The x-ray crystal structure of the cAMP-ligated T127L/S128A double mutant of cAMP receptor protein (CRP) was determined to a resolution of 2.2 Å. Although this structure is close to that of the x-ray crystal structure of cAMP-ligated CRP with one subunit in the open form and one subunit in the closed form, a bound syn-cAMP is clearly observed in the closed subunit in a third binding site in the C-terminal domain. In addition, water-mediated interactions replace the hydrogen bonding interactions between the N6 of domain. In addition, water-mediated interactions replace the closed subunit in a third binding site in the C-terminal domain. This may be induced by the unique flexibility at Ala128 of the allosterically activated structure than cAMP-ligated CRP. From the Center for Advanced Research in Biotechnology of the National Institute of Standards and Technology and the University of Maryland Biotechnology Institute, Rockville, Maryland 20850

Seung Y. Chu, Maria Tordova, Gary L. Gilliland‡, Inna Gorshkova, Ying Shi, Shenglun Wang, and Frederick P. Schwarz§

The allosteric protein cAMP receptor protein (CRP)1 is activated by the binding of cAMP to enhance the transcription of over 25 genes, which code for enzymes involved in carbohydrate metabolism in Escherichia coli. The x-ray crystal structure of cAMP-ligated CRP consists of two 210-amino acid residue subunits, with the N-terminal domain of each subunit containing a central β-barrel connected to an α/β C-terminal domain via an α-helix, which also is the primary interface between the two subunits (1). The N-terminal regulatory domain β-strands form an eight-stranded β-barrel that binds anti-cAMP, and the C-terminal domain contains a helix-turn-helix motif followed by a small β-sheet that binds to the promoter site. One subunit (A) has a “closed” form, where the C-terminal domain is swung away from the helical interface, and the other subunit (B) has an “open” form, where the C-terminal domain is swung away from the interface (1). In addition to interactions between the CRP binding site and the phosphate ribose of cAMP, Thr127 at the α-helical interface forms a hydrogen bond with the N6 of the bound cAMP, and Ser128 forms a hydrogen bond with the bound cAMP in the other subunit (1). The nature of the conformational change to the allosterically activated conformation has not been determined because the x-ray crystal structure of unligated CRP is not known. Small angle neutron scattering measurements on unligated CRP solutions show that the data best fit the simulated scattering data from a minimized energy structure of CRP with both units in the open form (2). Minimum energy calculations starting with the x-ray structure of both anti-cAMP-ligated subunits in the closed form and both subunits in the open form in solution show that the minimum energy conformation of anti-cAMP-ligated CRP is with both subunits in the closed form (3). In NMR measurements, five sets of histidine resonances are observed for CRP alone and in its complexes with cyclic nucleotides, which also implies that both subunits are in the same conformation in solution (4). The x-ray structure of the cAMP-ligated complex of CRP bound to a 30-base pair DNA duplex, which has the same sequence as that of a promoter site, shows that both subunits are in the closed conformation (5, 6). The implication of these results is that the allosteric activation of CRP to specifically bind to the promoter site involves conversion of both subunits from the open form to the closed form in solution upon cAMP binding.

Mutants of CRP have revealed some interesting alterations of the allosteric activation of CRP, which should exhibit some correlation with this change to the allosterically active conformation of CRP. The T127L (7) and T127C (8) CRP mutants remove the specificity of the activation of CRP by cAMP so that CRP is also activated by the binding of cGMP. A CRP double

1 The abbreviations used are: CRP, cAMP receptor protein; CRP*, the T127L/S128A double mutant of CRP; cGMP, 3′-5′ cyclic GMP; cIMP, 3′-5′ cyclic IMP; DSC, differential scanning calorimetry.
X-ray Crystal Structure of T127L/S128A CRP Mutant

mutant containing the T127L mutation and a S128A mutation (CRP*) activates in vivo and in vitro transcription in the absence of a cyclic nucleotide monophosphate (7, 9). The Thr^{127} mutations appear to alter the conformation of CRP to a conformation close to that of the allosterically activated CRP so that binding of the cAMP is not necessary (10). This is supported by small angle neutron scattering measurements on unligated and cAMP-ligated conformations of CRP* in solution, which show that there is very little conformational difference between the two species, in contrast to shrinkage of the radius of gyration of CRP upon ligation to cAMP in solution (2).

