Kinetic Studies of cAMP-induced Allosteric Changes in Mutants T127I, S128A, and T127I/S128A of the cAMP Receptor Protein from Escherichia coli*

The cAMP receptor protein (CRP) regulates the expression of several genes in Escherichia coli. The protein is a homodimer, and each monomer is folded into two distinct structural domains. After allosteric transitions resulting from the binding of cAMP, CRP specifically binds to DNA and activates transcription. We have used stopped-flow fluorometry measurements of CRP mutants bearing amino acid substitutions T127I, S128A, and T127I/S128A to study the kinetics of conformational changes in the protein induced by cAMP binding. Amino acid substitutions at positions 127 and 128 were chosen because these residues play a crucial role in interdomain and intersubunit communication during allosteric transition. Using N-iodoacetamidinoethyl-5-naphthylamine-1-sulfonic acid-labeled Cys128, localized in the protein helix-turn-helix motif, we observed conformational changes in the helix-turn-helix, localized in the C-terminal domain, upon binding of cAMP to high affinity sites (CRP-cAMP) in the N-terminal domain of CRP. The rate constants for the forward and backward conformational changes depend on the amino acid substitution: \( k_c = 3.62 \text{ s}^{-1} \) and \( k_c = 3.13 \text{ s}^{-1} \) for CRP T127I and \( k_c = 0.42 \text{ s}^{-1} \) and \( k_c = 0.78 \text{ s}^{-1} \) for CRP S128A. These values can be compared with \( k_c = 9.7 \text{ s}^{-1} \) and \( k_c = 0.31 \text{ s}^{-1} \) for wild-type CRP. The observed conformational changes can be described by the sequential model of allosteroy, with the amino acid substitutions influencing the allosteric changes. In the case of the double mutant, the observed rate constant of cAMP binding supports the suggestion that this unligated mutant possesses the structure that is close to the allosteric conformation necessary for promoter binding. The results of intrinsic fluorescence measurements suggest that the formation of the CRP-cAMP complex results from displacement of equilibrium between the two forms of the CRP-cAMP complex in the mutants studied, similar to wild-type CRP. The observed conformational changes occur according to a concerted model of allosteroy, and isomerization equilibrium between the two CRP states depends on the amino acid substitution. The data presented in this study indicate that Ser128 and Thr127 in CRP play an important role in the kinetics of intramolecular transitions triggered by cAMP.

