# Probing the Mechanism of CRP Activation by Site-Directed Mutagenesis: The Role of Serine 128 in the Allosteric Pathway of cAMP Receptor Protein Activation[†,‡]

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ABSTRACT: Upon activation by cAMP, *Escherichia coli* cAMP receptor protein (CRP) controls the expression of a network of catabolite sensitive genes. The activation of CRP by cAMP involves conformational changes such as realignments between subunits and domains within the protein. To understand the molecular events that lead to the activation of CRP, point mutations at position 128 were introduced *via* site-directed mutagenesis in an attempt to specifically affect the subunit interfacial interactions, as well as the ligand-binding reaction. The biochemical and biophysical properties of these mutants were rigorously tested with the goal of identifying the partial reactions in the activation pathway that are perturbed by this specific mutation. Results from this study suggest that mutation of Ser 128 to Ala or Pro does not significantly disturb the overall secondary structure as monitored by circular dichroism. The energetics of subunit−subunit interaction and protein stability were monitored by sedimentation and spectroscopic techniques. Although these mutants were designed to interrupt intersubunit interactions, the energetics of subunit association and protein stability remain quantitatively the same as those of the wild-type CRP. Nevertheless, the ability of the subunit to be realigned to the DNA-binding form is significantly affected as reflected by the pronounced decrease in the susceptibility of mutant CRP to proteolytic digestion in the presence of cAMP. In addition, the binding affinity of cAMP to the first ligand site in mutants S128A and S128P is the same as that of the wild type, but the affinity to the second ligand site is reduced. This observation indicates that mutation at position 128 affects ligand binding by amplifying the magnitude of negative cooperativity. Mutation at residue 128 does not impair the ability of interdomain interactions as indicated by the quantitative response of a spectroscopic probe in the DNA-binding domain to the binding of cAMP to the ligand-binding domain. The S128A mutant binds to a specific DNA sequence about 50-fold weaker than the wild-type CRP, while the mutant S128P has no measurable DNA affinity under the same conditions. This observation is consistent with the *in vivo* result that both mutants display an inactive CRP phenotype ($CRP^-$). In summary, these results suggest that communication between domains induced by cAMP binding can be dissociated from the proper subunit realignment of the CRP dimer that is crucial for the activation of CRP. Apparently, serine 128 is not vital for interdomainn communication, but plays an important role in mediating the interactions between the two subunits and discriminating between cAMP and cGMP.

In response to elevated intracellular cAMP levels, cAMP receptor protein (CRP)[1] controls the expression of a large number of genes (de Crombrugghe *et al.*, 1984). During this process, CRP binds to specific DNA sequences near promoters and induces significant structural changes in DNA. Binding of CRP to these DNA sites usually enhances transcription initiation by RNA polymerase (Kolb *et al.*, 1993).

The functional CRP is a dimer of two identical subunits. Each subunit further contains two interacting domains. The larger N-terminal domain binds one molecule of cAMP, while the smaller C-terminal domain interacts with DNA through the helix−turn−helix motif (McKay *et al.*, 1982; Weber & Steitz, 1987). Although the two cAMP-binding sites in the CRP dimer seem to be identical, the binding of cAMP to CRP is characterized by negative cooperativity. Among the three CRP conformational states, CRP, CRP−$(cAMP)_1$, and CRP−$(cAMP)_2$, the active conformer is the monoliganded CRP. Both free CRP and CRP−$(cAMP)_2$ bind DNA with very low affinity (Heyduk & Lee, 1989, 1990). The conformational changes induced by cAMP during the activation process have been observed by different techniques such as proteolytic digestion, chemical modification, cross-linking, fluorescence, and fluorine NMR spectroscopy (Krakow & Pastan, 1973; Wu *et al.*, 1974; Wu & Wu, 1974; Eilen & Krakow, 1977; Pampeno & Krakow, 1979; Tsugita *et al.*, 1982; Heyduk & Lee, 1989; Lee *et al.*, 1990; Sixl *et al.*, 1990; Hinds *et al.*, 1992). However, Raman and NMR spectroscopic analyses indicate that the structural differences between CRP and CRP−$(cAMP)_1$ are small, and no significant changes are observed in the secondary structure

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[‡] Dedicated to the memory of Genevieve Ching-Wen Lee (1974−1994).

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; CPM, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; CRP, cAMP receptor protein; GuHCl, guanidine hydrochloride; IAF, iodoacetamidofluorescein; PMSF, phenylmethanesulfonyl fluoride; buffer A, 50 mM Tris, 0.1 M KCl, and 1 mM EDTA at pH 7.8.

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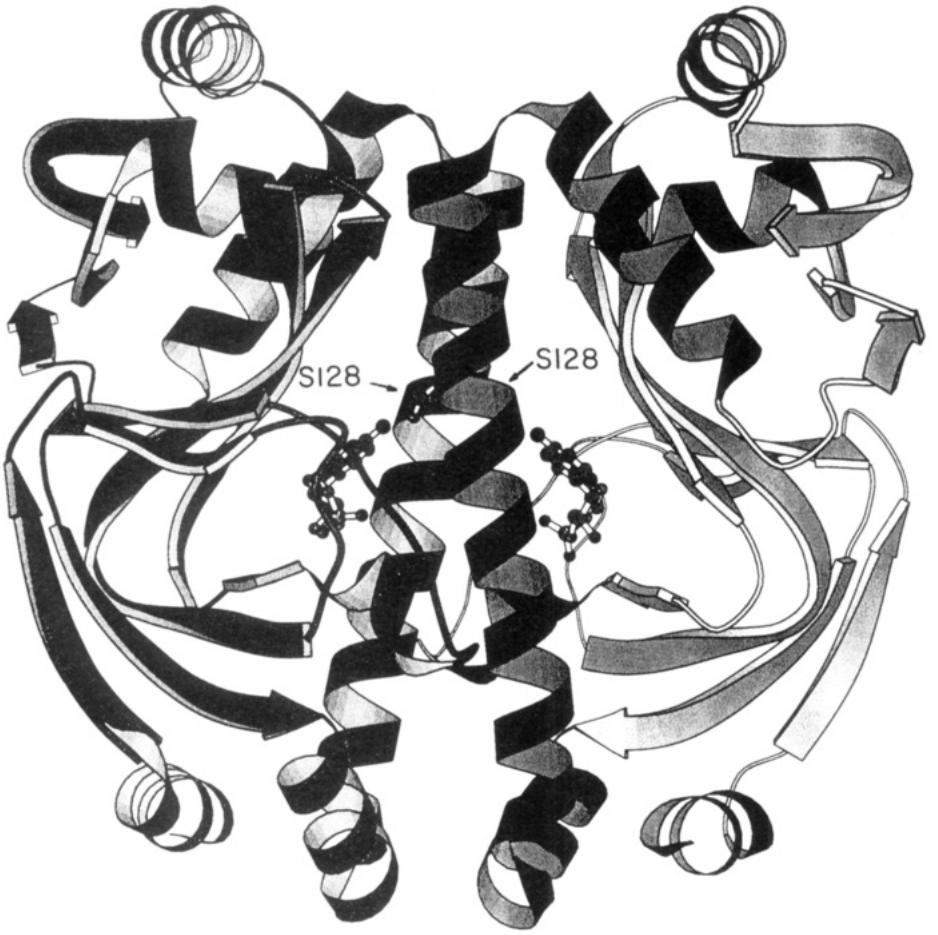


FIGURE 1: Structure of the CRP dimer. The locations of residues 128 are marked.

(Gronenborn & Clore, 1982; Lee *et al.*, 1990; Tan *et al.*, 1991). Using analytical gel chromatography, Heyduk *et al.* (1992) did not detect any significant change in the hydrodynamic properties of CRP upon the binding of the first cAMP molecule. This information implies that conformational changes induced by the binding of one cAMP to CRP are most likely represented by rigid body movements between subunits and domains. Hence, an intriguing question is the following: How is the signal transduced from the cAMP-binding site to the DNA-binding site?

