Interactive and Dominant Effects of Residues 128 and 141 on Cyclic Nucleotide and DNA Bindings in Escherichia coli cAMP Receptor Protein

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The molecular events in the cAMP-induced allosteric activation of cAMP receptor protein (CRP) involve interfacing communications between subunits and domains. However, the roles of intersubunit and interdomain interactions in defining the selectivity of cAMP against other cyclic nucleotides and cooperativity in ligand binding are still not known. Natural occurring CRP mutants with different phenotypes were employed to address these issues. Thermodynamic analyses of subunit association, protein stability, and cAMP and DNA binding as well as conformational studies of the mutants and wild-type CRPs lead to an identification of the apparently dominant roles of residues 128 and 141 in the cAMP-modulated DNA binding activity of CRP. Serine 128 and the C-helix were implicated as playing a critical role in modulating negative cooperativity of cyclic nucleotide binding. A correlation was established between a weak affinity for subunit assembly and the relaxation of cyclic nucleotide selectivity in the G141Q and S128A/G141Q mutants. These results imply that intersubunit interaction is important for cyclic nucleotide discrimination in CRP. The double mutant S128A/G141Q, constructed from two single mutations of S128A and G141Q, which exhibit opposite phenotypic characteristics of CRP<sup>−</sup> and CRP<sup>+</sup>, respectively, assumes a CRP<sup>+</sup> phenotype and has biochemical properties similar to those of the G141Q mutant. These observations suggest that mutation G141Q exerts a dominant effect over mutation S128A and that the subunit realignment induced by the G141Q mutation can override the local structural disruption created by mutation S128A.

The expression of many genes involved in different cellular functions in Escherichia coli is regulated by cAMP receptor protein (CRP)<sup>−</sup> and cAMP (1–3). The molecular events associated with the allosteric activation of CRP are characterized by negative cooperativity in cAMP binding and a differentiation among the various cyclic nucleotides present in the cell. Although the CRP-cAMP system has served as a paradigm of transcriptional regulation in procaryotes for many years, an understanding of this allosteric activation event at the structural level is still missing.

CRP is a 47,238-Da protein made up of two identical subunits, each of which is composed of two domains connected by a hinge region. The small carboxyl-terminal domain contains a helix-turn-helix DNA binding motif. The large amino-terminal domain is responsible for cyclic nucleotide binding (4). Biochemical and biophysical evidences show that binding of cAMP allosterically induces CRP to assume a conformation that exhibits a high affinity for specific DNA sequences (5–13). However, a comparison between the active, monoligated CRP and apo-CRP shows no significant secondary structural changes. The only observable major structural changes are associated with the doubly liganded CRP (11–16). These observations imply that conformational changes during CRP activation most likely involve rigid body movements between subunits and domains without major structural changes or a change in the dynamics of various structural elements without any significant conversion of secondary structures. In CRP these rigid body movements can be envisioned as subunit realignment and domain rearrangement that are mediated by interactions involving the subunit and domain interfaces, respectively. It has been shown that various locations in the CRP molecule all respond quantitatively to the binding of cAMP (17). These observations demonstrate that CRP responds to the binding of cAMP in a global manner. A global conformational switch is consistent with the proposed mechanisms involving either subunit realignment and domain rearrangement or a change in structural dynamics.

There is still no information to identify the structural elements in CRP that are responsible for imposing negative cooperativity in cAMP binding. How does CRP differentiate among the various cyclic nucleotides? What structural element is responsible for establishing a tight coupling between intersubunit and interdomain interactions? CRP mutants generated by site-specific mutagenesis are employed to address these outstanding issues. Studies of point mutations at residue 128 show that serine 128 is not vital for interdomain communication but plays an important role in mediating the interactions between the two subunits (18). In a recent study of mutation at residue 141, it was shown that the Gly → Gln mutation differentially perturbs the two interfacial interactions (19, 20). Structural and functional studies reveal that the G141Q mutant assumes a conformation state that has a realigned subunit interface. The G141Q mutant does not bind to specific DNA sequence without cyclic nucleotide (19). Complete activation of the mutant requires binding of cyclic nucleotide, which induces the reorientation of domains (20).