In the present investigation, the x-ray crystal structure of cAMP-ligated CRP* has been determined at 2.2-Å resolution. Although this structure is close to that of the x-ray crystal structure of cAMP-ligated CRP with one subunit in the open form and one subunit in the closed form (1, 11), an additional cAMP (syn-cAMP) is clearly observed bound on the surface of the C-terminal domain of the closed subunit. A similar syn-cAMP binding site on the two closed subunits of a cAMP-ligated CRP-DNA complex was recently reported by Passner and Steitz (12). Previously, only anti-cAMP bound to the sites in the N-terminal domains had been observed in the x-ray crystal structure of cAMP-ligated CRP (1, 11), although an interaction between syn-cAMP and CRP in solution had been earlier observed in NMR measurements on the cAMP-ligated CRP complex in solution (4). The bound syn-cAMP in the cAMP-ligated CRP-DNA crystal structure, which does not appear in the cAMP-ligated CRP structure alone, indicates that the final state of the CRP-DNA complex is one where syn-cAMP is bound between the C-terminal domain and the DNA. Thus, binding of a syn-cAMP to this site on the closed subunit of CRP* in the absence of bound DNA would imply that cAMP-ligated CRP* is in a conformation that is more accommodating for DNA binding than that of cAMP-ligated CRP. Differences between the conformations of cAMP-ligated CRP* and cAMP-ligated CRP are also evident in the binding of cAMP. As the pH is decreased from 7.0 to 5.2, the binding mechanism undergoes a change from an exothermic to an endothermic binding mechanism, an effect similar to that observed by the T127L mutant but not with CRP (13), which retains its endothermic cAMP binding mechanism over this pH range. The thermal denaturation of CRP* exhibits a broad transition similar to the superimposed multi-transition peaks observed for cAMP-ligated CRP but different than that of CRP (14) and its S128A mutant (13), which exhibit a relatively very narrow, single transition peak.

EXPERIMENTAL PROCEDURES

Materials—The production from E. coli of CRP and mutants and their purification have been described previously (7), and their activities were checked by an in vitro transcription assay as described by Zhang et al. (15). The concentrations of CRP and CRP* were determined from UV measurements at 280 nm using an extinction coefficient of 3.5 × 10^4 M^-1 cm^-1 (16). The glycerol, potassium phosphate salts, KCl, HEPES, Tris, MgCl2, and sodium salt of cAMP were reagent grade from Sigma.2 The diithiothreitol was Ultra Pure brand from Life Technologies, Inc. and does not imply that the materials, instruments, or equipment identified is necessarily the best available for the purpose.

X-ray Data Collection and Processing—The crystal was picked from a crystallization droplet using a CryoLoop (Hampton Research) and dipped in a cryostabilizing solution containing 30% (v/v) glycerol and placed directly in a nitrogen cryostream (Oxford Cryosystems) operating at 100 K. Crystals were placed on a Bruker Analytical x-ray sytextms, Inc. goniostat mounted on a Bruker Analytical X-ray Systems, Inc. rotating anode x-ray generator operating at 80 kV and 40 mA. The diffraction images were collected with the HI-STAR electronic area detector placed 13 cm from the crystal positioned with 2° per bin to intercept diffraction data from θ = ~1.8 Å resolution. The electronic images were collected using the Bruker FRAMBO program and were processed using the X-GEN program package (Molecular Simulations Inc.). The crystal was orthorhombic with space group P2₁2₁2₁, and unit cell dimensions a = 46.1 (Å), b = 93.1 (Å), and c = 104.4 (Å). A total of 177,444 observations were reduced to 38,054 unique reflections with the R_{free} = 0.088 with a redundancy of 4.7 at 1.92 Å, the completeness to 97.7% to 2.0 Å resolution. The average reflection I/σ(I) values were 5 and 2 at 2.69 and 2.22 Å resolution, respectively.

Crystallographic Refinement—The initial phases for the structure determination were obtained from an automated molecular replacement (17) molecular replacement solution using a previously reported CRP structure (11). Protein Data Bank (15) identifier 3GAP, which has the same space group but different unit cell parameters, a = 46.5 (Å), b = 96.7 (Å), and c = 105.3 (Å). Rigid body refinement was applied to the molecular replacement solution followed by maximum likelihood torsion angle refinement with a simulated annealing protocol using Crystalllography and NMR System Program version 0.9a (19). The resulting structure had an r of 0.336 and an R_{free} of 0.379. The phases from this model were used with the automated refinement package ARW/wARP (20) to continue the maximum likelihood refinement. The resulting model (r = 20.7 and R_{free} = 32.1) was subsequently refined using Shel97 (21) to r = 0.227 and R_{free} = 0.300. A final refinement was carried out that included all reflections even the reflections excluded for R_{free} to generate the final model with an r = 0.228. The same set of reflections was used for R_{free} monitoring for different refinement packages so that no bias in R_{free} values was introduced.