Serine 128, the location of which in the CRP structure is shown in Figure 1, has been implicated to be directly involved in cAMP binding as well as intersubunit interactions in the CRP−$(cAMP)_2$ crystal structure (Weber & Steitz, 1987). Thus, serine 128 seems to occupy a crucial location in the CRP activation pathway. In order to understand the allosteric changes in CRP induced by cAMP binding and the mechanism of CRP activation, site-specific mutagenesis was employed to perturb this functionally important residue at the subunit interface. The structural and functional properties of these mutants and the wild type were studied and compared. The results of this study provide a quantitative dissection of the partial reactions of CRP activation and establish the effects of serine 128 on these reactions.

## MATERIALS AND METHODS

*Materials.* Ultrapure guanidine hydrochloride was a product of ICN Biochemical. MacConkey agar, bactotryptone, and yeast extract were obtained from Difco Laboratories. Chymotrypsin A and cGMP were purchased from Boehringer Mannheim. Mutagenesis kit (Altered Sites *in vitro* Mutagenesis System) was obtained from Promega. Sequencing kit (Sequenase version 2.0) was from United States Biochemical Corporation, Inc. ANS (Kodak Lab) was purified as described previously (York *et al.*, 1978). CPM and IAF were purchased from Molecular Probes. Oligonucleotides were synthesized by Genosys. Restriction endonucleases were from Promega, Gibco BRL, United States Biochemical Corporation, Inc., or Boehringer Mannheim.

*Bacterial Strains and Plasmid Vectors. Escherichia coli* CA8445 (*HfrH Δcrp-45 Δcya-854 strA thi*) (Brickman *et al.*, 1973) containing plasmid pRK248cI$^{ts}$ (Bernard & Helinski, 1979; kindly provided by Dr. Peterkofsky of the National Heart, Lung and Blood Institute), which encodes a temperature sensitive $\lambda$ cI repressor, was the host for the expression and determination of the phenotype of mutant CRP. pPLcCRP1, a pPLc28 derivative that contains the wild-type *crp* gene under the control of $\lambda P_L$ promoter, was a gift from Drs. Gronenborn and Clore of NIH. Other *E. coli* strains and plasmids used in cloning and mutagenesis are from Promega and American Type Culture Collection.

*Methods.* All experiments were conducted in 50 mM Tris, 0.1 M KCl, and 1 mM EDTA at pH 7.8 (buffer A). The concentrations of protein, cyclic nucleotides, and fluorescence probes were determined by absorption spectroscopy using the following absorption coefficients: 20 400 $M^{-1}$ $cm^{-1}$ at 278 nm for CRP monomer (Takahashi *et al.*, 1980); 14 650 $M^{-1}$ $cm^{-1}$ at 259 nm and 12 950 $M^{-1}$ $cm^{-1}$ at 254 nm for cAMP and cGMP (*The Merck Index*, 1976), respectively; 6240 $M^{-1}$ $cm^{-1}$ at 351 nm for ANS (Ferguson *et al.*, 1975);

30 000 $M^{-1}$ $cm^{-1}$ at 387 nm for CPM (Sippel, 1981); 70 800 $M^{-1}$ $cm^{-1}$ at 494 nm for fluorescein (Cerione *et al.*, 1983).

*Site-Directed Mutagenesis and Expression of Mutants.* The protocol of the Promega Altered Site *in vitro* Mutagenesis System technical manual was employed to introduce specific point mutations into the *crp* gene (Cheng, 1994; Cheng & Lee, 1994). Mutagenic oligonucleotide used for constructing S128A and S128P mutants was CAAGTCAC $T^{G}_{C}$CA(TCA)GAGAAAG. The highlighted triplet sequences are codons for alanine/proline with the wild-type codon for serine in parentheses. The mutagenic oligonucleotide for S128A contains a recognition site for restriction enzyme *Pst*I. The desired mutant was directly screened by DNA sequencing. For expression of the mutant CRP, the mutant allele was cloned into the *Hin*dIII site of the expression vector pPLc28, and the recombinant plasmid was introduced into the *crp*$^-$/*cya*$^-$ strain *E. coli* CA8445/pRK248cI$^{ts}$. The mutant protein expression was induced in the host bacterium by shifting the incubation temperature from 28 to 42 °C.

*lac Operon Activation in Vivo.* To test the effects of mutation at position 128 of CRP on *lac* operon expression, *E. coli* CA8445/pRK248cI$^{ts}$ transformed with plasmid pPLc28 that encodes the appropriate *crp* mutant gene was streaked on MacConkey lactose indicator plates in the absence or presence of 0.5 mM cAMP. The plates were incubated at 37 °C overnight. The fermentation response of the mutant was scored according to the color of the colonies on the plates.

*Protein Purification.* The wild-type and mutant CRPs were purified from *E. coli* strains K-12 ΔH1 and CA8445/pRK248cI$^{ts}$ using a previously described protocol (Heyduk & Lee, 1989). Purified wild-type and mutant CRPs are >99% homogeneous as judged by SDS−polyacrylamide gel electrophoresis. Typical yields of protein are about 15 and 2 mg per liter of culture for wild-type and mutant CRPs, respectively. The LB medium can be replaced by terrific broth to improve the yield of mutant CRP.

*Fluorescence Labeling of CRP.* IAF modification of Cys 178 was carried out in 50 mM Tris, 0.3 M KCl, and 1 mM EDTA (pH 7.8) with a CRP concentration of 25 μM and a molar ratio of 1:10 for protein to dye at 4 °C overnight in the dark. Fluorescein-labeled CRP was purified by Sephadex G-25 spin columns and dialyzed extensively against buffer A. The extent of modification was determined as previously described (Heyduk *et al.*, 1992a,b) and was in the range of 1.5−2.0 mole of probe per mol of CRP dimer.

*Labeling of DNA with Fluorescent Probe.* DNA used for the CRP−DNA interaction study is the 40 bp fragment of the *lac* promoter with the sequence 5′-CAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCC-3′. The underlined sequence is the primary binding site for CRP. A complementary strand of this fragment was synthesized and purified separately by PAGE in denaturing conditions. One of the single-stranded polynucleotides was then labeled with CPM at the 5′-end as described previously (Heyduk & Lee, 1990, 1992). Modified single-stranded DNA was hybridized with equimoles of its complementary strand to give the fluorescent-labeled double-stranded DNA.

*Proteolytic Digestion.* Proteolytic digestion of CRP was employed as a tool to monitor the conformational changes in CRP (Cheng *et al.*, 1993). A typical reaction mixture (25 μL) contains CRP (0.4 mg/mL) and chymotrypsin or subtilisin (4.8 μg/mL) and different concentrations of cAMP in buffer A. The reaction was carried out at 25 °C for 80 min. The reaction was stopped by adding 0.25 μL of 100 mM PMSF in 2-propanol, and an aliquot of the reaction mixture (10 μL) was withdrawn, mixed with 10 μL of sample buffer, and loaded onto a 15% SDS−PAGE slab gel for analysis using a Bio-Rad mini protean II electrophoresis system.

*cAMP-Binding Assays.* cAMP binding to CRP was measured by three different techniques: namely, by following the intrinsic tryptophan fluorescence of CRP ($\lambda_{ex}$ = 300 nm, $\lambda_{em}$ = 345 nm), the quenching of ANS−CRP fluorescence ($\lambda_{ex}$ = 375 nm, $\lambda_{em}$ = 480 nm), and the fluorescence signal change of the CRP−IAF complexes ($\lambda_{ex}$ = 495 nm, $\lambda_{em}$ = 520 nm). The basic protocol employed has been described previously (Heyduk & Lee, 1989), but a minor modification was made: 1300−1800 μL instead of 220 μL of the initial reaction mixture was used in this study in order to improve the precision of the measurement. All fluorescence measurements were performed with an SLM 8000C spectrofluorometer equipped with a temperature-controlled water bath at 25 °C. Fluorescence spectra were collected with excitation and emission polarizers set at 90° and 54.7° (magic angle), respectively. All titration points were corrected for dilution and inner filter effects using the following formula (Burstein, 1968):

$$F_{icor} = (F_i - B_i)(V_i/V_0)\frac{P_0 + \Delta A}{P_0}\frac{1 - 10^{-P_0}}{1 - 10^{-(P_0+\Delta A)}} \quad (1)$$

where $F_{icor}$, $F_i$, $B_i$, $V_0$, and $V_i$ are the corrected values of the fluorescence intensity, observed fluorescence intensity, background, volume of the initial sample mixture, and volume of the sample at a given point of titration $i$, respectively. $P_0$ and $\Delta A$ denote the initial sample absorption at the excitation wavelength and the absorption change introduced by the titration, respectively.