In this study, the role of residue 141 in defining cyclic nucleotide specificity and the mutual influence of residues 128 and 141 in the allosteric control of CRP toward binding to
specific DNA are addressed. The in vivo and in vitro functional properties of mutant CRPs were monitored, with special attention to the subunit and domain interactions in CRP.

MATERIALS AND METHODS

Chymotrypsin A and cGMP were purchased from Boehringer Mannheim. A mutagenesis kit (Altered Sites in vitro Mutagenesis System) was obtained from Promega, and a sequencing kit (Sequenase version 2.0) was from U.S. Biochemical Corp. Subtilisin (protease type XXVII) and cAMP were purchased from Sigma. Ultrapure guanidine HCl was a product of ICN Biochemical, MacConkey agar, bacteriophage, and yeast extract were obtained from Difco. CPM, fluorescein 5-isothiocyanate, and IAF were purchased from Molecular Probes. Oligonucleotides and cAMP were purchased from Sigma. Ultrapure guanidine HCl was a product of ICN Biochemical. MacConkey lactose plate (identification of the various bacterial strains and plasmids can be found in Cheng et al. (18). Methods—All experiments, except as specifically indicated, were conducted in buffer A (50 mM Tris, 0.1 M KCl, and 1 mM EDTA, pH 7.8). The concentration of protein, cyclic nucleotides, and fluorescence probes was determined by absorption spectroscopy using the following absorption coefficients: 2,400 M⁻¹ cm⁻¹ at 278 nm for cAMP (17), 4,600 M⁻¹ cm⁻¹ at 278 nm for cGMP (22), respectively; 30,000 M⁻¹ cm⁻¹ at 387 nm for CPM (23); 70,800 M⁻¹ cm⁻¹ at 494 nm for fluorescein (24).

Site-directed Mutagenesis—The G141Q point mutation was generated as described previously (18–20). For constructing the double mutant SI258A/G141Q, the G141Q crp gene was subcloned into the unique HincII site of pALTER-1 vector. The same protocol described above was then used for the second round of mutagenesis. With the new Promega Altered Sites II in vitro Mutagenesis System, multiple rounds of mutation can be introduced into the same gene without subcloning.

Lac Operon Activation in Vivo—To test the effects of mutation on the lac operon expression, E. coli CA8445 (pPRK248cIts) harboring plasmid H1/pPlcCRP1 cells grown in LB (17). Mutant CRP CA8445/pPRK248cIts, transformed with pAL-3 containing the appropriate mutant crp gene, was subcloned into the unrestricted HindIII site of pALTER-1 vector. The same protocol described above was then employed with a minor modification (18).

DNA Binding Study—Fluorescence anisotropy measurements were used for qualitative measurements of CRP-DNA interactions using a previously published protocol (17).

Allosteric Activation of CRP

where $A_m$, $\Delta_l$, and $\Delta_s$ are the values of change in fluorescence intensity and the normalized values of change in going from free CRP to CRP-cAMP, and CRP-cAMP$_2$, respectively; $K_1$ and $K_2$ are Adair constants for the formation of CRP-cAMP, and CRP-cAMP$_2$; and [cAMP] is the free cAMP concentration.

Therefore, the apparent DNA binding affinity of CRP ($K_r$) is a function of cAMP concentration, which in accordance to the above scheme can be expressed as follows.

$K = K_r[K_1[cAMP] + K_2K_r[cAMP]^2] + K_1[K_1[cAMP] + K_2K_r[cAMP]^2]$ (Eq. 3)

$K_1$ and $K_2$ can be determined independently from the cAMP binding assay. The binding affinity of CRP-cAMP$_2$ ($K_2$) is very weak and cannot be precisely determined (17). However, the apparent DNA binding affinity of the CRP in the presence of 100–200 µM cAMP, where most of the CRP exists in the monomeric form, has been shown to be a good estimation of the intrinsic DNA binding affinity of the CRP-cAMP complex (28). Therefore, in this study the apparent DNA affinity of CRP in the presence of 200 µM cAMP was assumed to represent $K_r$. Sedimentation Equilibrium—The quaternary structure of CRP was monitored by sedimentation equilibrium as described previously (29). Experiments were conducted in a Beckman-Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner, an electronic speed control, and an RTIC temperature control. The high speed, me-mulus depletion procedure was employed (30). The loading cAMP concentrations were between 0.2 and 0.4 mg/ml. Sedimentation data were acquired (10 scans) and then averaged after reaching equilibrium. The density of the solution was determined with a Mettler-Paar Precision...
DMA-02D density meter. Values of the partial specific volume of the wild type and mutant CRPs in native buffer and 6.0 M GuHCl were calculated based on the amino acid composition of CRP (31, 32) using the procedure of Cohn and Edsall (33) and Lee and Timasheff (34), respectively.