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The cAMP receptor protein (CRP) regulates the expression of >100 genes in Escherichia coli. CRP is a dimeric protein composed of two identical subunits of 209 amino acid residues with a molecular mass of 22.6 kDa (1, 2). Each subunit is folded into two distinct domains (3). The larger N-terminal domain contains a binding site for cAMP in the anti-conformation, and the smaller C-terminal domain contains a helix-turn-helix (HTH) motif responsible for DNA recognition and binding. The two domains are connected with a short hinge region composed of residues 135–138. Crystal structure studies have shown that the protein additionally possesses a second binding site (located between the hinge and the turn of HTH) that binds cAMP in the syn-conformation (4). Indeed, a recent microcalorimetric isothermal titration calorimetry study has shown that, in solution, CRP sequentially binds two molecules of cAMP with negative cooperativity and high affinity; and additionally, at least one additional molecule binds to a low affinity binding site (5). It is believed that, at ~100 µM cAMP, CRP is able to recognize and bind specific DNA sequences and to stimulate transcription (6), and this active form of the protein is represented by the CRP-cAMP complex with two anti-sites occupied (7, 8). However, in the presence of millimolar concentrations of cAMP, at which the CRP-cAMP complex predominates, there is a loss of affinity for DNA and sequence specificity for its recognition and transcription regulation (7). Therefore, the occupancy of cAMP-binding anti- or syn-sites of CRP may have different effects on the regulatory mechanism of CRP associated with interactions between the protein subunits and domains. The idea of four cAMP-binding sites, viz. two in its anti-conformation operating at low concentrations of the ligand and two additional binding sites active at high cAMP concentrations, has also been used to describe the results of our fast kinetic studies (8) as well as time-resolved anisotropy (9) and fluorescence energy transfer measurements (10). A variety of biochemical and biophysical studies (2) have demonstrated that binding of cAMP allosterically induces CRP to assume a conformation that binds to DNA and that interacts with RNA polymerase (1). However, the details of the mechanism, which mediates the allosteric activation of CRP, remain obscure because the crystal structure of unligated CRP has not yet been elucidated. It is believed that the binding of cAMP involves subunit realignment and a reorientation of the hinge between the protein domains (11). Our recent fluorescence energy transfer studies support this idea and show that the binding of...
Anti-cAMP in the CRP-cAMP$_2$ complex results in the movement of the C-terminal domain of CRP by -8 Å toward the N-terminal domain (10). The binding of cAMP in the CRP-cAMP$_4$ complex effects only a very small increase in fluorescence energy transfer efficiency between fluorescently labeled Cys$_{378}$ and Trp$_{335}$. Biochemical and biophysical studies focused on the identification of structural changes accompanying binding of cAMP to CRP have been mostly performed under equilibrium conditions, and much less attention has been paid to the description of the kinetic mechanism of the ligand interaction with CRP. Our previous stopped-flow studies investigating the kinetics of conformational changes in CRP induced by cAMP binding had shown that, at micromolar cAMP concentrations, allosteric changes take place according to a sequential (Koshland-Némethy-Filmer (KNF)) model, whereas conformational changes observed at millimolar concentrations can be described by a concerted (Monod-Wyman-Changeux (MWC)) model (8). The conformational change observed upon binding of cAMP to anti-sites, described by the KNF model, clearly indicates long-range structural communication between the N- and C-terminal domains of CRP remaining under kinetic control (8). On the other hand, binding of cAMP to syn-sites of CRP, described by the MWC model, results from displacement of equilibrium between the two structural forms of CRP-cAMP$_2$, where only one form is able to form a CRP-cAMP$_4$ complex. Because the four cAMP-binding sites of CRP possess different cooperative affinity for the ligand (8), it can be expected that the kinetically controlled domain-domain and subunit-subunit communication may play a crucial role in the molecular regulatory mechanism of CRP action. In this study, we report a further kinetic description of cAMP-induced conformational changes in CRP based on the use of different mutants (T127I, S128A, and T127I/S128A). It is well known (12–14) that all of these mutants exhibit altered allosteric activation of CRP both in vitro and in vivo (15). Because Thr$_{127}$ and Ser$_{128}$ are directly involved in cAMP binding as well as in intersubunit and interdomain interactions in CRP-cAMP complexes (4, 16), fast kinetic measurements can provide further insight into the allosteric activation of CRP.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-Iodoacetylaminoethyl-5-naphthylamine-1-sulfonic acid (I-EDANS) was purchased from Molecular Probes, Inc. EDTA, dithiothreitol, phenylmethylsulfonyl fluoride, Tris, and cAMP were obtained from Sigma. Fractogel EMD SO$_3^-$, 650 Mw was from Merck, and Q-Sepharose Fast Flow, Sephacryl S-200HR, and Sephadex G-25 were from Amersham Biosciences. The nutrients for bacterial growth were obtained from ICN. All measurements were performed in buffers prepared in water purified with the Millipore system.

**Protein Purification**—CRP S128A was isolated from E. coli strain CA8445 transformed with plasmid pMlc28, which encodes a mutant crp gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene. The mutation at position 127 (threonine replaced by isoleucine) as well as the double mutation T127I/S128A were performed using the gene.

**Stopped-flow Fluorescence Measurements**—The stopped-flow fluorescence experiments were performed using an SX-17 MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK) in the two-slit mode. The dead time of mixing was determined to be <2 ms.