CRP exists as a dimer in solution and can bind two molecules of cAMP per dimer. The binding of cAMP to CRP can be described in accordance with the following equations:

$$\text{CRP} + \text{cAMP} \overset{K_1}{\rightleftharpoons} \text{CRP}-(\text{cAMP})_1 \quad (2)$$

$$\text{CRP} + 2\text{cAMP} \overset{K_2}{\rightleftharpoons} \text{CRP}-(\text{cAMP})_2 \quad (3)$$

with $K_1$ = [CRP−(cAMP)$_1$]/[CRP][cAMP] and $K_2$ = [CRP−(cAMP)$_2$]/[CRP][cAMP]$^2$.

Therefore, all of the data in this study can be fit to the following equation by nonlinear least-squares procedure to yield the thermodynamic parameters $K_1$, $K_2$, $\Delta P_1$, and $\Delta P_2$ (Heyduk & Lee, 1989):

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

where $\Delta P_{obs}$, $\Delta P_1$, and $\Delta P_2$ are the values of change in measured fluorescence intensity and the normalized values of change in fluorescence intensity in going from free CRP to CRP−(cAMP)$_1$ and CRP−(cAMP)$_2$, respectively; $K_1$ and

$K_2$ are Adair constants for the formation of CRP−$(cAMP)_1$ and CRP−$(cAMP)_2$, and [cAMP] is the free cAMP concentration.

*DNA-Binding Study.* Gel retardation assay was performed using the 40 bp *lac*−DNA fragment. Reaction mixtures of 20 μL each contained 0.1 μM DNA and various amounts of CRP (0−2 μM), cAMP, and 3% Ficoll in buffer A. An aliquot of 2 μL of sample buffer containing the tracking dyes was added to and gently mixed with the samples that had been equilibrated at room temperature for 1 h. Aliquots of 11 μL were then immediately loaded onto a 10% polyacrylamide gel that was prerun for 20 min at 150 V. Electrophoresis was carried out at 150 V in TBE buffer supplemented with the same cAMP concentration that was used in the binding reaction. After electrophoresis, gels were stained with ethidium bromide and DNA bands were visualized under UV light (Cheng *et al.*, 1993).

The fluorescence anisotropy experiment was used for quantitative measurements of the CRP−DNA interaction (Heyduk & Lee, 1990). Typically, a reaction mixture contained 12 nM CPM-labeled *lac*-40 DNA and a fixed amount of cAMP. Small volumes of concentrated CRP (1−5 μL) were then titrated into the solution, and the anisotropy of the sample was measured after each addition of CRP. The CRP samples were supplemented with the same concentration of cAMP that was used for the reaction mixture to keep the concentration of cAMP constant during titration. The total volume change that occurred in one titration curve was usually below 5%. The excitation and emission wavelengths were 380 and 470 nm (KV cutoff filter), respectively, for anisotropy measurements performed with CPM-labeled DNA.

The measured anisotropy data of the CRP−DNA interaction were fit to eq 5 as previously described (Heyduk & Lee, 1990) to estimate the apparent association constant ($K$) for binding CRP to DNA at the cAMP concentration used for the measurement:

$$A = A_D + (A_{PD} - A_D)\frac{KD_T + KP_T + 1 - \sqrt{(KD_T + KP_T + 1)^2 - 4K^2D_TP_T}}{2KD_T} \quad (5)$$

where $A$ is the measured value of anisotropy, $A_D$ and $A_{PD}$ are values of anisotropy associated with free DNA and CRP−DNA complex, respectively, and $D_T$ and $P_T$ are the total concentrations of DNA and protein, respectively.

*Sedimentation Equilibrium.* The quaternary structure of CRP was monitored by sedimentation equilibrium as previously described (Cheng *et al.*, 1993). Experiments were conducted at 20 °C. The loading CRP concentrations were between 0.2 and 0.3 mg/mL. Sedimentation data were acquired (10 scans) and then averaged.

Sedimentation equilibrium data were fit by nonlinear least-squares analysis to the dimer assembly schemes according to

$$C = \Delta C + \exp[\ln C_0\, \sigma(r^2/2 - r_0^2/2)] + \exp[2 \ln C_0 + 2\sigma(r^2/2 - r_0^2/2) + \ln K] \quad (6)$$

where $C$ is the CRP concentration observed at radial position $r$, $\Delta C$ is the baseline offset, $C_0$ is the concentration of CRP at the meniscus ($r_0$), $K$ is the dimer equilibrium association constant, and $\sigma$ is the reduced molecular weight given by $\sigma = M_r(1 - \bar{\nu}\varrho)\omega^2/2RT$, where $M_r$ is the monomer molecular weight, $\bar{\nu}$ is the partial specific volume, $\varrho$ is the solution density, $\omega$ is the angular velocity, and $R$ and $T$ are the gas constant and temperature in kelvin, respectively.

*Circular Dichroism Measurement.* CD spectra of the wild-type and mutant CRPs were collected according to a previously described procedure (Cheng *et al.*, 1993) over the range 200−300 nm using an Aviv Model 60 DS spectropolarimeter.

## RESULTS

*Mutagenesis and Expression of Mutant CRP.* Mutants S128A and S128P were generated by site-directed mutagenesis. A mutation efficiency of greater than 70% was usually obtained. For the expression of mutant CRP, the mutant *crp* allele was cloned into the expression vector pPLc28, and the recombinant plasmid was used for transformation of *crp* deficient *E. coli* strain CA8445/pRK248CI$^{ts}$. Mutant protein expression was induced in the host by increasing the incubation temperature to 42 °C. The expressed mutant protein is stable *in vivo*. Figure 2 showed the level of expression of CRP S128A as a function of time of induction. The cellular level of the mutant protein is still increasing after 6 h of induction.

*In Vivo Genetic Characterization of CRP Mutants.* The expression of many catabolic genes in *E. coli* is dependent upon the presence of an active CRP. Thus, the phenotype of CRP mutants can be tested by using MacConkey agar indicator plates. When a mutant *crp* allele is expressed in a *crp*$^-$/*cya*$^-$ strain such as *E. coli* CA8445, a *crp** phenotype is defined as showing purple colonies on MacConkey lactose plates (*Lac*$^+$) in the absence of exogenous cAMP or in the presence of cGMP. A *crp*$^+$ phenotype displays purple colonies only in the presence of cAMP, while a *crp*$^-$ phenotype is a mutant that always shows white colonies (*Lac*$^-$) on MacConkey plates. Mutants S128A and S128P both showed a *crp*$^-$ phenotype.

*Secondary Structure Characterization of Mutants S128A and S128P by CD Measurement.* To provide a more complete understanding of the significance and properties of the mutant proteins, the S128A and S128P mutants were purified to homogeneity for further biochemical and biophysical characterization. Proper interpretation of the functional data of site-specific mutants requires the correct folding of the mutant protein. To examine the structural integrity of S128A and S128P, the overall secondary structure of S128A and S128P was determined by CD measurements and the results are shown in Figure 3. Identical CD spectra were obtained for S128A and the wild-type protein, as would be expected for such a conservative replacement. In the case of S128P, due to the presence of a Pro residue within the C-helix, structural changes were predicted. However, the CD spectrum of S128P is essentially identical to those of S128A and the wild-type CRP. This observation indicates that either no secondary structural changes have occurred in S128P or there are structural changes but they do not lead to a net change. In any case, the structural changes cannot be major.