Structure Analysis—The electron density maps were visualized, and the model was adjust/built according to the density using TURBO-FRODO (22). The stereochemistry of the final model was checked using PROCHECK (23), and the alignments of the structures used in comparison were done using ALIGN program (24). Molecular images for the figures were generated using programs MolScript (25) and Raster3D (26).

Isothermal Titration Calorimetry—All calorimetric titrations were performed according to the methods of Wiseman et al. (27) and Gorshkov et al. (28) using a Microcal Omega titration calorimeter. The Omega titration calorimeter consists of a sample vessel (1.374 ml) containing the protein solution and a matched reference vessel (1.374 ml) containing the buffer solution. A separate titration of the ligand solution into the buffer was performed to determine any slight volume changes between the protein concentration of 0.05–0.1 mM in the sample vessel were added 3–4 min apart. A separate titration of the ligand solution into the buffer was performed to determine any ligand heat of dilution, which was then subtracted from the heats obtained during the titration of the ligand solution into the protein solution.

A nonlinear, least squares minimization performed by Microcal Origin scientific plotting software was used to fit the incremental heat of the ith titration (ΔH(i)) of the total heat, Q (29)

\[ \Delta H(i) = Q(i) = \Delta CV(i) [Q(i) + Q(i-1)] - Q(i-1) \] (Eq. 1)

where V is the volume of the sample solution. For an independent two-site binding model 3

\[ nC_2 + \Delta H_2/V = 1 + [X]/[nC_2] + 1nK_2C_2([X]/[nC_2])^3/2 \] (Eq. 2)

where n = 2, the stoichiometry of the binding reaction, [C] is the total concentration in the sample vessel, \( \Delta H_2 \) is the binding enthalpy, \([X]\) is the total cAMP concentration, and \( K_2 \) is the binding constant. For the interacting two-site model,

\[ Q_3 = C_3[V(K_3[X]/[nC_3]) + 1 + K_3/[2 + ([X]/[nC_3])^3/2] \] (Eq. 3)
X-ray Crystal Structure of T127L/S128A CRP Mutant

**Table I**

| CRP     | cAMP | pH | Temperature | $K_b(1)/K_b(2)^a$ | $-\Delta G_o^o$ | $\Delta H_o^o$ | $\Delta S_o^o$ |
|---------|------|----|-------------|-------------------|-----------------|----------------|----------------|
| CRP     | cAMP | 5.2| 24.5        | 2.0 ± 0.3         | 24.4 ± 0.3      | -5.9 ± 0.5     | 62 ± 2         |
| CRP     | cAMP | 5.2| 24.0        | 0.82 ± 0.07       | 22.3 ± 0.2      | 30.4 ± 0.9     | 177 ± 3        |
| CRP     | cAMP | 7.0| 24.0        | 0.19 ± 0.03       | 18.6 ± 0.3      | 4.8 ± 0.4      | 78 ± 2         |
| CRP     | cAMP | 5.2| 24.0        | 0.71 ± 0.03       | 22.1 ± 0.3      | 8.6 ± 0.6      | 103 ± 2        |
| CRP     | cAMP | 7.0| 24.0        | 2.9 ± 0.4         | 25.5 ± 0.3      | -5.6 ± 0.6     | 67 ± 18        |

*These are the on-site binding constants where $K_b(1)$ is to the first site and $K_b(2)$ is to the second site. The first line of data for each cAMP-ligated CRP mutant complex contains the binding data to the first site, and the second line contains the binding data to the second site. The CRP entries are from Ref. 13.*

where

$$P = 1 + K_o(1)[X] + K_o(1)K_o(2)[X]^2$$

(Eq. 4)

$K_o(1)$ and $\Delta H_o^o(1)$ are the binding constant and the enthalpy for binding to the first site, and $K_o(2)$ and $\Delta H_o^o(2)$ are the binding constant and enthalpy for binding to the second site. The Origin program yields the on-site binding constants and, thus, the binding constants reported in this paper are the on-site binding constants. The macroscopic binding constants to the first site and to the second site are, respectively, $2K_o(1)$ and $K_o(2)/2$ where for the identical site model, $K_o(1) = K_o(2)$. The binding entropies, $\Delta S_o^o$, were calculated using the following equation of thermodynamics:

$$\Delta S_o^o = (\Delta H_o^o - \Delta G_o^o)/T$$

(Eq. 5)

The combined standard uncertainties in the $K_o$ and in $\Delta H_o^o$ were estimated to be each 1.1% from standard uncertainties in $[C]$, $[X]$, $Q$, and $V$. This accounts for the S.D. in the mean value of $\Delta H_o^o$ from several titration scans. However, this combined estimated uncertainty is less than the S.D. in the mean values of $K_o$ from several titration runs as shown in Table I.

