k_1$ | $\Delta P_1$ | $K_2$[a] ($M^{-1}$) | $k_2$ | $\Delta P_2$ |
|---|---|---|---|---|---|---|
| proteolysis | $2.9 \times 10^4$ [$(1.4, 6.7) \times 10^4$] | $1.5 \times 10^4$ | 1.48 (1.2, 2.0) | $1.8 \times 10^7$ [$(1.0, 3.6) \times 10^7$] | $1.2 \times 10^3$ | 0 (1.8, −4) |
| Cys-178 reactivity | $3.0 \times 10^4$ [$(1.2, 8.6) \times 10^4$] | $1.5 \times 10^4$ | 1.03 (0.8, 1.4) | $1.1 \times 10^7$ [$(0.4, 2.5) \times 10^3$] | $0.7 \times 10^3$ | −1.00 (−0.5, −4) |
| Trp fluorescence | nd[c] | | nd | $1.6 \times 10^7$[b] [$(1.1, 2.4) \times 10^7$] | $1.0 \times 10^3$ | 0.57 (0.5, 0.7) |
| ANS–CRP fluorescence | $4.6 \times 10^4$ [$(3.9, 5.7) \times 10^4$] | $2.3 \times 10^4$ | 0.61 (0.58, 0.64) | $1.2 \times 10^7$ [$(0.2, 1.6) \times 10^7$] | $0.5 \times 10^3$ | 0.92 (0.88, 0.99) |
| direct binding | $9.2 \times 10^4$ [$(5.8, 15 \times 10^4$] | $4.6 \times 10^4$ | | $7.6 \times 10^7$ [$(2.2, 15) \times 10^7$] | $1.7 \times 10^3$ | |

[a] Adair constnats. The values in brackets or parentheses are limits within the 90% confidence level. [b] Value obtained by assuming that $k_1$ is $1.7 \times 10^4$ $M^{-1}$ (a medium value of $k_1$ obtained by three other methods). [c] Not determined.

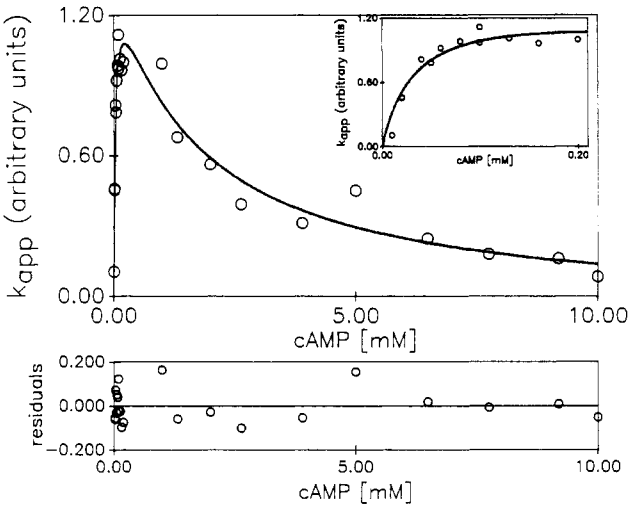



FIGURE 2: Rate of proteolytic digestion of CRP as a function of cAMP concentration. Rates were obtained as described under Experimental Procedures. The data correspond to four separate experiments, and the values of rate constants were normalized by assuming that at 200 $\mu$M cAMP the rate constant is 1. The solid line represents the best fit to the data as described in the text. Inset: Plot of the data in the range of low cAMP concentrations.

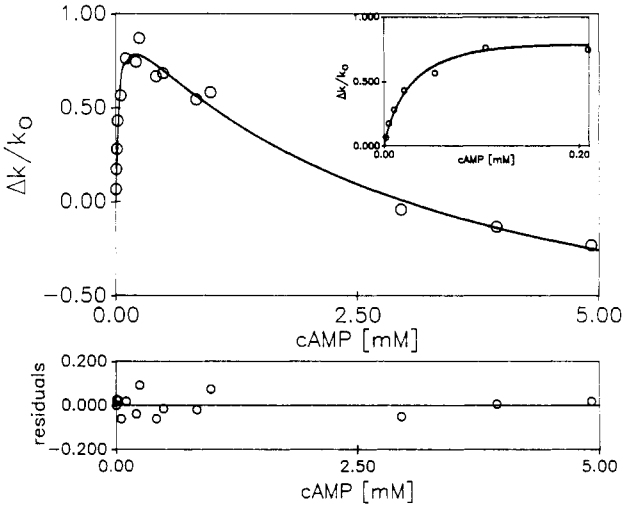



FIGURE 3: Rate of Cys-178 modification by DTNB as a function of cAMP concentration. Reactivity of Cys-178 is expressed as $\Delta k/k_0$ where $\Delta k$ is the difference in the rate of Cys-178 modification in the presence and absence of cAMP and $k_0$ is the rate constant for free protein. The solid line represents the best fit to the data as described in the text. Inset: Plot of the data in the range of low cAMP concentrations.

with subtilisin are reflecting the changes in protein conformation and not modulation of subtilisin activity by cAMP. To obtain a more quantitative measure of this effect, the rate of proteolysis was examined as a function of cAMP concentration. When these rates are plotted as a function of cAMP concentration (Figure 2), the biphasic nature of this relationship is apparent. At low cAMP concentrations (0 to about 200 $\mu$M), there is an increase in the rate (Figure 2, inset), whereas at cAMP concentrations of >1 mM the rate decreases. cGMP does not change the sensitivity of CRP to subtilisin digestion. The same pattern for the rates of digestion was obtained when trypsin was used. When the results were analyzed in accordance to eq 4, a good fit was obtained, yielding values of $2.9 \times 10^4$ $M^{-1}$ (90% confidence interval of $1.4 \times 10^4$ $M^{-1}$ and $6.7 \times 10^4$ $M^{-1}$) and $1.8 \times 10^7$ $M^{-2}$ (90% confidence interval of $1.0 \times 10^7$ $M^{-2}$ and $3.6 \times 10^7$ $M^{-2}$) for $K_1$ and $K_2$, respectively, as shown in Figure 2. In addition, the two ligation states show significant differences in digestion properties, as summarized in Table I.