*Subunit Association.* Having determined that the secondary structure of the wild-type CRP is maintained in both S128A and S128P, the quaternary structures of S128A and

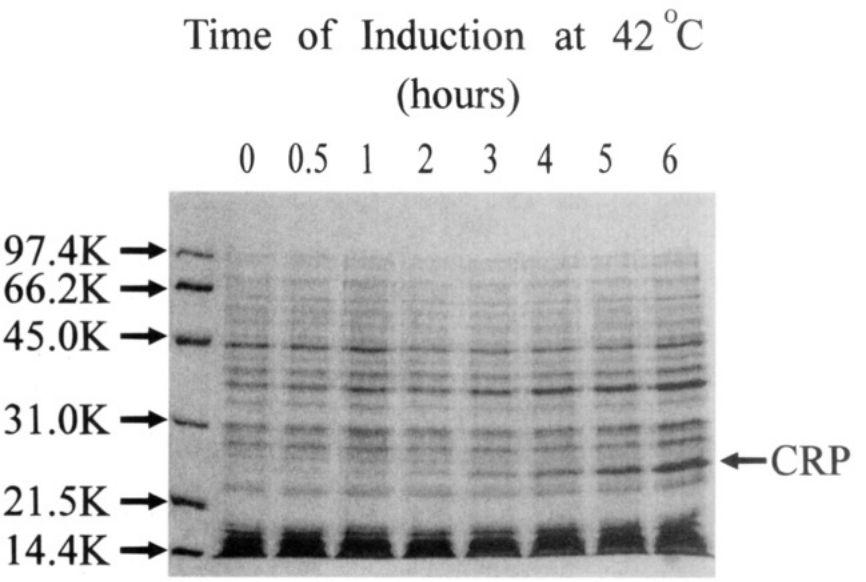


FIGURE 2: Expression of the CRP mutant S128A in *E. coli* CA8445/pRK248cI$^{ts}$ host. Bacteria with expression vector pPLc28 encoding the S128A *crp* gene were grown in LB medium containing the antibiotics ampicillin and tetracycline at 28 °C. When the optical density of the culture at 600 nm reached 0.6−0.8, the incubation temperature was shifted to 42 °C to inactivate the temperature sensitive cI repressor and, therefore, to induce the expression of the mutant gene under the control of the $\lambda P_L$ promoter. At certain time points, 100 $\mu$L of the culture was withdrawn and mixed with 100 $\mu$L of SDS sample buffer. The samples were boiled in water for 10 min, and then 20 $\mu$L of the sample was analyzed by 12% SDS−PAGE.

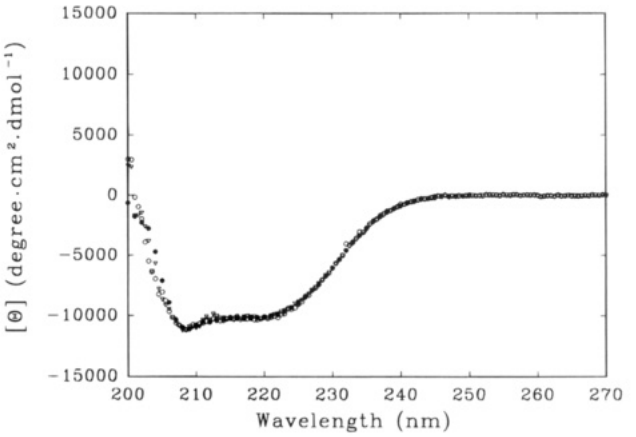


FIGURE 3: CD spectra of the wild-type CRP (○), S128P mutant (▽), and S128A mutant (●) in buffer A. Protein concentrations used for the measurement were 0.66, 0.85, and 1.33 mg/mL for the wild-type CRP, S128A, and S128P, respectively. For each sample, five repetitive scans were acquired and averaged.

S128P were examined. Initial analytical gel filtration studies showed identical elution profiles for the wild-type CRP and mutants, suggesting that S128A and S128P exist as a dimer structure, as in the wild-type CRP. Sedimentation equilibrium experiments as a function of GuHCl concentration were performed to quantitatively assess the equilibrium constant for subunit association. Figure 4A shows the distribution of S128A concentrations as a function of the radial distance at three GuHCl concentrations. The apparent dimer association constant ($K_{a,app}$) at individual GuHCl concentrations was determined by fitting the sedimentation profile to eq 6. A plot of ln $K_{a,app}$ *versus* GuHCl concentration displays an apparent linear relationship, as shown in Figure 4B. A linear extrapolation of ln $K_{a,app}$ to zero concentration of GuHCl yields a value for the dimer association constant for S128A in buffer A. The observed dimer association constant for S128A is 4.3(2.3,6.6) × $10^8$ $M^{-1}$, which is similar to that of the wild type (1.0(0.3,2.4) × $10^9$ $M^{-1}$). The sedimentation behavior of S128P in the presence of 2.26 M GuHCl was analyzed at 20 000 and 34 000 rpm to estimate the apparent association constant. The value of 8.5(6.3,11.5) × $10^4$ $M^{-1}$ was determined. The apparent association constants for wild-type and S128A CRPs are 2.8(1.7,4.7) × $10^5$ and 1.9(1.5,2.6) × $10^5$ $M^{-1}$, respectively, under the same experimental conditions. This suggests that S128P has a dimer stability similar to those of the wild-type and S128A CRPs. Therefore, mutation at residue 128, which is located at the subunit interface, does not lead to significant perturbation of the energetics of subunit interactions.

*Protein Stability of S128A.* An equilibrium denaturation study can provide information on protein stability as well as subunit association. Results of chemical denaturation studies of CRP show that CRP undergoes reversible dissociation and unfolding when exposed to denaturant (Cheng *et al.*, 1993). The GuHCl-induced chemical denaturation profiles of S128A, determined by CD at 27 $\mu$M protein concentration and by fluorescence anisotropy at 1 $\mu$M protein concentration, are almost identical to those of the wild type, as shown in Figure 5. Cheng *et al.* (1993) have shown that data such as those shown in Figure 5 are composites of subunit dissociation and protein unfolding. Given that the dissociation constants are identical for both wild-type and mutant CRPs and the denaturation profiles are identical, these results suggest that S128A and wild-type CRP are of equal stability. This conclusion is supported by the observation that the simulated denaturation profile, as indicated by the solid line in Figure 5 using all of the parameters for the wild-type CRP, represents very well the denaturation profiles of wild-type and mutant CRPs.

*cAMP Binding.* cAMP binding to CRP was monitored by following the quenching of the fluorescence intensity of the CRP−ANS complex and the increase in tryptophan fluorescence intensity of CRP as a function of cAMP concentration. When the wild-type CRP−ANS complex is titrated with cAMP, biphasic quenching of the fluorescence signal is observed. The biphasic behavior corresponds to

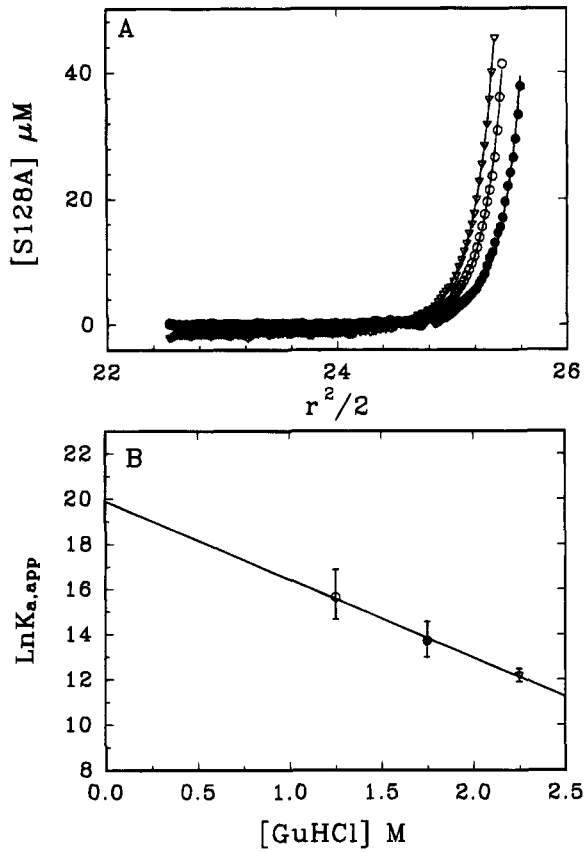


FIGURE 4: Subunit association of the S128A mutant monitored by sedimentation equilibrium. (A) Sedimentation equilibrium profiles of S128A. The experiments were conducted at 32 000 rpm and different GuHCl concentrations: (○) 1.25, (●) 1.75, and (▽) 2.25 M. The solid lines represent the best fits of the experimental data to eq 6. (B) Apparent dimer equilibrium association constants (determined from panel A) as a function of GuHCl concentration. The equilibrium constant of the subunit association in buffer that does not contain denaturant is determined by linear extrapolation. The symbols used in (B) are the same as those in (A).