Sedimentation equilibrium data were fit by nonlinear least squares to a dimer assembly scheme according to the equation,

\[ C = \Delta C + \exp(2\ln C_0 \sigma r^2/2 - r^2/2) + \exp(2\ln C_0 + 2\sigma r^2/2 - r^2/2) + \ln K \]

where \( C \) is the CRP concentration observed at radial position \( r \), \( \Delta C \) is the base-line 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 \( \sigma = M_r(1 - \tilde{\psi}p\omega^2/2RT) \), where \( M_r \) is the monomer molecular weight, \( \tilde{\psi} \) is the partial specific volume, \( p \) is the solution density, \( \omega \) is the angular velocity, and \( R \) and \( T \) are the gas constant and the temperature in Kelvin, respectively.

**Denaturation of CRP—** The effect of amino acid substitutions on the folding and domain-domain interactions in CRP was probed by monitoring the unfolding of CRP by GuHCl using fluorescence anisotropy. A detailed description of the protocol has been published (29). Briefly, the denaturation of CRP can best be described by a three-state model,

\[ K_u = \frac{D}{2} \]

\[ N_u = 2N \]

\[ F \]

**REACTION 1**

in which \( N_u, N, \) and \( U \) are folded dimer, folded monomer, and unfolded monomer of CRP, respectively. \( K_u \) and \( K_f \) are the equilibrium constants of dissociation and unfolding, respectively.

Unlike wild type CRP, mutant S128A/G141Q exhibits a weakened subunit association, which leads to a complete uncoupling of dimer dissociation and monomer unfolding. Therefore, these two processes can be studied separately. Subunit association of S128A/G141Q was measured by sedimentation equilibrium as described above. CRP unfolding was monitored by fluorescence anisotropy at the excitation and emission wavelengths of 280 and 345 nm, respectively.

To determine the equilibrium constant of unfolding, the fraction of CRP protein that is in the folded state, \( f \), was calculated by the equation,

\[ f = \frac{\epsilon - \epsilon_{\text{unf}}}{\epsilon_{\text{fold}} - \epsilon_{\text{unf}}} \]

where \( \epsilon, \epsilon_{\text{fold}}, \) and \( \epsilon_{\text{unf}} \) represent the measured fluorescence anisotropy signal of the protein sample and the values of fluorescence anisotropy of folded and unfolded monomer CRP at a given GuHCl concentration, respectively. \( \epsilon_{\text{fold}} \) and \( \epsilon_{\text{unf}} \) at the unfolding transition zone were derived by linear extrapolation of the measured signals at the predenaturation and postdenaturation zones, respectively.

Once \( f \) was determined, the equivalent \( K_u \) at different GuHCl concentrations can be calculated as follows.

\[ K_u = \frac{[U]}{[N]_0} - \frac{1 - f}{f} \]

(K_u) values were converted to \( \Delta G_u \) by \( \Delta G_u = -RT\ln \left( \frac{K_u}{2} \right) \), the free energy of unfolding in the absence of denaturant, was subsequently determined by linear extrapolation of \( \Delta G_u \) to zero denaturant (35).

**RESULTS**

**In Vivo Genetic Characterization of CRP Mutants—** G141Q displayed purple colonies on MacConkey plates in the absence of external cAMP and in the presence of cGMP as reported earlier (15, 19, 36, 37). Thus, this mutant is characterized by a CRP* phenotype. Interestingly, while the S128A mutant showed a CRP* phenotype (18), the double mutant S128A/G141Q is a CRP* mutant in vivo. This in vivo observation indicates that mutation of G141Q exerts a dominant effect over mutation S128A.

**In Vitro Structural Characterization of the S128A/G141Q Mutant**

**Near- and Far-UV CD—** Near- and far-UV CD spectra of the double mutant, S128A/G141Q are identical to that of the wild type CRP and S128A and G141Q single mutants (Fig. 1). These results indicate that no major secondary or tertiary structural changes have been introduced in CRP by these mutations.