*Cys-178 Reactivity.* Each subunit of CRP possesses three cysteine residues, two in the N-terminal domain (Cys-18, -92) and one (Cys-178) in the C-terminal domain (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982). Only Cys-178 can be chemically modified under native conditions while Cys-18 and Cys-92 seem to be buried (Ebright et al., 1985; Eilen & Krakow, 1977b). Cys-178 is located on the stretch of polypeptide connecting two helices in the helix–turn–helix motif of CRP (McKay & Steitz, 1981; McKay et al., 1982; Weber & Steitz, 1987), only three residues apart from Glu-181, which makes direct contacts with DNA (Ebright et al., 1984, 1987). Therefore, changes in the microenvironment of this residue upon binding of cAMP should reflect changes in the DNA binding domain in response to cAMP binding.

In this study, the microenvironment of Cys-178 was monitored by measuring the rate of Cys-178 reaction with DTNB (at high DTNB concentration, i.e., under pseudo-first-order conditions) as a function of cAMP concentration. Under these conditions, the number of sulfhydryl residues modified and the presence of intersubunit disulfide bridges were determined. CRP was modified with 1.5 mM DTNB in Tris buffer at pH 7.8. The modified protein was purified on a Sephadex G-25 column equilibrated with Bis-Tris buffer at pH 6.0, a pH which inhibits –SH ⇌ –SS– interchange reactions (Parker & Kharasch, 1959). Then the amount of TNB per mole of CRP was determined spectrophotometrically as described in Heyduk et al. (1986) or by measuring the amount of TNB released by DTT treatment of the modified protein. In both cases, 1.9 TNB/mol of CRP was obtained, and, therefore, no intersubunit disulfide bridges were formed in this case. When the same procedure was repeated in the presence of 100 $\mu$M cAMP, around 1.5 TNB/mol of CRP was detected. This indicates some disulfide formation, but the simple modification of Cys-178 by DTNB is still the predominant reaction.

In an attempt to fit the data to double-exponential kinetics, no convergence could be obtained or the fits converged to single-exponential solution. This result indicates that Cys-178 in each subunit does not reside in an intrinsically asymmetric environment. Since the kinetic data could be reasonably well fitted to simple pseudo-first-order kinetics, the apparent pseudo-first-order rate constants were chosen as a representation of Cys-178 reactivity.

The plot of the dependence of these rate constants on cAMP concentration is presented in Figure 3. As in the case of proteolysis data, the plot is clearly biphasic. At cAMP con-

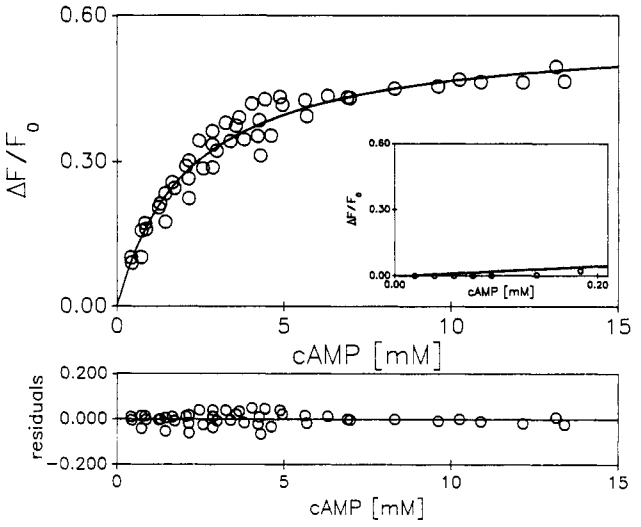



FIGURE 4: Fluorescence of CRP tryptophan residues as a function of cAMP concentration. The changes of fluorescence are expressed as $\Delta F/F_0$ where $\Delta F$ is the difference in fluorescence intensity observed in the presence and absence of cAMP and $F_0$ is the fluorescence intensity of free protein. The solid line represents the best fit of the data as described in text. Inset: Plot of the data in the range of low cAMP concentrations. The data presented correspond to four separate experiments performed with two different CRP preparations.

centrations in the range from 0 to about 200 $\mu$M, an increase in reactivity of Cys-178 is observed (Figure 3, inset), whereas at higher cAMP concentrations the cysteine reactivity decreases. Results from control experiments show that cGMP does not induce any significant changes in Cys-178 reactivity, even at millimolar concentration, and cAMP does not change the rate of reaction between DTNB and cysteine. Hence, the observed structural change in CRP is induced specifically by cAMP. The results shown in Figure 3 were analyzed in accordance to eq 4. It is evident that again two conformational states corresponding to the two ligation states of CRP can be resolved, as shown in Figure 3 by the solid curve. Values of $3 \times 10^4$ $M^{-1}$ and $1.1 \times 10^7$ $M^{-2}$ were determined for $K_1$ and $K_2$, respectively, in good agreement with those determined by proteolysis. Their respective 90% confidence intervals are $1.2 \times 10^4$ $M^{-1}$, $8.6 \times 10^4$ $M^{-1}$, and $0.4 \times 10^7$ $M^{-2}$, $2.5 \times 10^7$ $M^{-2}$. Again, the two ligation states exhibit different Cys-178 reactivity, as summarized in Table I.