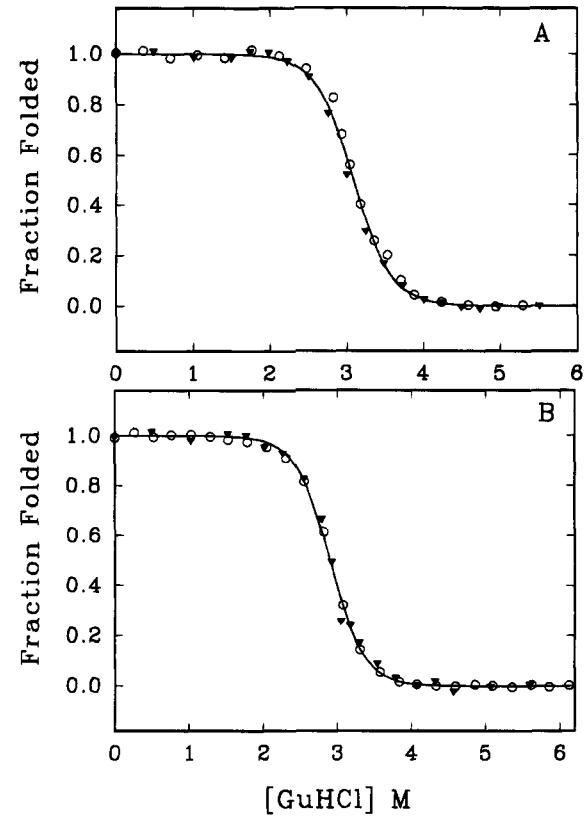


FIGURE 5: GuHCl-induced chemical denaturation of the S128A mutant and the wild-type CRP: (A) fraction of 27 μM S128A (○) and 22 μM CRP (▼) in the folded states determined by CD; (B) fraction of 1 μM S128A (○) and 1 μM CRP (▼) in the folded states determined by fluorescence anisotropy measurements.

the two events associated with the binding of two molecules of cAMP. From such experiments it is possible to extract valid thermodynamic parameters of cAMP binding to CRP (Heyduk & Lee, 1989). Although in the earlier study Heyduk and Lee (1989) did not observe biphasic behavior in monitoring cAMP binding with changes in tryptophan fluorescence, with a slight modification of the procedures as stated in Materials and Methods, the fluorescence intensity of tryptophan also shows a two-step increase. The first phase occurs at low (micromolar) cAMP concentrations and is characterized by a small increase (about 5% change) in fluorescence intensity. This event is ascribed to the binding of the first cAMP. At high (millimolar) cAMP concentrations there is a pronounced increase in the tryptophan fluorescence intensity, as shown in Figure 6A. This second phase corresponds to the binding of the second cAMP. This biphasic behavior suggests that when the first cAMP interacts with CRP it induces a structural change so that the environment of the tryptophan residues in CRP undergoes a small but noticeable change, while the binding of the second cAMP affects the tryptophan environment significantly. This observation is consistent with the results derived from a Raman spectroscopic study (Tan *et al.*, 1991), which indicates that only minor structural changes are associated with the binding of the first molecule of cAMP but substantial structural perturbations occur upon the binding of the second ligand.

The binding of cAMP to S128A showed a two-step pattern similar to that of the wild-type CRP, as shown in Figure 6A. The second phase of the cAMP-binding isotherm is shifted to higher cAMP concentrations than that of the wild-type CRP. This shift indicates that the binding of the second cAMP is weaker in S128A. The binding of cAMP to S128A can also be determined by measuring the quenching of fluorescence intensity of the S128A−ANS complex, as shown in Figure 6B. The observed quenching is due to the dissociation of ANS probe from the hydrophobic area of CRP accompanying cAMP binding (Heyduk & Lee, 1989). The fitted parameters of $k_1 = 2.1 \times 10^4$ M$^{-1}$ and $k_2 = 19$ M$^{-1}$ reflect a similar binding constant $k_1$ and a much weaker $k_2$ for S128A than those of wild-type CRP, whose cAMP-binding affinities are $k_1 = 2.6 \times 10^4$ M$^{-1}$ and $k_2 = 0.5 \times 10^3$ M$^{-1}$. These results are summarized in Table 1.

The binding of cAMP to S128P exhibits characteristics similar to that of S128A, with an identical $k_1$ and a very weak $k_2$ (Table 1). The $k_2$ is so weak that the second phase of the typical two-step cAMP-binding isotherm for CRP was not observed in S128P. Interestingly, the binding of cAMP to S128P leads to quenching of the intensity of the S128P intrinsic fluorescence. A quenching of the signal is opposite of the enhancement effect shown in both CRP and S128A. This observation implies that the conformation of the S128P−(cAMP)$_1$ complex is different from those of S128A−(cAMP)$_1$ and CRP−(cAMP)$_1$. The identity of the values of $k_1$ for wild-type and mutant CRPs indicates that replacing serine 128 does not significantly alter the N-terminal cAMP-

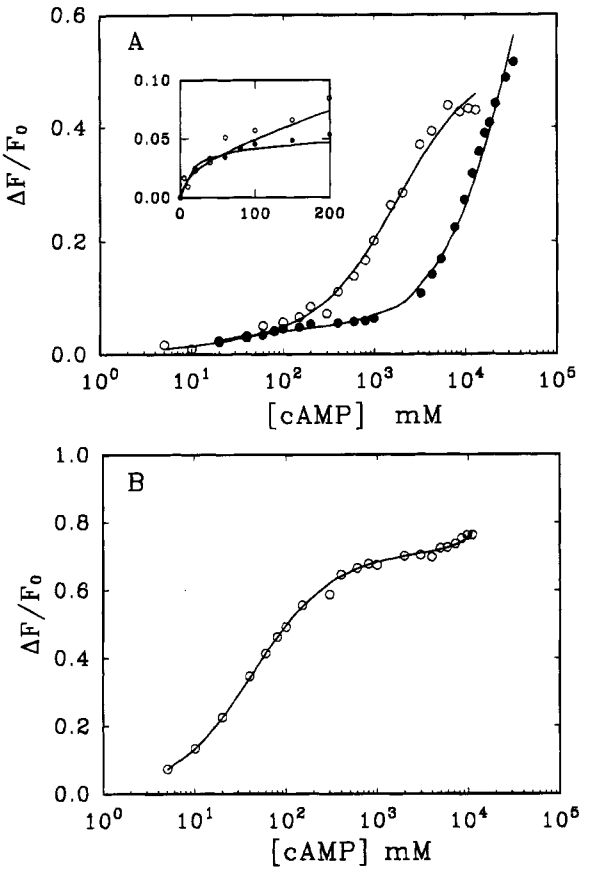


FIGURE 6: Binding of cAMP to the wild-type CRP and S128A mutant: (A) changes in the intrinsic tryptophan fluorescence of the wild-type CRP (○) and S128A (●) as a function of cAMP concentration; (B) fluorescence quenching of the ANS−S128A complex as a function of cAMP concentration. For (A) and (B), the solid lines represent the best fits of the data to eq 4. Inset: Plot of the data at low cAMP concentrations.

binding domain or that the changes do not affect cAMP binding to the first site. On the other hand, the subunit cross-talking, as reflected by the cooperativity between the two cAMP-binding sites, was disrupted by substituting serine 128 with alanine or proline, as indicated by the very small values of $k_2$ in these mutants.

*Subunit Conformation as Monitored by Protease Sensitivity.* The sensitivity of CRP to proteolytic digestion is closely related to the CRP activation process. CRP is resistant to the digestion of many proteases in the absence of cAMP. It has also been shown that the response of CRP to protease digestion in the presence of cAMP is biphasic. At low (micromolar) cAMP concentrations the rate of proteolytic digestion increases as the concentration of cAMP increases, whereas at high (millimolar) cAMP concentrations the rate of digestion decreases as the cAMP concentration increases. Therefore, the susceptibility of CRP to protease digestion is a sensitive probe for monitoring conformational changes in the molecule (Heyduk & Lee, 1989).