**Subunit Association—** Like the wild type, S128A, and G141Q CRPs, the S128A/G141Q mutant exists in solution as an apparent dimer in the mg/ml concentration regime. However, one way of probing the effect of mutation on intersubunit communication is by monitoring the energetics of dimer formation of each mutant. Sedimentation equilibrium experiments of S128A/G141Q at different concentrations of GuHCl ranging from 0 to 1.5 M were conducted to determine the dimer dissociation constant for this double mutant. The sedimentation equilibrium experiments were performed at multiple speeds of 20,000, 28,000, and 34,000 rpm for each GuHCl concentration. The results are shown in Fig. 2A. The experimental data were then analyzed to estimate the apparent dimer association constant, \( K_{\text{app}} \), at each individual GuHCl concentration using the program NONLIN (38). A plot of Ln\( K_{\text{app}} \) versus GuHCl concentration displays an apparent linear relationship. Extrapolation of the data to zero concentration of GuHCl yields a value.
of the dimer association constant for S128A/G141Q in buffer (Fig. 2). The extrapolated dimer association constant is similar to that of the G141Q mutant, i.e. weaker dimerization than that of the S128A mutant and wild type CRP. Table I summarized the results on subunit dissociation for all of the mutants and wild type CRP. Again, G141Q mutation exerts a dominating effect on subunit assembly.

Protein Stability—Protein stability studies in general can provide information on the folding stability of protein molecules and possibly domain-domain interaction. The stability of the double mutant was monitored by GuHCl denaturation studies using a published procedure (29). The unfolding curve of the S128A/G141Q mutant is characterized by a biphasic transition resembling that of the G141Q mutant, as shown in Fig. 3A. Again this reflects the uncoupling of the processes of dimer dissociation and monomer unfolding. Similarly, the unfolding free energy of the S128A/G141Q monomer was determined to be 7.4 kcal/mol, as shown in Fig. 3B. This value is similar to that of the wild type, S128A, and G141Q CRPs (Table I).

Protease Sensitivity—The proteolytic digestion pattern of wild type CRP is biphasic as a function of cAMP. An initial increase in the rate of proteolysis at low cAMP concentration was followed by a decrease in rate with a further increase in cAMP concentration (17). The S128A mutant shows a significant decrease in sensitivity to protease digestion in a wide range of cAMP concentrations (18), while mutant G141Q is sensitive to protease cleavage in the absence of cAMP. The digestion pattern of the S128A/G141Q double mutant is different from that of the wild type and S128A mutant but similar to that of the G141Q mutant (Fig. 4). The S128A/G141Q mutant is also susceptible to either chymotrypsin or subtilisin digestion in the absence of cAMP. The digestion rates of S128A/G141Q and G141Q CRP are dependent on cAMP concentration. Furthermore, the rate decreases in the order of increasing cAMP concentration. The effects of cGMP on the proteolytic digestion patterns of CRP and the mutants were also examined. At a low cGMP concentration (200 μM) no significant effect was observed, while at a high cGMP concentration (50

![Fig. 2. Subunit association of S128A/G141Q studied by sedimentation equilibrium. A, sedimentation equilibrium profiles of S128A/G141Q at different angular velocity at 0 M GuHCl concentration. ○, 34,000 rpm; □, 28,000 rpm; ▲, 20,000 rpm. The solid lines represent the best fits of the experimental data to Eq. 4. B, apparent dimer equilibrium association constants (determined from A) as a function of GuHCl concentration.](image)

![Fig. 3. GuHCl-induced chemical denaturation of S128A/G141Q. A, denaturation curve of S128A/G141Q (1 μM) measured by fluorescence anisotropy. B, apparent unfolding free energy of S128A/G141Q monomer as a function of GuHCl concentration.](image)

| CRP mutant       | Dimer dissociation constant \( * \) | \( \Delta G \) of monomer unfolding \( \text{kcal/mole} \) |
|------------------|------------------------------------|----------------------------------|
| Wild type        | \( 1.0 \times 10^{-6} \)          | 7.2                              |
| G141Q            | \( 1.4 \times 10^{-6} \)          | 7.7                              |
| S128A            | \( 2.3 \times 10^{-6} \)          | 7.7                              |
| S128A/G141Q      | \( 6.3 (1.7, 22.1) \times 10^{-7} \) | \( 7.4 (7.1, 7.7) \)            |

\( * \) Errors in parentheses are expressed in terms of 75% confidence intervals.