*Tryptophan Fluorescence.* The effect of cAMP on tryptophan fluorescence was monitored. When CRP is titrated with cAMP, the fluorescence intensity of protein tryptophan residues increases (Figure 4). However, this increase can only be detected in the millimolar range of cAMP concentration. At micromolar cAMP concentrations, there is no detectable change in fluorescence intensity, (Figure 4, inset). Even in millimolar concentrations, cGMP does not affect significantly the fluorescence intensity of CRP. Since the data apparently only reflect the binding of the second cAMP molecule, the data were fitted for $K_2$ alone. A value of $1.6 \times 10^7$ $M^{-2}$ with a 90% confidence interval of $(1.1–2.4) \times 10^7$ $M^{-2}$ was obtained, as shown in Figure 4. This value is consistent with those determined by other measurements reported in this study.

*ANS–CRP Fluorescence.* In the presence of CRP, the fluorescence intensity of ANS increases dramatically with a concomitant shift of the wavelength for maximum emission from about 530 nm to 480 nm. Such changes are characteristic for formation of the protein–ANS complex (Stryer, 1965; Weber & Lawrence, 1954). Having demonstrated that ANS binds to CRP, a quantitative analysis of the binding of ANS to CRP was performed. Figure 5 shows the results of CRP titration with ANS and also of ANS titration with CRP. When the data were analyzed as described by Horowitz and Criscimagna (1985), it was determined that there are two ANS binding sites per CRP dimer with a dissociation constant of 600 $\mu$M. At present, the localization of the ANS binding sites is uncertain, although it was found that ANS also binds to $\alpha$-CRP (E. Heyduk and J. C. Lee, unpublished data). Hence, it is possible that the ANS binding sites are also located in the N-terminal domains in intact protein.

When the ANS–CRP complex is titrated with cAMP, a quenching of the fluorescence signal is observed, as shown in Figure 6. The pattern of quenching is apparently biphasic. The first phase occurs approximately in the range from 0 to about 200 $\mu$M cAMP, as shown in the inset of Figure 6, whereas the second phase occurs at millimolar concentrations of cAMP. This biphasic change could be a manifestation of a change in the interaction of ANS to CRP; hence, the binding of ANS to CRP was studied in the presence of cAMP.

In the presence of 200 $\mu$M cAMP, the dissociation constant of ANS–CRP remains roughly unchanged (about 500 $\mu$M), but the number of binding sites is reduced to about 1.2 (Figure 5C,D). This suggests that the first phase of fluorescence quenching of ANS–CRP in the presence of cAMP corresponds to the dissociation of one ANS molecule from the ANS–CRP complex. It is tempting to ascribe the second phase of quenching to the dissociation of the second ANS molecule. However, this is difficult to prove experimentally. At high cAMP concentrations, there is some residual fluorescence of CRP–ANS which may be due to a small amount of ANS bound to other sites in protein or to ANS bound to that second site but with greatly reduced quantum yield. It is impossible to discriminate between these various possibilities at present.

The results shown in Figure 6 were analyzed in accordance to eq 4 and resulted in values of $4.6 \times 10^4$ $M^{-1}$ and $1.2 \times 10^7$ $M^{-2}$ for $K_1$ and $K_2$, respectively. Their respective 90% confidence intervals are $(3.9–5.7) \times 10^4$ $M^{-1}$ and $(0.2–1.6) \times 10^7$ $M^{-2}$. *It is worthwhile to note that the values of $K_1$ and $K_2$, determined in this study, are in total agreement regardless of the specific method of monitoring*, although the formations of the CRP–cAMP and CRP–$(cAMP)_2$ complexes exhibit very different behaviors, as summarized in Table I.

*Binding of cAMP to CRP.* Figure 7 shows binding data obtained by using three methods: equilibrium dialysis, non-equilibrium dialysis, and ultrafiltration. The data presented in Figure 7 correspond to 15 independent binding experiments performed with 5 different preparations of CRP. To obtain Adair constants (Adair, 1925) for cAMP binding, the data were fitted to eq 8 where $N_s$ is the ratio of cAMP bound per

$$N_s = \frac{K_1[\text{cAMP}] + 2K_2[\text{cAMP}]^2}{1 + K_1[\text{cAMP}] + K_2[\text{cAMP}]^2} \quad (8)$$

mole of CRP dimers, [cAMP] is the concentration of free ligand, and $K_1$ and $K_2$ are Adair constants of formation of CRP–cAMP and CRP–$(cAMP)_2$ complexes, respectively, in accordance to eq 5. All three methods yield very similar results. Hence, the data were analyzed in toto. Equilibrium constants obtained from fitting the data to eq 8 are $9.2 \times 10^4$ $M^{-1}$ (90% confidence interval of $5.8 \times 10^4$ $M^{-1}$ and $15 \times 10^4$ $M^{-1}$) and $7.6 \times 10^7$ $M^{-2}$ (90% confidence interval of $2.2 \times 10^7$ $M^{-2}$ and $15 \times 10^7$ $M^{-2}$) for $K_1$ and $K_2$, respectively. As a consequence of the stronger binding of the first cAMP, $K_1$ can be determined to a higher degree of accuracy than $K_2$. For a better estimation of $K_2$, data at higher concentrations of cAMP and CRP should be obtained. This, however, could not be accomplished under the present experimental conditions.