**Differential Scanning Calorimetry**—All DSC measurements were performed using a Hart 7707 DSC heat conduction scanning microcalorimeter as described by Schwarz and Kirchhoff (30). The Hart DSC consists of three removable vessels containing the protein solution and a fourth removable vessel containing just the buffer solution in an adiabatic enclosure. The sample size was 0.500 g, and the samples were scanned at a typical scan rate of 15 K h⁻¹ from 30 to 90 °C. The software program EXAM (31) was used to subtract the thermal power versus temperature scans of buffer versus buffer from the solution versus buffer scans and to divide the subtracted scans by the scan rate to obtain the net heat capacity versus temperature scans. EXAM was used to extrapolate a sigmoidal base line under the transition peak and to fit the two-state transition model to the data points to obtain the transition temperature ($T_m$, the temperature at half the peak area), a van’t Hoff enthalpy ($\Delta Hv H$) for the transition and an area for the transition peak. The transition peak area divided by the number of moles of protein in the sample yielded the calorimetric enthalpy, $\Delta Hv H$.

The standard uncertainty in $T_m$ determined from imprecision in the temperature readings and imprecision in the fractional areas under the transition peak is estimated to be ±0.1 K. The combined estimated uncertainty in the van’t Hogg enthalpy from imprecision in the fractional area under the transition peak is 3%. The combined estimated uncertainty in $\Delta Hv H$ contains uncertainty contributions from the area under the transition peak, the concentration of protein, the sample mass, and the heat calibration of the DSC and is 3.2%. As shown in Table II, the S.D. of the mean values of $\Delta Hv H$ and $\Delta Hv H$ is greater than the combined estimated uncertainties of these values.

**RESULTS**

**Three-dimensional Structure of CRP**—The final model of the CRP structure contains 208 and 205 of the possible 210 residues for the closed subunit A and the open subunit B, respectively. There are 216 water molecules and three cAMP molecules associated with the two subunits in the asymmetric unit of the crystal. Each subunit of the CRP molecule is composed of two domains, the C-terminal DNA-binding domain with a helix-turn-helix motif together with a four-strand β-sheet and the N-terminal domain composed of β-strands forming a β-barrel motif that binds anti-cAMP and a long helix forming the dimer interface. Fig. 1 shows the overall structure of CRP with the bound cAMP molecules. The two domains are connected via a flexible hinge comprised of residues Phe₁³⁸–Thr₁⁴₀. Each subunit has 10 residues at both the N and the C termini with weak electron density and correspondingly high temperature factors. The residues near Pro₁⁵⁵ and Met₁⁶³ in the closed conformation (subunit A) have weak electron density; thus, their interpretation is tenuous, whereas the same regions were clearly interpretable in the open conformation (subunit B). The density for two anti-cAMP molecules bound in the N-terminal regulatory domains of the subunits and an additional syn-cAMP at the hinge region were clearly observed. From the PROCHECK (23) analysis, the overall geometry of the polypeptide chain were within acceptable limits, and the Ramachandran plot (32) shows that 94.1% of the residues are within the most favored region and the remaining residues in the additionally allowed regions.

In the comparison of the two subunits of the CRP in Fig. 2, only the N-terminal domain of each subunit was used in alignment, because the N-terminal domains of both subunits are nearly identical, and the resulting rotation and translation matrixes were applied to the entire subunit. The apparent difference between the open and closed conformations is observed in the C-terminal domain and seems to result from rotation of the domains around residue 128. Alignment of just the C-terminal domains of each CRP subunit showed that the two C-terminal structures alone were nearly identical.

**Comparison with the Wild Type**—The structure reported here for CRP is the first CRP structure that includes the additional binding site occupied by syn-cAMP in the closed subunit in the absence of DNA. The initial concentration of cAMP is 5.0 mM, which is higher than the concentration of 0.5 mM used in the crystallization of CRP (11). Fig. 3 shows a comparison between the subunit structures of the 3GAP wild type (11) and the CRP structures using the N-terminal domains as the reference because the structures of the N-terminal domains of wild type CRP and CRP subunits are nearly identical. A comparison of the structure of the C-terminal domain of the CRP closed subunit with the structure of the C-terminal domain of the 3GAP closed subunit does not show any significant differences between the two domains. The regions showing any small differences between the C-terminal domains of the closed subunits of CRP and 3GAP contain residues with poor electron density. The largest difference between the overall CRP and the 3GAP structures is observed in the open subunits near the anti-cAMP binding site at residue 128. This difference, caused by the presence of a water molecule (discussed below) in the binding pocket at residue 128, swings the C-terminal domain of the open subunit in CRP closer in to the N-terminal domain.