\( b \) Ref. 29.

\( c \) Ref. 45.

\( d \) Ref. 18.
In Vitro Functional Characterization of CRP Mutants

Cyclic Nucleotide Binding—Unlike wild type CRP, mutant G141Q CRP can also be activated by cGMP and other cyclic nucleotides for specific DNA binding (19). Thus, the binding affinities of the G141Q mutant for cGMP and cAMP were determined by monitoring the fluorescence quenching of the IAF-labeled G141Q CRP as a function of cGMP concentration, and the results are shown in Fig. 5. The estimated values of the equilibrium binding constants for cGMP are $k_1 = 6.4 \times 10^4 \text{ M}^{-1}$ and $k_2 = 2.5 \times 10^3 \text{ M}^{-1}$. Thus, the binding constants of cAMP and cGMP in the G141Q mutant are essentially identical.

CAMP binding to S128A/G141Q CRP was monitored by fluorescence quenching of the S128A/G141Q-IAF complex (Fig. 6). Values of $1.4 \times 10^4 \text{ M}^{-1}$ and $1.9 \times 10^2 \text{ M}^{-1}$ were determined for $k_1$ and $k_2$, respectively. The binding constant of the first cAMP is slightly lower than but not significantly different from that of wild type, S128A, and G141Q CRP, whereas the binding constant for the second cAMP is about 6 and 14 times lower than that of wild type and G141Q CRP, respectively.

Negative cooperativity in cAMP binding has been observed in wild type CRP (17), and the ratio of the microscopic cAMP binding constants, $k_1$ and $k_2$, can be used to estimate the degree of cooperativity. For the wild type and G141Q CRPs, values of about 25 were determined (Table II), while the two binding constants differed more than 1000-fold in mutant S128A CRP, indicating that mutation at residue 128 leads to an even higher degree of negative cooperativity in CRP. Interestingly, a value of 74 for $k_1/k_2$ was observed for the double mutant. This result suggests that the strong negative cooperativity for cAMP binding observed in S128A is partially preserved in S128A/G141Q CRP.

DNA Binding—cGMP binds to but fails to activate wild type CRP (21, 39). Interestingly, cGMP not only binds to G141Q CRP with affinity similar to cAMP, but it can also replace cAMP in activating G141Q CRP to interact with DNA (19). When cGMP was titrated into a solution containing 13 nM lac-40-CPM DNA and 1 mM of G141Q CRP, the formation of a protein-DNA complex responded to the increase of cGMP concentration in a biphasic manner that is similar to the response of wild type CRP to cAMP (Fig. 7A). This biphasic behavior reflects the strong affinity of CRP-cGMP1 for lac-40 DNA but a decreased affinity of the CRP-cGMP2 species. Quantitative determination of the apparent binding constant of G141Q CRP to DNA in the presence of 100 mM cGMP yields a value of $3.0 \times 10^7 \text{ M}^{-1}$ (Fig. 7B), while wild type CRP binds to specific DNA with an apparent binding constant of less than $7 \times 10^4 \text{ M}^{-1}$ (Table III). Such high affinity of G141Q-cGMP, complex for specific DNA is close to the affinity of CRP-cAMP, complex to some naturally occurring CRP-dependent promoters, indicating a possible physiological significance, e.g., the activation of some CRP-dependent promoters in the absence of cAMP.

The binding of S128A/G141Q CRP to lac-40-CPM-labeled DNA in the presence of cAMP was also studied (Fig. 8). The
apparent DNA binding constant of S128A/G141Q CRP in the presence of 200 μM cAMP is $1.7 \times 10^7$ M$^{-1}$, which is stronger than that of S128A CRP but weaker than that of G141Q CRP. cGMP can also activate S128A/G141Q CRP for specific DNA binding (Fig. 8). The estimated apparent DNA binding constant of S128A/G141Q CRP in the presence of cGMP is about $3.0 \times 10^6$ M$^{-1}$. Under the same conditions, S128A CRP has no measurable binding affinity to lac-40 DNA.