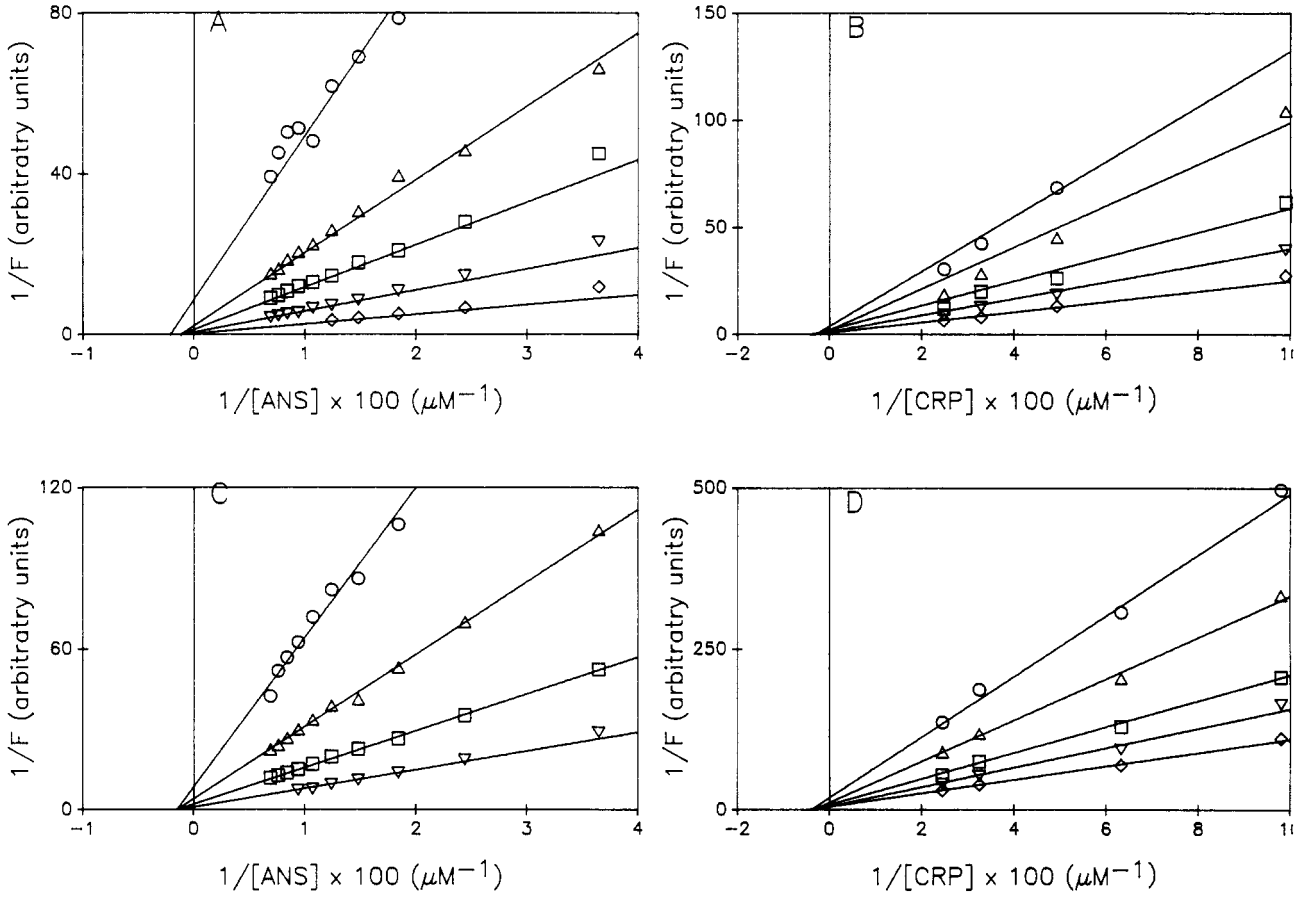



FIGURE 5: Characterization of the binding of ANS to CRP. (A and B) No cAMP; (C and D) 200 $\mu$M cAMP. (A) CRP concentration: (○) 0.74 $\mu$M; (△) 1.49 $\mu$M; (□) 2.79 $\mu$M; (▽) 5.02 $\mu$M; (◇) 9.8 $\mu$M. (B) ANS concentration: (○) 2.1 $\mu$M; (△) 3.2 $\mu$M; (□) 5.3 $\mu$M; (▽) 7.3 $\mu$M; (◇) 10.4 $\mu$M. (C) CRP concentration: (○) 1.49 $\mu$M; (△) 2.79 $\mu$M; (□) 5.02 $\mu$M; (▽) 9.85 $\mu$M. (D) ANS concentration: (○) 2.1 $\mu$M; (△) 3.2 $\mu$M; (□) 5.3 $\mu$M; (▽) 7.3 $\mu$M; (◇) 10.4 $\mu$M. Dissociation constants for the ANS–CRP complex and the number of binding sites for ANS were obtained from abscissa intercepts as described under Experimental Procedures.