Conformation changes in the proteins induced by cAMP binding were monitored by the fluorescence intensity of I-EDANS covalently attached to Cys$_{378}$ or by the fluorescence intensity of tryptophan residues (Trp$_{335}$ and Trp$_{338}$). CRP covalently labeled with I-EDANS at Cys$_{378}$ (I-EDANS-CRP) was excited at 340 nm, and fluorescence emission was monitored at wavelengths >475 nm using a cutoff filter. The fluorescence intensity of tryptophan residues was observed at >320 nm using a cutoff filter after excitation at 295 nm. All measurements were performed at 20 ± 0.1 °C, and the reaction mixtures contained final concentrations of 1 µM I-EDANS-CRP and ~2 µM cAMP (for tryptophan experiments). Background measurements were also carried out at the beginning of each experiment. The sample was incubated in a stopped-flow syringe for 5 min to allow for thermal equilibration.

Stopped-flow experiments were initiated by mixing equal volumes of protein (fixed concentration) and various concentrations of cAMP. Four-thousand data points were acquired in each stopped-flow experiment. Multiple kinetic runs were summed (10–14) to obtain adequate signal-to-noise ratios. Such averaged kinetic traces were fit to a single exponential or to a sum of such terms (Equation 1),

\[
F(t) = A \exp(-k t) + C
\]

where $F$ is the fluorescence intensity at time $t$; $A$ and $k$ are the amplitude and observed rate constant, respectively; and $C$ is the fluorescence at infinite time. All kinetic traces were analyzed using software supplied by Applied Photophysics. The validity of the fit was evaluated by an inspection of residuals and normalized variation parameters.

**RESULTS**

**Binding of cAMP Monitored by I-EDANS Fluorescence**—The kinetics of the binding of cAMP to the CRP T127I, CRP S128A, and CRP T127I/S128A mutants were measured as changes in the I-EDANS-CRP fluorescence using a stopped-flow method under pseudo first-order conditions. In the absence of cAMP, after excitation at 340 nm, the maximum fluorescence emission of I-EDANS was observed at ~490 nm for all of the labeled CRP mutants. In all cases, after adding cAMP, an increase in I-EDANS fluorescence was observed with increasing ligand concentrations. The maximum increases in the fluorescence intensity of the I-EDANS moiety observed in the kinetic experiments were ~16.5 and ~7% for CRP T127I and CRP S128A, respectively. In addition, a blue shift occurred in the emission spectra, shifting the maximum to ~478 and ~480 nm for CRP T127I and CRP S128A, respectively. The fluorescence intensity changes and the blue shift suggest that the conformational changes induced by cAMP in CRP T127I and CRP S128A are different. In the case of the CRP double mutant, the addition of the ligand did not cause significant changes in the fluorescence intensity of I-EDANS.

The progress curves of the reactions of CRP T127I and CRP S128A with cAMP fit well with the single-exponential model. The addition of a second exponent did not significantly alter the goodness of the fit. The normalized variance value (obtained with the single- and double-exponential fits) indicates that the two-component analysis is not significantly better than the one-component analysis. Also, the distribution of the residuals was not improved upon the addition of the second exponent. Fig. 1 shows a typical kinetic course of the binding reaction.
Allosteric Changes in T127I, S128A, and T127I/S128A of CRP

Fig. 1. Kinetics of cAMP binding to I-EDANS-CRP T127I measured by changes in the fluorescence intensity of the I-EDANS label. Measurements were performed at 20 °C in buffer A in the presence of 1.6 μM I-EDANS-CRP T127I and final cAMP concentrations of 0 μM (●), 30 μM (○), 70 μM (▲), 150 μM (□), and 2.5 mM (■). The solid lines are the single-exponential fits.

between cAMP and CRP T127I. For the CRP T127I and CRP S128A mutants, the observed rate constants (k_{obs}) increased with increasing ligand concentrations and approached a constant value at high millimolar concentrations of cAMP, as shown in Figs. 2 and 3. Thus, they are consistent with our previous kinetic studies using wild-type CRP (8).