While the wild-type CRP responds to chymotrypsin digestion in a biphasic manner as a function of cAMP, both S128A and S128P are resistant to chymotrypsin cleavage in the presence of a broad range of cAMP concentrations tested (0−50 mM) under the same experimental conditions, as shown in Figure 7. A much higher protease concentration is needed in order to obtain a digestion pattern similar to that of wild-type CRP. In addition, the digestion of both S128A and S128P by chymotrypsin at high protease concentrations does not show the typical biphasic response observed in the wild-type CRP. In contrast, the rate of digestion levels off at high cAMP concentrations. This observation is consistent with the results of the cAMP-binding study, which shows that the second cAMP binds to S128A and S128P with a much weaker affinity than that of the wild-type CRP.

*DNA Binding.* One of the central functions of CRP is to recognize specific DNA sequences. Wild-type CRP interacts with DNA in a cAMP dependent manner. Among the three conformational states, CRP shows only a very weak affinity for nonspecific DNA, while the monoliganded state, CRP−$(cAMP)_1$, interacts with specific DNA very strongly, and CRP−$(cAMP)_2$ binds to DNA at least 100 times weaker than CRP−$(cAMP)_1$ (Heyduk & Lee, 1989, 1990).

Mutants S128A and S128P both show the *crp*$^-$ phenotype *in vivo*, implying that these mutant CRPs do not bind to specific DNA sites; hence, *in vitro* DNA-binding studies were conducted. S128A does not bind to DNA in the absence of cAMP and interacts with DNA with only very low affinity even in the presence of cAMP. The measured apparent equilibrium constant was $6.6 \times 10^6$ $M^{-1}$ in the presence of 200 $\mu$M cAMP, as shown in Figure 8. This result reflects a 50-fold reduction in DNA-binding affinity compared to the wild-type CRP (Table 2). In addition, the relative binding affinities of the monoliganded and doubly liganded CRPs to DNA were monitored by fluorescence anisotropy, as shown in Figure 9. The signal increases with increasing cAMP concentration. After reaching a plateau value, the signal decreases. This biphasic behavior implies that CRP−$(cAMP)_1$ is the species that recognizes a specific DNA site, whereas the CRP−$(cAMP)_2$ species exhibits a much weaker affinity. Thus, these results indicate that S128A interacts with specific DNA in a cAMP dependent manner similar to that of the wild-type CRP. Unlike the more conservative alanine mutation, S128P has no measurable binding affinity toward specific DNA in the presence or absence of cAMP under the same experimental conditions.

*Domain Cross-Talking Monitored by CRP−IAF Fluorescence.* There are three cysteine residues in each CRP subunit: Cys 18, 91, and 178 (Aiba *et al.*, 1982; Cossart & Gicquel-Sanzey, 1982). Under native conditions only Cys 178 is accessible to chemical modification, while Cys 18 and 92 seem to be buried (Eilen & Krakow, 1977; Ebright *et al.*, 1985). In the crystal structure Cys 178 is located in the middle of the helix−turn−helix DNA-binding motif, which is only two residues away from the DNA recognition F-helix. Therefore, changes in the microenvironment of Cys 178 upon the binding of cyclic nucleotides, as monitored by changes in the fluorescence intensity of the Cys 178−IAF probe, should reflect communication between the DNA and the cAMP-binding domain in response to cyclic nucleotide binding. If an analysis of the binding isotherm obtained by this method yields binding constants for cAMP that are identical to those determined by other methodologies, then this is taken as evidence for interdomain interaction.

The binding of cAMP to IAF-labeled wild-type CRP and S128A mutant was studied by monitoring the change in fluorescence intensity of the IAF probe, and the results are shown in Figure 10A. The data are expressed as $(F_0 - F)/F_0$ *versus* ligand concentration, where $F_0$ and $F$ are fluorescence intensities in the absence and presence of ligand,

Table 1: Summary of the Fitted Parameters for cAMP Binding to Wild-Type and Mutant CRPs

| CRP mutant | | $K_1$ ($M^{-1}$)[a] | $k_1$($M^{-1}$) | $K_2$ ($M^{-2}$) | $k_2$ ($M^{-1}$) | $k_1/k_2$ |
|---|---|---|---|---|---|---|
| wild type | averaged[b] | | $2.6 \times 10^4$ | | $0.5 \times 10^3$ | 52 |
| S128A | tryptophan | $5.9(3.1,8.7) \times 10^4$ | $3.0 \times 10^4$ | $1.1(0.07,2.1) \times 10^6$ | $1.9 \times 10^1$ | 1105 |
| | ANS | $2.5(2.3,2.6) \times 10^4$ | $1.2 \times 10^4$ | nd[c] | | |
| | averaged | | $2.1 \times 10^4$ | | $1.9 \times 10^1$ | |
| S128P | tryptophan | $4.0(3.5,4.5) \times 10^4$ | $2.0 \times 10^4$ | nd | | |
| | fluorescein | $2.2(1.6,2.8) \times 10^4$ | $1.1 \times 10^4$ | nd | | |
| | averaged | | $1.6 \times 10^4$ | | | |

[a] The values in parentheses are limits within a 75% confidence level. [b] Heyduk & Lee, 1989. [c] Not able to determine.

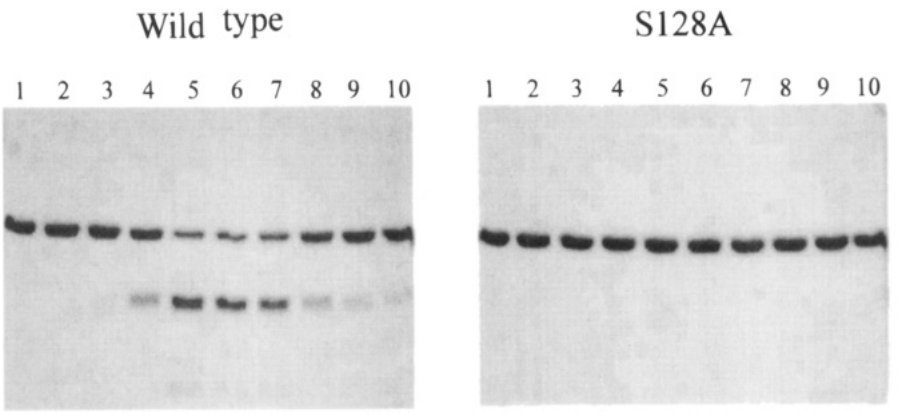


FIGURE 7: Proteolytic digestion of the wild-type CRP and S128A mutant by chymotrypsin at different cAMP concentrations. Lane 1 is CRP alone, and lanes 2−10 contain protease and various concentrations of cAMP: 2, 0 $\mu$M; 3, 5 $\mu$M; 4, 20 $\mu$M; 5, 200 $\mu$M; 6, 2 mM; 7, 5 mM; 8, 10 mM; 9, 25 mM; 10, 50 mM.

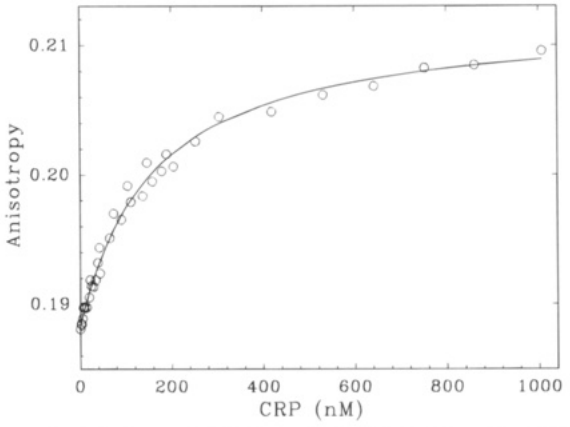


FIGURE 8: Binding of S128A to CPM-labeled *lac*-40 DNA as monitored by fluorescence anisotropy in buffer A containing 12 nM DNA and 200 $\mu$M cAMP. The solid line is the best fit of the data using eq 5.