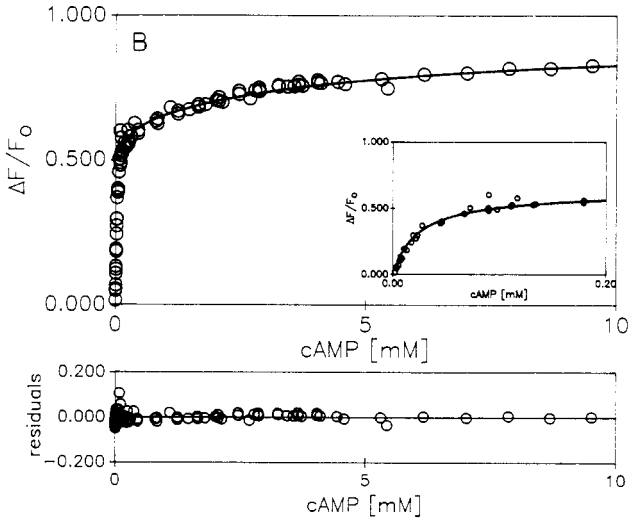



FIGURE 6: Fluorescence of the ANS–CRP complex as a function of cAMP concentration. The data are expressed as $\Delta F/F_0$ where $\Delta F$ ($F_0 - F$) is the difference in fluorescence between the ANS–CRP complex in the absence and presence of cAMP. The solid line represents the best fit to experimental data as described in the text. Inset: Plot of the data in the range of low cAMP concentrations. The data presented correspond to seven separate titrations performed with two different CRP preparations.

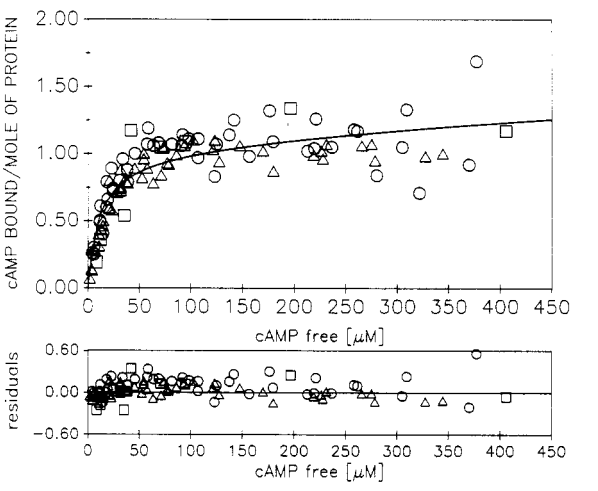



FIGURE 7: [$^3$H]cAMP binding to CRP. The data were obtained as described under Experimental Procedures. The methods used are (○) equilibrium dialysis, (△) nonequilibrium dialysis, and (□) ultrafiltration.

Concentrated solutions of CRP at high concentrations of cAMP have a tendency to precipitate out of solutions. Precipitation of CRP under similar conditions was reported by others (Fried, 1982; Kumar et al., 1980; Ghosaini et al., 1988). In order to evaluate the effect of CRP aggregation on the binding data, sedimentation velocity experiments were conducted as a function of CRP concentration in a Beckman Model E analytical ultracentrifuge. At CRP concentrations below 1 mg/mL, there was no indication of the presence of higher aggregates with or without 200 $\mu$M cAMP. These results are consistent with those reported by Takahashi et al. (1980) and Saxe and Revzin (1979). Having ruled out the presence of a significant amount of CRP aggregates, it is valid to analyze the cAMP binding data as simple binding isotherms

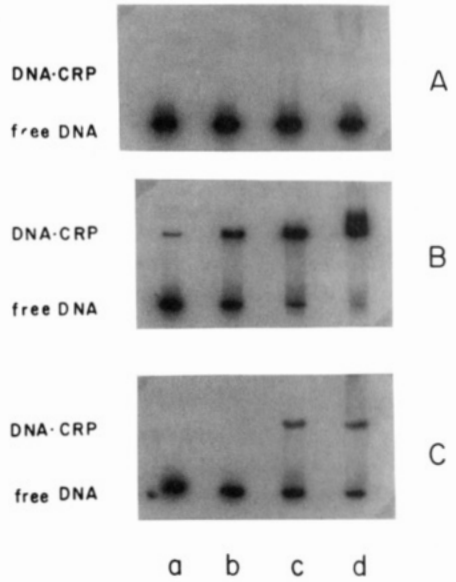



FIGURE 8: Binding of CRP to the 203 bp fragment of *lac* promoter DNA at low and high cAMP concentrations using the gel retardation assay. Conditions of the assay are described under Experimental Procedures. Lane a, $5 \times 10^{-8}$ M CRP; lane b, $2 \times 10^{-7}$ M CRP; lane c, $2.4 \times 10^{-7}$ M CRP; lane d, $9.6 \times 10^{-7}$ M CRP.

without linkage to protein aggregation. It is more meaningful to express binding constants as microscopic constants, and the relationship between Adair, $K_i$, and microscopic constants, $k_i$, for two cAMP binding sites in CRP is

$$K_1 = 2k_1 \qquad K_2 = k_1k_2 \tag{9}$$

This formulation assumes that both cAMP binding sites are initially identical. The values of microscopic constants are summarized in Table I. In addition, the values of $k_i$ determined by indirect methods are also tabulated. It is evident that the equilibrium constants of formation for CRP–cAMP and CRP–$(cAMP)_2$ complexes obtained in this work are in reasonable good agreement with the corresponding equilibrium constants estimated from the structural studies. Hence, these results demonstrate convincingly that CRP exhibits two cAMP-dependent conformations which are directly related to the occupancy of the first and second site in CRP by cAMP.