As we described previously (8), hyperbolic saturation of k_{obs} versus the cAMP concentration would indicate a two-step binding process. After a very fast binding process, the double-ligated protein undergoes conformational changes, which were observed in our measurements. Thus, the increase in rate constants accompanying the binding of cAMP at micromolar concentrations to CRP S128A and CRP T127I was analyzed in terms of a sequential (KNF) model of allostery, represented by Scheme 1,

\[
K_1 \quad \text{CRP} + \text{cAMP} \rightleftharpoons \text{CRP-cAMP}
\]

\[
K_2 \quad \text{CRP-cAMP} + \text{cAMP} \rightleftharpoons \text{CRP-cAMP}_2
\]

where \( K_1 \) and \( K_2 \) are intrinsic dissociation constants for binding of the first and second cAMP molecules to high affinity (anti) sites, respectively; and CRP and CRP^cAMP represent the protein before and after the conformational change, respectively. \( K_C \) is the equilibrium constant between these two forms, whereas \( k_+ \) and \( k_- \) are rate constants that describe the conformational change step.

If cAMP binding occurs with a simple two-step mechanism, assuming independent binding, as shown in Scheme 1, then the observed rate constant (k_{obs}) should increase with increasing cAMP concentrations according to Equation 2.

\[
k_{\text{obs}} = k_+ + k_2 \frac{[\text{cAMP}]^2}{K_{\text{anti}} + 2K_{\text{anti}}[\text{cAMP}] + [\text{cAMP}]^2}
\]  
(Eq. 2)

In Equation 2, \( K_{\text{anti}} \) has been considered as a geometric average of \( K_1 \) and \( K_2 \). The resultant parameters of fitting the data to Equation 2 are shown in Table I.

Binding of cAMP Monitored by Fluorescence of Tryptophan Residues—The rate of cAMP binding to CRP T127I, CRP S128A, and CRP T127I/S128A was also measured by fluorescence intensity of tryptophan residues using a stopped-flow method. As we have shown previously (8), binding of cAMP to wild-type CRP caused significant changes in the Trp intensity only at high ligand concentrations (>300 μM). Similar observations were made with the CRP S128A mutant. However, pronounced changes in the fluorescence intensity of the tryptophan residues of CRP T127I and CRP T127I/S128A were detected only at very high concentrations of cAMP, viz. 3 and 10 mM, respectively. In all cases, mixing cAMP with CRP T127I, CRP S128A, and CRP T127I/S128A resulted in a time-dependent increase in fluorescence emission by the tryptophan residues. The fluorescence intensity increased by ~5, 9, and 3% for CRP T127I, CRP S128A, and CRP T127I/S128A, respectively. This suggests that the conformational changes induced by cAMP in CRP T127I, CRP S128A, and CRP T127I/S128A are different.

Fig. 4 presents the progress curves for association of CRP T127I, CRP S128A, and CRP T127I/S128A with 10 mM cAMP. For all concentrations of cAMP, the kinetic traces could be fitted well using a single-exponential curve; double-exponential fitting did not improve the goodness of the fit. The rate constants (k_{obs}) calculated from the kinetic traces were plotted against the concentration of the ligand. For all investigated CRP mutants, as the cAMP concentration increased, the observed rate constants decreased, reaching a plateau at very high ligand concentrations, as shown in Fig. 5. A good descrip-
tion of the CRP T127I, CRP S128A, and CRP T127I/S128A interactions with cAMP was obtained using a model that we described in our previous study, where we analyzed the binding of cAMP to wild-type CRP (8). As we have reported, at millimolar cAMP concentrations, CRP undergoes a conformational change according to a concerted (MWC) model of allostery, which can be represented by Scheme 2,

\[
P \xrightarrow[k_{on}]{[cAMP]} P' \xrightarrow[k_{off}]{[H_1]} K_0 \xrightarrow{[cAMP]} P_{[H_2]}\]

where \(P'\) and \(P\) are the binding and non-binding forms of protein; \(K_0\) is the equilibrium constant between these two states; \(k_{on}\) and \(k_{off}\) are rate constants that describe the isomerization step; and \(K_3\) and \(K_4\) are intrinsic dissociation constants for binding of the first and second cAMP molecules to low affinity (syn) sites, respectively. The association of cAMP with CRP is a very fast process, and \(P\) and \(P'\) are two interconvertible forms of the CRP-cAMP\(_2\) complex. The changes in fluorescence intensity of Trp residues upon the addition of cAMP observed in our experiment must be associated with the slow isomerization of the CRP-cAMP\(_2\) complex after its formation. Moreover, as these changes occur at a millimolar concentration