When the DNA binding constants for CRP in the presence of cAMP or cGMP are compared, a more than 5,000-fold difference is obtained (Table III), suggesting that CRP is highly selective for cyclic nucleotides. Mutation S128A does not seem to affect the selectivity, while mutation G141Q leads to a dramatic relaxation in cyclic nucleotide selectivity, decreasing the 5,000-fold difference in binding affinity to only about 8-fold. Moreover, the effect of the G141Q mutation is preserved in the double mutant (Table III). These results imply that the G141Q mutation exhibits a dominant effect over the S128A mutation in conferring recognition to specific DNA sequence and discriminatory ability toward the various cyclic nucleotides.

### DISCUSSION

In vitro studies of CRP-DNA interaction show that the species that interacts with specific DNA sequences is CRP-cAMP$_1$ (17, 18, 20, 26, 28, 40, 41). The CRP-cAMP$_1$ complex exhibits much lower or no affinity for these DNA sequences. There is no exception for all of the nine DNA fragments of natural or artificial sequences studied (28, 41). One possible rationale for this behavior is the requirement of recognition between two asymmetric macromolecules, namely CRP-cAMP$_1$ and CRP-dependent promoter, as proposed by Heyduk and Lee (17). The natural DNA sequences of CRP binding sites are not palindromic (1, 3), and RNA polymerase seems to recognize a DNA-bound CRP subunit in a particular orientation (42, 43). Hence, it is conceivable that CRP binds to the asymmetric DNA site in a specific orientation. Binding of one cAMP molecule to the dimeric CRP molecule would render the protein molecule asymmetric. If such an interpretation is valid, then what are the structural elements in the CRP molecule that impart these functional properties? What is

### TABLE II

Summary of fitted parameters for cyclic nucleotide binding to CRP mutants

| CRP mutant   | Ligand | $K_1$ | $k_1$ | $K_2$ | $k_2$ | $k_1/k_2$ |
|--------------|--------|-------|-------|-------|-------|-----------|
| Wild type    | cAMP   | $2.5 \times 10^4$ | $1.0 \times 10^3$ | 25     |
| S128A        | cAMP   | $2.2 \times 10^4$ | $2.0 \times 10^3$ | 1100   |
| G141Q        | cAMP   | $6.4 \times 10^4$ | $2.5 \times 10^3$ | 26     |
| S128A/G141Q  | cGMP   | $9.4 (8.6, 10.5) \times 10^4$ | $1.5 (1.2, 2.0) \times 10^6$ | 15     |
| S128A/G141Q  | cAMP   | $2.8 (2.7, 4.1) \times 10^4$ | $2.6 (1.2, 14.1) \times 10^6$ | 74     |

$K_1$ and $K_2$ are Adair equilibrium constants for binding of the first and second cAMP, respectively. $k_1$ and $k_2$ are the corresponding microscopic equilibrium constants. Errors in parentheses are expressed in terms of 75% confidence intervals.

*Ref. 17.*
*Ref. 18.*
*Ref. 45.*

### FIG. 6

Binding of cAMP to S128A/G141Q as monitored by fluorescence quenching of S128A/G141Q-IAF. The solid lines represent the best fits of the data to Eq. 1.

### FIG. 7

Interaction of G141Q with lac-40 DNA in the presence of cGMP. A, G141Q-DNA interaction as a function of cGMP concentration. B, binding of G141Q to lac-40 DNA in the presence of 100 μM cGMP. The solid line represents the best fit of the data to Eq. 2.
Role of Residue 128 and C-helix in the Negative Cooperativity of cAMP Binding—An earlier study on cAMP binding to α-CRP, the ligand binding domain of CRP generated by proteolytic digestion, led to the observation that the degree of negative cooperativity in cAMP binding is related to the amount of C-helix retained in CRP cooperativity in cAMP binding is related to the amount of digestion, led to the observation that the degree of negative cooperativity in cAMP binding is partially retained in the double mutant S128A/G141Q. The results of this study of a limited number of mutants indicate that the C-helix apparently plays an important role in imparting negative cooperativity in cAMP binding to CRP and that residue 128 apparently exhibits a dominant effect over residue 141 in maintaining negative cooperativity in ligand binding.