*Binding of CRP to DNA.* In order to investigate the effect of cAMP concentration on the binding of CRP to DNA, two types of experiments were performed. The binding of CRP to a specific DNA site was monitored. A 203 bp fragment of *lac* operon DNA was incubated with increasing amounts of CRP at either 100 $\mu$M or 10 mM cAMP, i.e., under the conditions where CRP–cAMP and CRP–$(cAMP)_2$ would be the prevailing species in solution, respectively. Figure 8 shows the results of these experiments. In the absence of cAMP, only free DNA fragment was detected, indicating that CRP alone does not bind to the *lac* operon, as shown in Figure 8A. In the presence of 100 $\mu$M cAMP, a significant amount of CRP–DNA complex can be detected even at the lowest concentration of CRP employed. With increasing amount of CRP, more complex can be detected with a concomitant decrease in free [DNA], as shown in Figure 8B. These results indicate that 100 $\mu$M cAMP mediates the formation of a CRP–DNA complex. At the higher concentration of cAMP of 10 mM, there is a significant decrease in the amount of complex formed, as shown in Figure 8C. These results clearly demonstrate that the formation of the CRP–DNA complex is cAMP concentration dependent, and, more importantly, the dependence is biphasic; i.e., complex formation is favored at an intermediate concentration of cAMP and disfavored at lower or higher concentrations of the ligand.

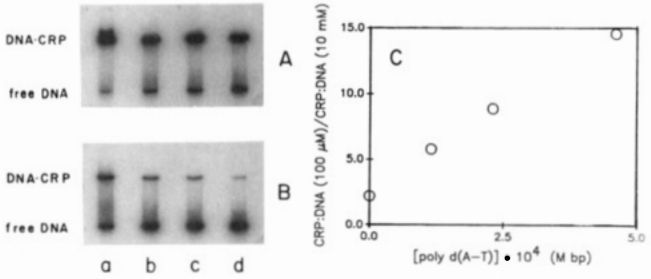



FIGURE 9: Dissociation of CRP–DNA complexes with nonspecific DNA. Conditions of the assay as described under Experimental Procedures. The concentrations of cAMP were (A) 100 $\mu$M and (B) 10 $\mu$M. CRP concentration in both (A) and (B) was $5.3 \times 10^{-7}$ M. Concentrations (in bp) of poly[d(A-T)] were as follows: lane a, 0; lane b, $1.15 \times 10^{-4}$ M; lane c, $2.3 \times 10^{-4}$ M; lane d, $4.6 \times 10^{-4}$ M. (C) Ratio of the amount of specific DNA–protein complexes formed at 100 $\mu$M cAMP and 10 mM cAMP as a function of poly[d(A-T)] concentration. The amount of complexes was determined by densitometric scanning of the autoradiogram.

The second type of experiment involved dissociation of preformed protein–DNA complex with increasing concentrations of nonspecific DNA, poly[d(A-T)], in either 100 $\mu$M or 10 mM cAMP. This experiment gives information about the relative affinity of the protein for specific and nonspecific DNA. At all concentrations of poly[d(A-T)] tested, there are less CRP–DNA complexes formed at 10 mM cAMP, as shown in Figure 9. The ratio of complexes formed at 100 $\mu$M and 10 mM cAMP is greater with increasing concentration of poly[d(A-T)]. These results indicated that at the higher concentration of cAMP the nonspecific DNA is much more efficient in competing with the *lac* promoter for CRP. Increased affinity of CRP for nonspecific DNA at high cAMP concentration was noted by Taniguchi et al. (1979).

Control experiments were performed to check if the decrease in DNA binding at 10 mM cAMP is related to a specific effect of a high concentration of cAMP. In those experiments, the binding of CRP to DNA was compared at 100 $\mu$M cAMP, with no additive, 10 mM AMP, 10 mM GMP, or 10 mM cAMP. Only in the presence of 10 mM cAMP, a significant decrease in the amount of CRP–DNA complex was detected; therefore, this effect is specifically associated with cAMP.

## Discussion

In the literature, the currently favored concept for CRP is that it exists in two conformational states, namely, an "inactive" and an "active" conformation. The latter is more sensitive to proteolysis and disulfide cross-linking, and it binds to cAMP and DNA [see de Crombrugghe et al. (1984) for a review]. This simple two-state model, however, *cannot* account for a significant amount of structural and functional observations, e.g., the presence of more than two types of cAMP analogues, namely, one mimics the effects of cAMP in inducing the typical structural changes in CRP and activates transcription; another type which binds to CRP and induces the same typical structural changes in CRP but *does not* activate transcription; a third type which binds to CRP but *does not* induce structural changes nor activate transcription (Ebright et al., 1985). These results imply that cAMP must induce CRP to assume more than one conformational state. The most important conclusion which can be drawn from this work is that CRP exists in at least three conformational states, two of which are cAMP dependent. One very interesting feature of the two cAMP-dependent conformational states of CRP is that the one which is observed at high cAMP concentration. It has properties resembling more of the free