**Table I**

| Protein                 | \(k_{on}\) | \(k_{off}\) | \(K_0\) | \(K_{3\text{syn}}\) |
|------------------------|------------|-------------|---------|------------------|
| Wild-type CRP\(^a\)   | 9.7 ± 0.1  | 0.31 ± 0.05 | 0.032   | 27.5 ± 1.0       |
| CRP T127I              | 3.62 ± 0.25| 3.13 ± 0.28 | 0.86    | 20.1 ± 2.5       |
| CRP S128A              | 0.42 ± 0.12| 0.78 ± 0.13 | 1.86    | 36 ± 24          |

\(^a\) Data are from Ref. 8.

FIG. 3. Observed rate constants \((k_{obs})\) for the association of CRP S128A with cAMP measured by changes in the fluorescence intensity of the I-EDANS label. The solid line is the best fit according to Equation 2, with the parameters of the fit summarized in Table I.

**FIG. 4.** Kinetics of cAMP binding to CRP T127I, CRP S128A, and CRP T127I/S128A measured by changes in the fluorescence intensity of Trp residues. Measurements were performed at 20 °C in buffer B in the presence of ~2 μM protein and a final cAMP concentration of 10 mM. ○, CRP T127I/S128A; □, CRP S128A; ▽, CRP T127I. The solid lines are the single-exponential fits.
of cAMP, they must be associated with binding of the ligand to low affinity sites of the protein.

The observed rate constants ($k_{obs}$) derived from kinetic experiments performed at millimolar cAMP concentrations can be fitted to Equation 3 to yield the kinetic and thermodynamic parameters $k_{on}$, $k_{off}$, and $K_{syn}$.

$$k_{obs} = k_{on} + k_{off} K_{syn} [cAMP] + [cAMP]^2$$  \hspace{1cm} (Eq. 3)

In Equation 3, $K_{syn}$ has been considered as a geometric average of $K_s$ and $K_a$. The kinetic and thermodynamic parameters obtained from fitting the data to Equation 3 are reported in Table II.

**DISCUSSION**

The conformational changes induced by cAMP binding to high affinity anti-sites result in a switch in the CRP from a low affinity nonspecific DNA-binding protein to a state of the protein that binds DNA with high affinity and sequence specificity. It has been accepted that this phenomenon is associated with CRP subunit-subunit realignment and hinge reorientation (11), and our recent experiments have shown that this change is accompanied by an $\sim$8-Å decrease in the distance between the two domains of the protein (10). Our previous kinetic studies have demonstrated that the formation of the CRP-cAMP$_2$ complex, after saturation of high affinity sites, is followed by a conformational change that occurs according to the sequential model of allostery (8). The binding of cAMP to the lower affinity syn-sites in the CRP-cAMP$_4$ complex was accompanied by conformational changes that resulted from displacement of equilibrium between the two forms of CRPcAMP$_2$ and that occurred according to the concerted model of allostery (8). In the present kinetic studies, we used CRP muteins with the single substitutions S128A and T127I as well as a CRP T127I/S128A double mutant. These substitutions confer unique properties on the CRP structure and on the cAMP-mediated activation of CRP as a transcription factor (12–14).

The fluorescence data on CRP muteins presented here confirm and extend our previous kinetic studies on wild-type CRP (8) and show that cAMP binding in the anti-conformation to the high affinity sites of CRP with an amino acid substitution at position 127 or 128 causes an increase in the I-EDANS-labeled Cys$_{178}$ fluorescence, with an accompanying 10-nm blue shift in the maximum of the fluorescence emission at a micromolar concentration of cAMP. However, the increases in the fluorescence intensity of $\sim$16.5 and 7% for CRP T127I and CRP S128A, respectively, depend on the amino acid substitution, and the changes are smaller than those observed in the case of wild-type CRP, i.e. a 25% rise in fluorescence intensity and a 20-nm blue shift in its maximum (8). Therefore, it may be postulated that the environment of Cys$_{178}$, located within the HTH motif of the C-terminal domain of the protein, is perturbed upon binding of cAMP to anti-sites in the N-terminal domain of CRP. Because these two domains are spatially separated, this observation clearly indicates allosteric changes and interdomain cross-talking. However, the magnitude of the observed changes in fluorescence depends on the mutation in CRP. Binding of cAMP to anti-sites of the CRP muteins studied was associated with the 10-nm blue shift in the maximum of