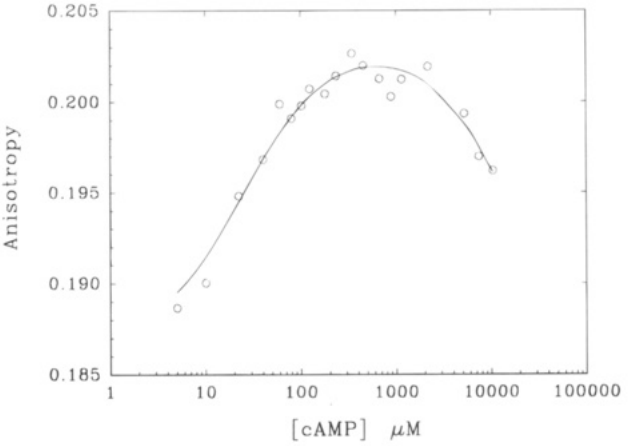


FIGURE 9: Interaction of S128A with *lac*-40 DNA as monitored by fluorescence anisotropy in buffer A as a function of cAMP. The experiment was done by titrating concentrated cAMP into a reaction mixture containing 12 nM DNA and 250 nM S128A. The line was drawn to indicate the trend of the data only.

Table 2: Apparent DNA-Binding Affinity of the Wild-Type and Mutant CRPs in the Presence of 200 $\mu$M cAMP

| CRP mutant | $K_{app}$ ($M^{-1}$)[a] |
|---|---|
| wild-type CRP | $3.5(2.1,5.0) \times 10^8$ |
| S128A | $6.6(5.6,7.8) \times 10^6$ |
| S128P | nm[b] |

[a] The values in parentheses are limits within a 75% confidence level. [b] No measurable affinity under the experimental conditions.

respectively. At low cAMP concentrations, the fluorescence intensity of the probe attached to the wild-type CRP increases with increasing cAMP concentrations, leading to the appearance of a concave curve. Conversely, a quenching of fluorescence is observed for S128A−IAF. The binding isotherm was fitted to eq 6 to determine the binding constants of cAMP to these proteins. The results of such an analysis are summarized in Table 3. It is interesting to note that the binding constants for the first cAMP molecule are essentially identical. Furthermore, these values are in good agreement with the binding constants determined by other independent techniques in this study (Table 2) and in the literature (Heyduk & Lee, 1989; Takahashi *et al.*, 1980). These results indicate that there is a quantitative response of the DNA-binding domain to the binding of cAMP to the ligand-binding domain in the wild-type and mutant CRPs.

CRP is known to bind cGMP without being activated for DNA binding. Hence, it is of interest to monitor the response of the DNA-binding domain to the binding of cGMP. IAF-labeled wild-type CRP and S128A mutant were subjected to this study, and the results are shown in Figure 10B. Binding of cGMP causes a quenching of fluorescence intensity in both the wild-type and S128A CRPs. Fitting of

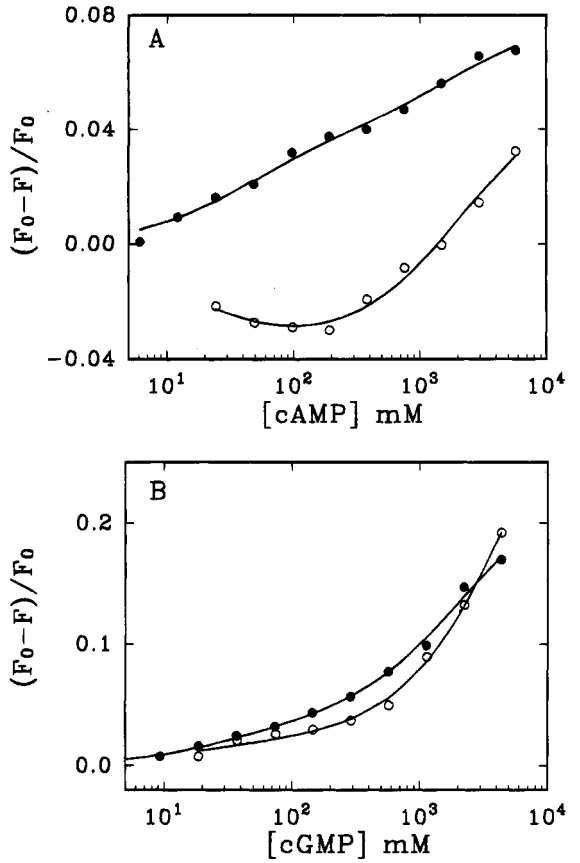


FIGURE 10: Fluorescence intensity change of the fluorescein probe attached to cysteine residue 178 of CRP as a function of (A) cAMP and (B) cGMP concentrations: (○) wild-type and (●) S128A. The solid lines represent the best fits of the data to eq 4, and the estimated parameters are summarized in Table 3.

Table 3: Summary of the Fitted Parameters for Cyclic Nucleotide Binding to Wild-Type and Mutant CRPs Measured by CRP−IAF Fluorescence

| CRP mutant | | $K_1$ ($M^{-1}$) | $k_1$ ($M^{-1}$) | $K_2$ ($M^{-2}$) | $k_2$ ($M^{-1}$) |
|---|---|---|---|---|---|
| wild type | cAMP | $7.2 \times 10^4$ | $3.6 \times 10^4$ | $3.4 \times 10^7$ | $4.7 \times 10^2$ |
| | cGMP | $6.1 \times 10^4$ | $3.0 \times 10^4$ | $1.0 \times 10^7$ | $1.6 \times 10^2$ |
| S128A | cAMP | $2.4 \times 10^4$ | $1.2 \times 10^4$ | $1.1 \times 10^7$ | $4.6 \times 10^2$ |
| | cGMP | $3.1 \times 10^4$ | $1.5 \times 10^4$ | $1.4 \times 10^7$ | $4.5 \times 10^2$ |

the binding isotherms to eq 6 resulted in values of $k_1$ that are very similar, as summarized in Table 3.

## DISCUSSION

The activated species that binds to the specific DNA site is CRP−$(cAMP)_1$. This conclusion is valid for all of the CRP-regulated operons tested (E. A. Pyles and J. C. Lee, unpublished data). Apparently, the negatively cooperative cAMP-binding feature is a mechanism to ensure the presence of such an asymmetric species that plays a key role in the molecular mechanism of CRP activity. The binding of this single cAMP molecule leads to realignments between subunits and domains. There is ample evidence to support these structural changes (Krakow & Pastan, 1973; Wu *et al.*, 1974; Wu & Wu, 1974; Eilen & Krakow, 1977; Pampeno & Krakow, 1979; Tsugita *et al.*, 1982, Heyduk & Lee, 1989; Lee *et al.*, 1990; Sixl *et al.*, 1990; Hinds *et al.*, 1992). Thus, the crucial information required to establish a structure−function relationship for CRP is to identify the structural element(s) in CRP that is (are) responsible for imposing negative cooperativity. Does that structural element also participate in inducing these realignments of subunits and domains?

In order to address these issues, it is important to be able not only to perturb the CRP structure by site-directed mutagenesis but also to devise the appropriate measurements to monitor functions, such as cAMP and DNA bindings as well as structural perturbations. In this study, multiple approaches were employed in essentially every assay for function or structure. For example, the effect of mutation on the structural stability of CRP was monitored by circular dichroism and determination of the $\Delta G$ of CRP unfolding in the presence of chemical denaturant. The spectroscopic technique provides information on the structural features in global folding of the protein molecule, while the chemical denaturation study yields the energetics of CRP stability. The mutation introduced into CRP may not lead to an observable change in the circular dichroic spectrum, but may be manifested in a change in the energetics of unfolding or *vice versa*. The same rationale applies to the monitoring of subunit−subunit interactions, namely, employing both proteolysis and sedimentation to provide structural and energetic information. Thus, this study was designed to maximize the chance of dissecting the effect of point mutation to elucidate the mechanism of CRP activation by the binding of cAMP.

Both alanine and proline substitutions at position 128 result in the *crp*$^-$ phenotype *in vivo*, suggesting that serine 128 is important for CRP function. This observation is consistent with previous studies showing that the S128A mutant is a poor activator of *lac*P or exhibits lower affinity for a specific DNA site (Gent *et al.*, 1987; Lee *et al.*, 1994). However, without in-depth *in vitro* characterization of the mutants, it is impossible to know the exact cause for CRP to exhibit an inactive phenotype. Furthermore, the activation of CRP by cAMP is a complicated process that involves multistep conformational changes. In order to understand the mechanism of CRP activation, it is important to know which conformational step is disturbed by individual mutation. A systematic analysis of various mutants will enable one to identify the allosteric pathway of CRP activation, as well as to correlate the mutation with a specific conformational step(s) involved in the process.