**Cyclic AMP Binding Sites**—In a comparison of the two anti-
cAMP binding sites in the regulatory N-terminal domains with other structures (5, 6, 11) in Fig. 4, the only observable differences in the anti-cAMP binding site are at the mutation sites. In the wild type CRP molecule, the N\textsuperscript{a} atom of the purine ring of cAMP is coordinated by OG1 atom of Thr\textsuperscript{127} and OG atom of Ser\textsuperscript{128} residues. However, in the CRP* structure, these residues are changed to leucine and alanine, respectively, resulting in a loss of the hydrogen bonding interactions between the protein and the cAMP. Partially compensating for the loss of these interactions is a water molecule bound at the position of the OG atom of the wild type Thr\textsuperscript{128} residue coordinating the cAMP ligands are the OG atom of the wild type Thr128 residue coordinating the OG1 atom of Thr127 and OG atom of Ser128 residues. However, in the CRP* structure, these residues are changed to leucine and alanine, respectively, resulting in a loss of the hydrogen bonding interactions between the protein and the cAMP. Partially compensating for the loss of these interactions is a water molecule bound at the position of the OG atom of the wild type Thr\textsuperscript{128} residue coordinating the N\textsuperscript{a} atom of cAMP. To accommodate this water molecule, the residues near Ala\textsuperscript{129} of the mutant protein have shifted resulting in flexibility of the C α-helix that is responsible for the difference in the C helices between the wild type and the mutant shown in Fig. 3.

In CRP*, the syn-AMP binding site is only observed in the closed subunit and is composed of the residues Gly\textsuperscript{56} and Lys\textsuperscript{57} to Met\textsuperscript{199} and Gln\textsuperscript{170}, Gln\textsuperscript{174}, and Gln\textsuperscript{177} to Arg\textsuperscript{180} (Fig. 5). The nitrogen atoms, N and NE, from Arg\textsuperscript{180} coordinate the two axial oxygen atoms of the phosphate group of the syn-cAMP. Additional interactions are observed between an axial oxygen atom of the ligand and the NE2 atom of Gln\textsuperscript{170}. The N\textsuperscript{a} atom of the purine ring of the syn-cAMP interacts with the backbone carbonyl O atoms from the residues Gly\textsuperscript{177}, Ala\textsuperscript{135} and Phe\textsuperscript{136} where Ala\textsuperscript{135} and Phe\textsuperscript{136} are from the hinge region of the other subunit. The O\textsubscript{\textsuperscript{a}} atom of the ribose of syn-cAMP interacts with the N atom of Phe\textsuperscript{58}. The same region in the open subunit could not accommodate a syn-cAMP molecule because residues Gly\textsuperscript{177} and Cys\textsuperscript{178} would sterically hinder binding. In a comparison of the closed subunit of the CRP* structure with the 2CGP structure from Passner and Steitz (12), very few differences were observed in the N-terminal regulatory domains and only small differences were observed in the four \(β\)-strands in the C-terminal DNA-binding domains. The same residues interacting with the syn-cAMP in the two closed subunits of the 2CGP structure are also observed to interact with the syn-cAMP in the closed subunit of CRP*. When DNA is bound to the CRP molecule, the syn-cAMP molecule is positioned to interact with the backbone of the DNA in 2CGP (12).

**Isothermal Titration Calorimetry Scans**—A comparison of the binding isotherms in Fig. 6 shows that the cAMP binding reaction to CRP* changes from an exothermic binding process at pH 7.0 to an endothermic binding process at pH 5.2. This unique allosteric dependence on pH was also observed for the T127L mutant but not with the wild type CRP and its S128A mutant (13). The solid lines in the binding isotherms indicate that the binding of cAMP to CRP* at pH 7.0 follows an identical, independent two-site mechanism as observed for S128A at both pH levels, while at pH 5.2, the binding mechanism follows an interacting two-site binding mechanism, as observed for CRP at both pH levels (13). Each solid line generated by the binding mechanism (Equations 1–3) fitted quite well to the binding isotherms as shown in Fig. 6, although they were performed without considering the third cAMP binding site in CRP*. Apparently, there is either a very small contribution of the binding enthalpy from this third site or the binding affinity is much weaker than to the cAMP binding sites in the N-terminal domains. The binding parameters for CRP* and CRP are presented in Table I. At pH 5.2, CRP*, like that of CRP at pH 7.0 (28), the interacting binding site mechanism exhibits positive cooperativity in that binding to the second site is