![Image](263x439 to 563x737)

**FIG. 5.** Dependence of observed rate constants ($k_{obs}$) for cAMP-induced changes in the Trp fluorescence intensity of CRP T127I, CRP S128A, and CRP T127I/S128A on the final cAMP concentration. The solid lines are the best fit according to Equation 3, with the parameters of the fit summarized in Table II.

**TABLE II**

| Protein            | $k_{on}$ | $k_{off}$ | $K_s$ | $K_{syn}$ |
|--------------------|---------|-----------|------|-----------|
| Wild-type CRP$^a$  | 28 ± 1.5| 75 ± 5.5  | 2.7  | 2.0 ± 0.2 |
| CRP T127I          | 1.4 ± 0.2| 70 ± 2.1  | 10.1 | 50 ± 2.3  |
| CRP S128A          | 0.6 ± 0.9| 47 ± 5.7  | 7.7  | 2.7 ± 0.7 |
| CRP T127I/S128A    | 8.4 ± 2.5| 50 ± 4.4  | 2.3  | 9.1 ± 7.7 |

$^a$ Data are from Ref. 8.
the I-EDANS-labeled Cys<sup>178</sup> emission, suggesting a decrease in internal mobility and/or increased hydrophobicity of the local surroundings of Cys<sup>178</sup> upon ligand binding.

The fluorescence of EDANS-CRP was used to study the kinetics of the conformational change in the HTH motif due to cAMP binding to high affinity anti-sites of the CRP muteins. The process was studied under pseudo first-order conditions and can be described by a simple exponential. In the case of amino acid substitutions S128A and T127I, the rates of the observed processes increased with increasing ligand concentrations, reaching asymptotic values at cAMP concentrations <1 mM. Such behavior is usually interpreted as evidence of a two-step process, where an association step is followed by a unimolecular conformational change step (21, 24). Therefore, one would expect that, in such a case, the bimodal response should be observed: one step associated with cAMP binding to the tight binding site and the second step associated with the conformational change in CRP. Such an interpretation results from the fact that the association step is much faster than the conformational change step and that the reactions occur on a different time scale and can be treated separately (21). The temperature jump fast kinetic studies (22) of CRP-cAMP interactions show that the bimolecular rate constant for cAMP binding with the high affinity anti-sites is estimated to be \( k_1 = 9.7 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \). This value corresponds to the values of association constants ranging from \( 10^2 \) to \( 10^9 \text{ M}^{-1} \text{s}^{-1} \) that have been found for the binding of several various ligands to a number of different proteins (21, 24). Because the association of cAMP with CRP can be considered a fast process, which is much faster than the stopped-flow device dead time of \( 2 \) ms, one can assume that the observed conformational changes in the CRP S128A and T127I mutants will start from the double-ligated CRP-cAMP<sub>2</sub> complex. Therefore, the observed process of ligand binding to the tight sites can be described as a unimodal conformational change in CRP mutants that occurs according to the sequential (KNF) model of allostery described by Scheme 1. For this process, the calculated rate constant \( k_{\text{obs}} \) should increase with increasing cAMP concentrations, according to Equation 2. In this calculation, because of difficulties with the fitting procedure, the dissociation constant of cAMP \( K_{\text{anti}} \) for high affinity binding sites has been considered as a geometric average of the dissociation constants \( K_1 \) and \( K_2 \) for two cAMP molecules sequentially bound to high affinity sites of CRP. The microscopic association constants \( K_1 \) and \( K_2 \), recently determined (5) by isothermal titration calorimetry to be \( 8 \times 10^4 \) and \( 3.5 \times 10^4 \text{ M}^{-1} \), respectively, give a geometric average dissociation constant \( K_{\text{anti}} \) of \( 19 \mu \text{M} \). This value (determined at \( 25^\circ \text{C} \)) corresponds very well to the average \( K_{\text{anti}} \) values determined in this study (Table I). This agreement further supports the proposed mechanism of the sequential KNF model of cAMP binding to CRP. An analysis of the data according to Equation 2 gives the values for the equilibrium constants \( K_c \) between the two conformational forms of CRP-cAMP<sub>2</sub> as 0.86 and 1.86 for CRP T127I and CRP S128A, respectively. Because the estimated \( K_c \) value for wild-type CRP is low and equal to 0.032, one can expect that, in the wild-type protein, almost all double-ligated CRP would exist in the active conformation (8). Therefore, the amino acid substitution at position 127 can shift the equilibrium between the two conformational forms of CRP toward the low activity conformation. The CRP S128A mutation further switches CRP-cAMP<sub>2</sub> into a much less active conformation. The increase in \( K_c \) upon substitution of Thr<sup>127</sup> and Ser<sup>128</sup> suggests that these mutations led to a decrease in the positive cooperativity of ligand binding. The values of the \( K_{\text{anti}} \) thermo-dynamic constants (Table I) estimated for wild-type CRP as well as for CRP T127I and CRP S128A are similar and further support the validity of the employed model. In the case of the CRP T127I/S128A double mutant, the fluorescence intensity of EDANS-CRP did not change significantly at any cAMP concentration. This suggests that the protein possesses the conformation of the HTH motif, which is able to recognize the specific sequences of the promoter DNA. This suggestion is supported by the observation that a CRP double mutant containing both the T127I and S128A mutations activates transcription \textit{in vivo} and \textit{in vitro} in the absence of cyclic nucleotide monophosphate (14).