The substitution of serine 128 by alanine is designed to eliminate the hydrogen bonding between cAMP in one subunit and serine in the adjacent subunit, while the substitution of proline is aimed to disrupt the C-helix, which is involved in intersubunit contacts. However, both substitutions do not induce any observable structural changes in free CRP, as monitored by circular dichroism, nor in the energetics of protein folding. In addition, the dimer association constant of both S128A and S128P is not significantly perturbed in comparison with that of the wild-type CRP. All of these results suggest that mutation at residue 128 does not significantly perturb the structural integrity of free CRP.

The effects of mutation are amplified in cAMP and DNA binding. Binding of the first cAMP to S128A and S128P is identical to that of the wild-type CRP, demonstrating that despite the functional importance of the intersubunit hydrogen bonding between the hydroxyl group of serine 128 and the N-6 amino group of cAMP this specific interaction is not critical for the energetics of CRP−cAMP association. This observation is consistent with the results of the study employing cAMP analogues. While alterations in the ribose

3′,5′-cyclic phosphate moiety of cAMP significantly affect cAMP binding to CRP, binding *per se* is not highly sensitive to modification in the adenine part of cAMP (Ebright & Wong, 1981; Ebright *et al.*, 1985). Although S128A and S128P bind the first molecule of cAMP as well as wild-type CRP, these mutants display a significant decrease in protease sensitivity in the presence of a wide range of cAMP concentrations (0−50 mM). This observation suggests that serine 128 is important in mediating cAMP-induced allosteric conformational changes. Since essentially all of the proteolytic cleavage sites in CRP are localized at the C α-helices, it is conceivable that the sensitivity of CRP to proteolytic digestion monitors the conformational changes along the C α-helices, which in turn reflects the realignment of subunit. Wild-type CRP is only sensitive to proteolytic digestion in the presence of cAMP, indicating that the subunit realignment is an integral step involved in cAMP-induced CRP activation. Replacement of serine 128, a functionally important residue in intersubunit interactions, destroys the ability of the S128A and S128P mutants to generate proper subunit realignment in response to cAMP binding. Thus, S128A and S128P are insensitive to protease digestion even in the presence of cAMP. These results, in combination with the fact that S128A and S128P are *crp*$^-$ *in vivo*, strongly imply that subunit realignment is a critical step in CRP activation. This is in complete agreement with the observation that, unlike cAMP, C-6-substituted cAMP analogues also fail to elicit the conformational changes in CRP needed for proteolytic digestion and activation of CRP for specific DNA binding (Ebright *et al.*, 1985). Mutation of serine 128 and modification of the C-6 of cAMP both affect the same interaction: hydrogen bonding between the hydroxyl group of serine 128 and the C-6 $NH_2$ group of cAMP.

Consistent with the *in vivo* observation, S128A binds to a specific DNA sequence with poor affinity in the presence of cAMP, and S128P displays no measurable affinity toward a specific DNA under same experimental conditions. These results again demonstrate that serine 128 is functionally essential for CRP activation. Having excluded the possibility of incorrect folding of the mutant proteins, the inactivation of CRP mutants could be due to one of the following possibilities: incapability of proper intersubunit realignment, loss of proper interdomain interactions, or both. It has been demonstrated that the subunit realignment ability of the serine mutants is perturbed; therefore, the question is whether the ability to undergo interdomain interaction is also disturbed in these mutants.

To address this question, the cysteine 178 residue was labeled in the wild-type CRP and mutants with a fluorescein probe. The change in emission intensity of the fluorescence probe in response to cyclic nucleotide binding was followed. Since cysteine 178 is located at a position just before the DNA-binding helix, and there is no direct contact between cyclic nucleotides and the DNA-binding domain, the fluorescence signal changes should reflect the interdomain communication induced by cyclic nucleotide binding. This is further confirmed by the fact that the changes in the fluorescence signal can be reversed by decoupling the covalent linkage between the cAMP- and DNA-binding domains by proteases that cleave the CRP at the C α-helix in solution. The quantitative and qualitative nature of the response of CRP−IAF to cAMP is very revealing. Equilibrium association constants for cAMP binding that were identical to those determined by other independent techniques were obtained by monitoring the change in fluorescence intensity of CRP−IAF. These results demonstrate that signal change of the fluorescein probe attached to cysteine 178 is reflective of a quantitative response of the DNA-binding domain to the binding of cAMP to the N-terminal domain. Interestingly, the fluorescence probe attached to cysteine 178 of the wild-type CRP also quantitatively responds to the binding of cGMP, yielding equilibrium binding constants that are in agreement with those previously reported (Takahashi *et al.*, 1980). These results suggest that cGMP can also induce domain−domain rearrangement in CRP. However, unlike cAMP, at low concentrations (<200 μM) cGMP causes a quenching of the fluorescence signal. This difference in the qualitative nature of the response to cAMP and cGMP implies that the specific conformational changes that these cyclic nucleotides impose on CRP are different. In addition to domain−domain rearrangement, cAMP can also induce subunit−subunit realignment in CRP, which cGMP is incapable of doing. In other words, the apparent differences in the change of fluorescence intensity induced by cAMP and cGMP binding reflect the difference in the conformational changes induced by cAMP and cGMP at the subunit interface.

If the preceding explanation were correct, one might predict the response of the fluorescence probe, attached to cysteine 178 of the S128A mutant, to the binding of cAMP and cGMP. Since the proteolytic digestion experiments show that the substitutions of serine 128 disrupt the ability of the subunit interfaces to be realigned by cAMP in the mutants, this implies that the S128A mutant−cAMP complex most likely would assume a conformation analogous to that of CRP−cGMP. The predicted response is then a quenching of the fluorescence signal at low cAMP concentrations. Indeed, that is exactly what was observed experimentally. These results indicate that mutation of residue 128 does not impair interdomain communication. Thus, the significantly reduced affinity of the mutants for DNA is the consequence of impaired intersubunit communication, preventing proper subunit realignment that is essential for DNA binding.

Although mutants S128A and S128P interrupt the hydrogen-bonding interaction between serine 128 and cAMP observed in wild-type CRP, this perturbation does not affect the binding of the first cAMP molecule. However, elimination of this hydrogen bonding by mutagenesis results in a significant increase in the negative cooperativity in the binding of the second cAMP molecule. This observation suggests that the hydrogen bonding between serine 128 and cAMP is important for subunit communication. Most previous cAMP-binding studies did not involve linkage analysis and, thus, failed to detect the change in cAMP-binding cooperativity that is important for CRP function (Gronenborn *et al.*, 1988; Lee *et al.*, 1994). In the search for a structural explanation for the observed increase in negative cooperativity, it is interesting to note that there is a hydrogen bond between the pair of serine 117 residues in the two subunits. Since serine 128 is exactly three turns away from serine 117 in the C-helix, serine 128 residues can also potentially form a hydrogen bond to each other in the free CRP. If this is the case, then serine 128 does not contribute energetically to the binding of the first cAMP simply because the binding of cAMP involves replacing the hydrogen bond between the serine 128 pair with a new

hydrogen bond between serine 128 and cAMP. However, binding of the second cAMP in the wild-type CRP results in the net formation of a hydrogen bond between cAMP and serine 128 that is lacking in both the S128A and S128P mutants. This difference could be used to explain why binding of the second cAMP is weaker in the serine 128 mutants than in the wild type. Experiments to test this hypothesis are currently in progress.

In summary, the results from this study suggest that, while the structural integrity and the ability for interdomain communication of the CRP are preserved in S128A and S128P, subunit communication is perturbed so much that the essential and correct subunit realignment cannot be executed. This impairment leads to the inactivation of the mutant proteins, further suggesting that the subunit realignment mediated by serine 128 is one of the crucial conformational steps involved in CRP activation. These studies on mutants S128A and S128P also imply that proper subunit communication is one of the mechanisms for CRP to discriminate between cyclic nucleotides such as cGMP and cAMP. Although cGMP can bind to CRP and induce domain−domain rearrangement, binding of cGMP does not provide the proper interactions required for subunit realignment, which is necessary for CRP activation.

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