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**Table II**

| CRP/Mutant | \(T_m\) (°C) | \(\Delta_m H_m\) (kJ mol\(^{-1}\)) | \(\Delta_m H_c\) (kJ mol\(^{-1}\)) | \(\Delta_m H / \Delta_m H_c\) |
|------------|----------------|-----------------|-----------------|-----------------|
| CRP        | 65.6 ± 0.3     | 1054 ± 104      | 328 ± 7         | 0.31 ± 0.03     |
| cAMP-ligated CRP\(^a\) | | | | |
| Transition 1 | 67.1–70.8     | 469 ± 12        | 602 ± 28        |                |
| Transition 2 | 71.5–82.6     | 795 ± 12        |                |                |
| Transition 3 | 75.6–89.4     | 477 ± 24        |                |                |
| CRP\(^b\) | (15 K h\(^{-1}\)) | 65.9 ± 0.1      | 630 ± 30        | 0.28 ± 0.03     |
|            | (8 K h\(^{-1}\)) | 64.8 ± 0.6      | 575 ± 30        | 0.35 ± 0.06     |
|            | (30 K h\(^{-1}\)) | 68.0 ± 2.0      | 600 ± 30        | 0.31 ± 0.04     |

\(^a\) Three transition peaks are observed for cAMP-ligated CRP, and these results are from Ref. 14.

\(^b\) For CRP*, first entry values were determined at different scan rates as indicated. Ghosaini et al. (14) found that the CRP DSC results were also independent of scan rate.
enhanced by binding to the first site. At pH 5.2, CRP, however, exhibits negative cooperativity, where binding to the second site is reduced by binding to the first site. The change in the cAMP binding mechanism to CRP* from an exothermic binding mechanism at pH 7.0 to an endothermic binding mechanism at pH 5.2 undoubtedly results from protonation of amino acid residues distributed on the surface of CRP*. The most likely amino acid residues that would protonate in this 5–7 pH range are the six histidine residues distributed on the surface of each subunit. In contrast, CRP retains its endothermic binding mechanism, and S128A retains its exothermic binding mechanism at both pH levels and, thus, protonation of the amino acid residues on the surface of the protein has little effect on the cAMP binding mechanism. The effect of protonation on CRP* is apparently coupled to the conformational changes on the surface of the C-terminal domain that also induce binding of the syn-cAMP ligand to the surface of the closed subunit.

DSC Scans—Typical DSC scans of CRP and CRP* are presented in Fig. 7, which were scanned at a concentration of 0.16 mM in 50 mM potassium phosphate buffer containing 0.5 M KCl and a scan rate of 15 K h\(^{-1}\). The transitions did not reappear upon a rescan of the samples, but the transition temperature and enthalpies were independent of scan rate from 5 K h\(^{-1}\) to 30 K h\(^{-1}\). This justified applying a two-state reversible thermodynamic transition model to the data to obtain the transition quantities in Table II. Also, presented in Table II are the

Fig. 2. Comparison of the two subunits of CRP* where green is the closed subunit and yellow is the open subunit. Two relative positions of the cAMP molecules are shown and the CA atom of the residue Ala\(^{128}\) is shown (CA 128).

FIG. 2. Comparison of the two subunits of CRP* where green is the closed subunit and yellow is the open subunit. Two relative positions of the cAMP molecules are shown and the CA atom of the residue Ala\(^{128}\) is shown (CA 128).

Fig. 3. Coordination of anti-cAMP by CRP and CRP*. The yellow trace is the 3GAP structure (11), and the blue trace is the CRP* structure. The Ala\(^{128}\) residue responsible for the different orientation of the open subunit of the CRP* structure is indicated by the sphere in the C\(\alpha\)-helix. The syn-cAMP observed in only the CRP* structure is shown at the top of the structures.

FIG. 3. Coordination of anti-cAMP by CRP and CRP*. The yellow trace is the 3GAP structure (11), and the blue trace is the CRP* structure. The Ala\(^{128}\) residue responsible for the different orientation of the open subunit of the CRP* structure is indicated by the sphere in the C\(\alpha\)-helix. The syn-cAMP observed in only the CRP* structure is shown at the top of the structures.

Fig. 4. Comparison of the anti-cAMP binding sites of the wild type CRP 1BER (6), 2CGP (12), and 3GAP (11) structures in light gray backbone tracings with that of CRP* in the dark opaque backbone tracing. The two cAMP molecules and the water molecules in red are from the CRP* structure.