Role of Residue 141 and Subunit Interface in the Discrimination of Cyclic Nucleotides—Another important issue in the mechanism of CRP function is its ability to bind and be activated only by cAMP. Although other cyclic nucleotides can bind to CRP, no activation in CRP toward recognition of specific DNA sequence is observed. Nevertheless, the G141Q mutant provides an opportunity to address the issue of cyclic nucleotide discrimination. The strict specificity requirement for cyclic nucleotide observed in wild type CRP is significantly relaxed in the G141Q mutant (19) as well as in the double mutant S128A/G141Q. These observations are unexpected. One may speculate that specificity will be imparted by amino acid residues within the binding site of cyclic nucleotides. Serine 128 resides in the cAMP domain and is a prime candidate for discriminating specific cyclic nucleotides, since the crystallographic data indicate hydrogen bonding between the hydroxyl group of serine 128 and N-6 of cAMP (4). However, elimination of the hydroxyl group by a Ser → Ala substitution does not affect the ability of the S128A mutant to discriminate between cAMP and cGMP. Yet, a substitution of glycine by glutamine in residue 141, which is neither located within the cAMP binding domain nor involved in the formation of intersubunit contacts, leads to a breakdown in the ability of CRP to discriminate cAMP from the other cyclic nucleotides.

Substitution of Gly → Gln does not lead to any change in the secondary or tertiary structure of CRP as monitored by CD, and this substitution does not alter the global folding of CRP as monitored by chemical denaturation (20, 45). Thus, spectroscopically and energetically, a Gly → Gln mutation does not produce any significant perturbation in the structure of CRP, and the loss of ability to discriminate cyclic nucleotides is not the result of detectable alteration in the global structure of CRP. Mutation G141Q results in a weakening in the energetics of intersubunit interaction. The consequence is an increase in susceptibility to protease digestion along the C-helices, an observation that mimics the effect of cAMP binding. The net result of this weaker interfacial interaction is a relaxation of cyclic nucleotide discrimination. Therefore, CRP maintains its cyclic nucleotide selectivity by imposing an energetic barrier along the subunit interface that only cAMP can overcome. Interestingly, the double mutant S128A/G141Q responds to protease digestion in a manner almost identical to the G141Q mutation can override the local structural changes created by the S128A mutation.

Role of Domain Interface in CRP Activation—A combination of subunit realignment and domain rearrangement activates CRP for specific DNA binding. The G141Q mutation only leads to a change in realignment of subunits without inducing the proper domain rearrangement. The latter structural change is only induced upon binding of cyclic nucleotide. That is the
underlying reason why the G141Q mutant cannot bind to a specific DNA sequence without cAMP. The supporting evidence for this interpretation is the response of the IAF-labeled G141Q CRP to the titration of cAMP or cGMP. The IAF is covalently attached to cysteine 178, which is located at a position just before the DNA-binding helix (F-helix). Quenching of fluorescence intensity of the covalently attached fluorescein probe was observed and must reflect the domain rearrangement in response to ligand binding. The estimated values of binding constants are in good agreement with the values determined by other approaches. This again suggests that the cAMP- or cGMP-induced fluorescence quenching reflects the intrinsic conformational changes in the DNA binding domain in response to cAMP or cGMP binding. The IAF-labeled S128A/G141Q CRP responds to the binding of cAMP in a manner similar to that of G141Q CRP. This effect further substantiates the involvement of domain-domain rearrangement in CRP activation by cAMP and the dominating effect of the G141Q mutation.

In summary, the results from this study imply that the cyclic nucleotide-induced activation process is linked to the energetics of overcoming an unfavorable intersubunit structure. A weaker interfacial interaction, such as those observed in the G141Q and S128A/G141Q mutants, makes it easier to switch over to the appropriate intersubunit structure and consequently to induce the proper interdomain orientation for specific DNA sequence recognition. Furthermore, this study reveals the apparent dominant effects of residues 128 and 141 in conferring functional characteristics in CRP. The influence of residue 128 is more significant in ligand binding (in particular, binding of the second cAMP). Residue 141 exerts more effects on the energetics of dimerization and all events apparently associated with intersubunit interactions, e.g., discrimination among various cyclic nucleotides in their ability to activate CRP for binding to specific DNA sequence.

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