protein. By combining the results of structural studies as a function of cAMP concentration and direct ligand binding measurements, it is evident that there is a direct quantitative correlation between structural change and the ligation of the protein, since the derived association constants for cAMP–CRP interactions are the same regardless of the method of measurements, be it direct or indirect. Binding of the first cAMP is about 1–2 orders of magnitude stronger than the second one. These observations enable one to establish a quantitative correlation between protein structure and function. One may address issues such as the following: Why do two identical binding sites exhibit such diverse affinity for cAMP? How would the occupancy of these sites affect DNA binding? Does the single-ligated CRP (CRP–cAMP) exhibit a different affinity for specific and nonspecific DNA sites than the double-ligated form [CRP–$(cAMP)_2$]? Let us examine the observations of this study in light of their relevance to the basic biological function of CRP, i.e., DNA binding and activation of gene expression.

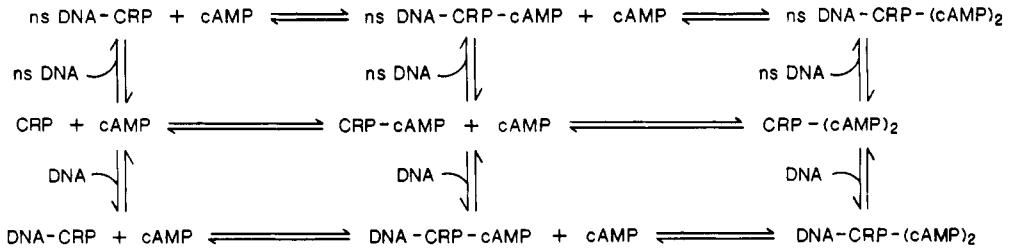
CRP can be considered as a protein that exhibits allosteric behavior. The interesting question that can be asked is as follows: What is the simplest allosteric model which can describe the behavior of this protein? The binding of cAMP to CRP seems to be characterized by strong negative cooperativity. Although the cAMP binding data can also be fitted for two nonidentical independent sites for cAMP, this is not a reasonable representation of the solution behavior of CRP since pronounced, sequential conformational changes in the protein were detected. There is, therefore, interaction between sites, and there is negative cooperativity in binding. These experimental results mean that a simple MWC model (Monod et al., 1965) is not adequate to represent the CRP system. The simplest allosteric model which can account for the observed data would be a sequential one (Koshland et al., 1966), in which there are three ligation and three conformational states of the protein. In this simplest case, the single-ligated protein would assume an intermediate conformation that is between that of the free and the double-ligated protein. This certainly is not consistent with the results shown in this study which demonstrated that the single-ligated state of CRP (for example, see Figures 2, 3, 4, and 7 and Table I) assumes a conformation that is *not* merely an intermediate state. Therefore, the CRP system may be represented by a more general model in which binding of cAMP to one subunit changes the conformation of this and its neighboring subunit. There are three ligation and three conformational states of the protein in this model, and it allows any relationship between the conformation of single- and double-ligated species. A possible extension of this model would be to take into account a preexisting asymmetry in subunit arrangement of the protein in solution. Such an asymmetric subunit orientation has been reported on the double-ligated protein in its crystalline state (McKay & Steitz, 1981). It is interesting to note that a similar model was proposed for the *lac* repressor system (O'Gorman et al., 1980).

Having established the linkage between the conformational states of CRP and occupancy of the cAMP binding sites, it is of interest to correlate this information with DNA binding. In this study, it was demonstrated that at cAMP concentrations at which the predominant species is the CRP–$(cAMP)_2$ complex, there is a significant decrease in the binding of CRP to a specific DNA site as compared to the CRP–cAMP complex. Hence, it can be concluded that of the three species-free CRP, CRP–cAMP, and CRP–$(cAMP)_2$, only CRP–cAMP is active in binding to specific DNA sequences. In contrast, free CRP and CRP–$(cAMP)_2$ do not bind to a specific DNA sequence or at least with significantly reduced affinity. This conclusion is consistent with the report by Garner and Revzin (1982) that in the CRP–DNA complex only one cAMP bound per protein molecule was observed. In addition, on the basis of the results of an extensive equilibrium study on the CRP–DNA interaction, Fried and Crothers (1984) concluded that the *net* binding of 1 equiv of cAMP per CRP dimer is sufficient to cause free CRP to bind to promoter [Figure 6 of Fried and Crothers (1984)]. On the other hand, after analysis of the effect of cAMP concentration on CRP binding to nonspecific DNA, the same authors concluded that the translocation of CRP from promoter to nonspecific DNA involves a *net release* of 1 mol of cAMP per mole of CRP dimer [Figure 3 of Fried and Crothers (1984)]. There is no a priori reason or evidence to support the interpretation that the net binding or release of cAMP is linked to a change in stoichiometry alone, since these values are derived from thermodynamic equilibrium measurements of a complex reaction involving multiple, linked equilibria. However, in combination with the results from this study, it can be concluded that the affinity of CRP for specific or nonspecific DNA depends on the ligation state of the protein. Results from this study also allow one to conclude that there is direct evidence to support the mechanism that CRP–cAMP binds to promoters whereas CRP and CRP–$(cAMP)_2$ do not and now this behavior may be understood in terms of the distribution of conformational states of CRP in solution. It may be of significance to note that saturation of half of the inducer binding sites in the *lac* repressor is sufficient for the induction process to occur under physiological conditions (O'Gorman et al., 1980). Thus, the CRP and *lac* repressor systems seem to share some common behaviors.