Fig. 5. Comparison of the residues of the syn-cAMP binding site with the lighter tracing for the 2CGP (12) structure and the darker tracing for the CRP* structure. The atoms involved in the interaction between the protein and the ligand are as indicated, and the bound DNA in the 2CGP structure is not shown.

FIG. 5. Comparison of the residues of the syn-cAMP binding site with the lighter tracing for the 2CGP (12) structure and the darker tracing for the CRP* structure. The atoms involved in the interaction between the protein and the ligand are as indicated, and the bound DNA in the 2CGP structure is not shown.
transition quantities for the multiphase transitions observed for cAMP-ligated CRP (14). As shown in Fig. 7, the transition peak observed for CRP is about twice as broad as that observed for wild type CRP and is indicated by the ratio of the van’t Hoff enthalpies of 1.6:1 for CRP:CRP*. The broadness of the transition peak may be due to two overlapping transitions in this temperature region, and in fact, the transition could be resolved into a small transition with \( \Delta_H = 800 \text{ kJ mol}^{-1} \) and \( T_m = 62.1 ^\circ \text{C} \) and a larger transition with \( \Delta_H = 722 \text{ kJ mol}^{-1} \) and \( T_m = 65.8 ^\circ \text{C} \). DSC scans of the T127L mutant at pH 5.2 exhibit two overlapping transitions, whereas CRP exhibits only a single transition peak at this pH (13). The broad transition of CRP does confirm that the structural interactions, such as domain-domain interactions, in CRP are weaker than those observed for the unligated wild type, where only a single narrow transition is observed, but stronger than those observed for cAMP-ligated CRP, where three transitions are observed. Thus, the conformational changes induced by the mutations produce structural interactions within the protein that approach those observed in the allosteric-activated conformation of cAMP-ligated CRP.

FIG. 6. Binding isotherms from isothermal titration calorimetry measurements at 24 °C on the binding of cAMP to CRP* at pH 7.0 with 5-μl aliquots of 2.6 mM cAMP titrated into 0.14 mM CRP* (a) and at pH 5.2 with 5-μl aliquots of 10 mM cAMP titrated into 0.15 mM CRP* (b). The molar ratio is the number of moles of cAMP per number of moles of CRP* in the sample vessel.

FIG. 7. Comparison of the DSC scans for 0.18 mM CRP* and 0.18 mM CRP in phosphate buffer at pH 7 and 0.5 M KCl. The areas under the transition peaks are equal for the 0.5-g samples, and the scan rate is 15 K h⁻¹.

X-ray Crystal Structure of T127L/S128A CRP Mutant

Because CRP* can be activated at low cAMP concentrations in vivo and in vitro, the implication is that in solution, CRP* is close to the allosteric conformation necessary for promoter binding (13). This is supported by small angle neutron scattering measurements, which exhibit little difference between the cAMP-ligated and unligated CRP* conformations in contrast to the difference observed between the cAMP-ligated and unligated wild type CRP conformations in solution (2). Although the overall x-ray crystal structure of cAMP-ligated CRP* is very similar to that of the wild type protein (11) with a root mean square deviation of 0.998 between the two structures and with one subunit in the open conformation and the other in the closed conformation, there are two subtle differences: (i) the closed conformation of CRP* is observed with an additional syn-cAMP bound in a site on the surface of the C-terminal domain and (ii) the C-terminal domain in CRP* is shifted closer to the N-terminal domain than in the corresponding open subunit of the cAMP-ligated wild type CRP structure. Because the final conformation of CRP in the 2CGP promoter-bound state (12) consists of both subunits in the closed conformation with syn-cAMP bound at the same site now observed in the CRP* structure, the conformation of cAMP-ligated CRP* in the crystal state is closer to the promoter-bound state than that of the cAMP-ligated CRP* in the crystal state.