It is known that substitutions T127I (13) and S128A (12) in CRP increase the negative cooperativity between cAMP-binding sites of the protein. The CRP S128A mutant binds to DNA sequence ~50-fold more weakly than wild-type CRP (12). Unfortunately, the x-ray crystal structure of unligated CRP is not known yet; however, it has been postulated that the cAMP-induced allosteric transition may involve a change in the relative orientation of the subunits and a change in the orientation of the C-terminal DNA-binding domain relative to the cAMP-binding domain (23). Indeed, our recent fluorescence energy transfer measurements support this idea and indicate an ~8-Å movement of the C-terminal domain toward the N-terminal domain upon binding of cAMP to anti-sites of the protein (10). Because Thr<sup>127</sup> forms a hydrogen bond with N-6 of the bound cAMP and Ser<sup>128</sup> forms a hydrogen bond with cAMP in the other subunit (16, 4, 23), the mutation of these residues can influence both domain-domain interaction as well as the interaction between the two subunits of CRP. The x-ray structure studies of the cAMP-ligated CRP T127I/S128A double mutant have shown that the S128A substitution induces an increase in flexibility in helix C of the protein, which in turn swings the C-terminal domain more toward the N-terminal domain of CRP (14). We suggest that the observed changes in the kinetic rate constants \( k_1 \) and \( k_2 \) as well as the changes in the equilibrium of conformational transitions \( K_c \) in the allosteric activation of the CRP T127I and CRP S128A mutants by cAMP result from conformational reorientation of the HTH motif in the C-terminal domain of the protein.