The results of this study are also relevant to the present understanding of the structure of the CRP–cAMP complex and its interaction with DNA. At present, the only form of CRP which forms crystals in solution is CRP–$(cAMP)_2$ (McKay & Steitz, 1981; McKay et al., 1982). Our data show that CRP–$(cAMP)_2$ seems to be inactive in specific binding to DNA and its solution conformation is quite different from that for free protein or CRP–cAMP. Such distribution of active (CRP–cAMP) and inactive [free CRP, CRP–$(cAMP)_2$] states of the protein can explain the inhibition of transcription of cAMP-regulated operons observed at high [cAMP] (Emmer et al., 1970; Lis & Schlief, 1973). A similar explanation was originally proposed by Lis and Schleif (1973) and Fried and Crothers (1984).

Is there any physiological significance in these results which show CRP–cAMP as the species that bind to the promoter whereas CRP and CRP–$(cAMP)_2$ do not? Let us consider the role(s) of CRP and cAMP in the regulation of gene expression in *E. coli*. As *E. coli* senses the presence of an alternative source of metabolites, such as lactose, the cellular level of cAMP increases, resulting in an increased amount of CRP–cAMP complex which then binds to the *lac* promoter to activate the transcription of mRNA coding for the metabolic enzymes responsible for utilizing the alternative energy source. Hence, conceptually it is acceptable to assume that both CRP–cAMP and CRP–$(cAMP)_2$ complexes can bind to the operon for metabolic enzymes. However, CRP is an autoregulator of the expression of its own gene (Aiba, 1983; Okamoto & Freundlich, 1986; Okamoto et al., 1988). The CRP–cAMP complex serves to repress the expression of the CRP gene. If both CRP–cAMP and CRP–$(cAMP)_2$ complexes can bind to the CRP operon, then the transcription of the CRP gene, thus the synthesis of CRP, is repressed when it is most needed. It would be logical that at higher concen-

Scheme I




trations of cAMP, the formation of CRP–$(cAMP)_2$ is favored. It leads to dissociation of the CRP–cAMP complex from the CRP operon, thus de-repressing the transcription of the CRP gene, resulting in an increase in the synthesis of more CRP to cope with the increased need of metabolic enzymes. This is a simplistic hypothesis of the physiological relevance of the results of our present in vitro study. Nevertheless, it provides a more logical model than to consider that the fully ligated form of CRP is also active in binding to promoters. The natural question which one can ask is the following: What is the feasibility of our proposal? More specifically, does the concentration of cAMP vary drastically to span the range predicted by the binding constants reported in this study? The concentration of cAMP in the *E. coli* cell is normallly in the micromolar range (Bernhohr et al., 1974; Pastan & Adhya, 1976). Therefore, CRP–$(cAMP)_2$ which is formed with an association constant of $k_2 = 16.5 \times 10^2$ $M^{-1}$ is almost not present in solution at all in the physiological range of cAMP concentration. The logical conclusion would be that there is no functional importance of CRP–$(cAMP)_2$ whatsoever. However, one has to remember that in the cell CRP exists in the presence of DNA and other proteins and therefore binding of cAMP to CRP is linked to binding of protein to DNA (specific and nonspecific) and possibly to binding to other proteins, e.g., RNA polymerase (Pinkey & Hoggett, 1988). These thermodynamic linkages can greatly change the distribution between free protein, CRP–cAMP, and CRP–$(cAMP)_2$. Takahashi et al. (1980) showed that the binding of cAMP to CRP is markedly affected by the presence of nonspecific DNA so much so that the apparent negative cooperativity of cAMP binding is altered to exhibit an apparent *positive* cooperative behavior. In addition, the concentration of cAMP which is necessary to induce half-maximal stimulation of transcription at the *lac* promoter is about 1 $\mu$M, a value substantially lower than the value of $k_1$ (Harman et al., 1986; Malan & McClure, 1984). The final assessment of the physiological significance of our findings should await determination of all linked equilibria involved in the system, an undertaking that is being pursued in this laboratory. The minimal scheme of thermodynamic linkages in the system containing CRP, cAMP, and specific and nonspecific DNA is shown in Scheme I where DNA and ns DNA denote specific and nonspecific DNA, respectively. Whatever the physiological significance of our results would be, one is certain that this system is not exempt from laws of thermodynamics and any analysis of its behavior has to be done with the cognizance of the many linked equilibria involved.

In addition to the thermodynamic consideration, another possible rationale for such distribution of active and inactive states of CRP is structural in nature. It is conceivable that for proper elicitation of its function the complex CRP–cAMP has to be asymmetric. Naturally, a singly ligated CRP, which has two sites for a ligand, is asymmetric. This asymmetry provides polarity in its interaction with DNA. The possible importance of polarity of the specific CRP–DNA complex was stressed by Fried and Crothers (1984) in view of the facts that all DNA sequences specifically binding CRP are asymmetric and that the crystalline structure of CRP–$(cAMP)_2$ shows asymmetry. Hence, the polar nature of the CRP–DNA complex seems to be an attractive model which needs to be addressed experimentally.

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