The alanine substitution at 128 introduces a flexibility in the C α-helix that is responsible for reorienting the C-terminal domain toward the N-terminal domain in the open subunit of CRP* (Figs. 2 and 3). There are water-mediated interactions between the N6 of anti-cAMP and the Leu¹²⁷ and Ala¹²⁸ mutations, which replace the hydrogen bonding interactions between N6 and the OH groups of Thr¹²⁷ and Ser¹²⁸. To accommodate the bound water molecule in CRP*, the residues near Ala¹²⁸ have shifted, resulting in the flexibility of the C α-helix that is responsible for the observed conformational difference between the open subunits of CRP* and CRP. Because N6 is replaced by a carbonyl group in cGMP and cIMP, these hydrogen bonding interactions are removed, and, indeed, cGMP and cIMP do not allosterically activate CRP, which further substantiates the importance of the interaction between cAMP N6 and Thr¹²⁷ and Ser¹²⁸ in shifting the conformation of CRP to the allosterically activated form. Because these water molecules can also accommodate cGMP and cIMP in CRP*, the specificity requirement for cAMP in the allosteric activation of CRP can be fulfilled by cGMP and cIMP provided that the allosteric conformational change is indeed induced by the flexibility in the C α-helix at Ala¹²⁸. The tendency for water molecules to maintain the structural integrity of ligated proteins that have been mutated at the binding site has also been observed in lysozyme-antigen-Fv antibody complexes (33) where replacement of tryptophan in the Fv binding site by smaller side chain amino acid residues induces water molecules to fill the void to maintain
In another scenario, the conformational differences between cAMP-ligated CRP* and cAMP-ligated CRP, which favor the active conformation can result from a shift in the C-terminal domain induced by the binding of syn-cAMP near the hinge region of CRP*. Observations that mutations at the hinge residue 138 and D-helix residues 141 and 144, which result in activation of CRP in the absence of cAMP, like that of CRP*, would indicate the direct involvement of the hinge region and D-helix in the allosteric conformational change (34–36). A close van der Waal's distance of 4.25 Å is observed between the C² atom of the cAMP purine ring and the O⁶ atom of the bound DNA backbone at the syn-cAMP binding site in the wild type cAMP-ligated CRP-DNA complex (12). In addition, the N⁶ atom of the syn-cAMP is tightly coordinated by the carbonyl O atom of the Ala¹³⁵ residue in this complex. Additional hydrogen bonding interactions are provided by the carbonyl O atom of the Gly¹⁷⁷ residue. The absence of allosteric activation of CRP by cGMP would result from lack of cGMP-binding to this site because the carbonyl oxygen on the C⁶ atom of the cGMP purine ring would result in an unfavorable hydrogen bonding interaction between cGMP and the O atom of the Ala¹³⁵ residue. Because the allosteric conformational change entails a shift in the hinge region and the rest of the C-terminal domain in CRP*, it is possible that such a large movement may result in the shift of the critical discriminating Ala¹³⁵ residue for cAMP specificity far enough away to accommodate the otherwise unacceptable cGMP ligand in the syn-cAMP binding site. The additional flexibility at the Ala¹²⁸ residue in CRP* would further favor a shift of the hinge and the C-terminal domain. Therefore, the key discriminatory interaction between the N⁶ atom of the cAMP and Ala¹³⁵ would be abolished in CRP* and, thus, either a cIMP or cGMP can be accommodated for activation. In this scenario, the anti-cAMP bound in the N-terminal domain of the CRP molecule may not be directly involved in the allosteric conformational change and thus may not be essential. In support of this scenario, there is evidence that only a single cAMP ligand is necessary for the allosteric activation of CRP (10, 37). However, the anti-cAMP ligands may be essential for subunit assembly because in CRP subunit exchange assays, Brown and Crothers (38) showed that CRP subunits did not undergo exchange in the presence of cAMP with or without bound DNA. Finally, it is possible that both scenarios would contribute to the allosteric activation of CRP*.

The isothermal titration calorimetry results tend to confirm that the conformation of CRP* is unique because a decrease in pH from 7.0 to 5.2 substantially alters the cAMP binding mechanism to CRP*, in contrast to CRP and S128A, which retain their binding mechanisms throughout this pH range. A similar unique dependence was reported for the T127L mutant and attributed to conformational changes in the C-terminal domain, which favor the closed conformation in the mutant (13). For CRP*, which contains the T127L mutation and an additional S128A mutation, the x-ray structure shows that the conformational difference is induced by the flexibility at Ala¹²⁸ and/or the binding of syn-cAMP in the hinge region. The DSC results are also indicative of a conformation for unliganded CRP* in solution that is intermediate between that of unligated CRP and cAMP-ligated CRP, i.e. the thermal unfolding of CRP* consists of a broad or two transitions, whereas that of CRP and S128A consists of one narrow transition and that of cAMP-ligated CRP consists of three transitions. These differences in the thermal unfolding of CRP*, CRP, and cAMP-ligated CRP would imply that the intramolecular interactions of CRP* in solution are intermediate between those of CRP and cAMP-ligated CRP.

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The Structure of the T127L/S128A Mutant of cAMP Receptor Protein Facilitates Promoter Site Binding

Seung Y. Chu, Maria Tordova, Gary L. Gilliland, Inna Gorshkova, Ying Shi, Shenglun Wang and Frederick P. Schwarz

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