Our previous kinetic studies have shown that, for wild-type CRP, the formation of the CRP-cAMP<sub>2</sub> complex with wild-type CRP can be described by displacement of equilibrium between the two forms of CRP-cAMP<sub>2</sub>, where only one form is able to bind cAMP to low affinity sites (8). This process is followed by conformational changes in CRP, and this mechanism can be described by the concerted (MWC) model of allostery. The kinetic mechanism of CRP conformational changes can be monitored by the Trp fluorescence intensity changes upon binding of cAMP to \textit{syn}-sites of CRP. We have also shown that the binding of cAMP induces a significant change in the fluorescence of Trp<sup>85</sup> only at a ligand concentration higher than ~300 \( \mu \text{M} \) (10). Because Trp<sup>85</sup> is located in the N-terminal domain of the protein, the observed conformational changes upon binding of cAMP to low affinity sites is restricted to the ligand-binding domain of the protein. At present, the kinetics of cAMP binding to all muteins studied were investigated under pseudo first-order conditions, and the experimental data could be fitted to single-exponential curves. The observed rate constants for CRP T127I, CRP S128A, and CRP T127I/S128A decreased with increases in cAMP concentration, and this relationship, similarly as interpreted for wild-type CRP (8), could be described as evidence of displacement of equilibrium between the two conformational states of CRP, of which only one is able to bind the ligand. The bimolecular rate constants of cAMP association with the low affinity sites of CRP are estimated the range of \( ~2 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) (8), and this process is much faster than the isomerization step calculated for the muteins studied in this
The single-exponential model was superior, as can be seen from the normalized variance and residual distribution. In all cases, as we mentioned above, the single-exponential model was superior, as can be judged from the normalized variance and residual distribution analysis. The observation that the calculated rate constants (k_{obs}) increased with increasing ligand concentrations led us to the conclusion that only the sequential KNF model of allosteric (Scheme 1) can describe these results. On the other hand, in the case of binding of cAMP to weaker binding sites, we observed decreases in the k_{obs} rate constants with increasing ligand concentrations, which, in turn, can lead to the MWC model of allosteric binding as described in Scheme 2. In conclusion, the KNF model can fit only the data for tight cAMP-binding sites, and the MWC model can fit only the data for weaker cAMP-binding sites.

The dependence of k_{obs} on the final cAMP concentration has been used to evaluate the rate constants k_{on} and k_{off}, which indicate that the CRP mutations influence mostly the rate of the isomerization step from the non-binding to binding form of the protein and that the magnitude of these changes depends on the mutation. The equilibrium constants of isomerization (K_i) between the two forms of the CRP-cAMP_2 complex, which are 50, 75, and 6 for the CRP T127I, CRP S128A, and CRP T127I/S128A mutants, respectively, are higher than the value of 2.7, which has been found for wild-type CRP (8). Because these two forms of the CRP-cAMP_2 complex can exist in different conformational states and only one of them (P') can bind cAMP, the observed increase in the K_i isomerization constant with the amino acid substitutions can result from the shifting of equilibrium from the inactive to active state of CRP, and the magnitude of this shift depends on substitutions. However, a smaller shift in this equilibrium has been observed in the case of the double mutant of CRP, which can activate transcription in the absence of cAMP, and (as it is believed) this mutant possesses the structure appropriate for DNA binding (14).

The isoleucine substitution for threonine, which had a small effect on the cAMP dissociation constant for anti-sites, had a significant effect on binding to syn-sites of CRP (Tables I and II), indicating the increase in negative cooperativity between these cAMP-binding sites. This observation is in agreement with the equilibrium measurements of cAMP binding to the CRP T127I mutant (13) as well as to the double mutant of CRP (14), where an increase in the ratio of apparent association constants for binding sites (k_{ant}/k_{syn}) was observed. The K_{syn} dissociation constant value (2.7 mM) did not significantly change with the substitution S128A (Table II) if we compare it with the value of 2.0 mM, which has been found for wild-type CRP.

To summarize, the results obtained in this study show that the mutations T127I and S128A in CRP do not affect significantly the dissociation constants of ligand binding to anti- and syn-sites of the protein. However, the mutations do quantitatively affect the kinetics of C- and N-terminal domain communication as well as subunit-subunit interactions. The observed intramolecular signal transmission (triggered by cAMP binding) indicates that CRP can exist in various conformational states and the distributions of these states can be shifted by mutations. Because these conformational states of CRP may differ in affinity for DNA and RNA polymerases, this phenomenon may play a crucial role in fine transcription regulation.

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