Cyclic AMP is a ubiquitous secondary message that regulates a large variety of functions. The protein structural motif that binds cAMP is highly conserved with the exception of loops 3 and 4, whose structure and length are variable. The cAMP receptor protein of Escherichia coli, CRP, was employed as a model system to elucidate the functional roles of these loops. Based on the sequence differences between CRP and cyclic nucleotide gated channel, three mutants of CRP were constructed: deletion (residues 54–56 in loop 3 were deleted), insertion (loop 4 was lengthened by 5 residues between Glu-78 and Gly-79) and double mutants. The effects of these mutations on the structure and function of CRP were monitored. Results show that the deletion and insertion mutations do not significantly change the secondary structure of CRP, although the tertiary and quaternary structures are perturbed. The functional data indicate that loop 3 modulates the binding affinities of cAMP and DNA. Although the lengthened loop 4 may have some fine-tuning functions, the specific function of the original loop 4 of CRP remains uncertain. The function consequences of mutation in loop 3 of CRP are similar to that of site A and site B in the regulatory subunits of cyclic AMP-dependent protein kinases. Thus, the roles played by loop 3 in CRP may represent a more common mechanism employed by cyclic nucleotide binding domain in modulating ligand binding affinity and intramolecular communication.

Cyclic AMP serves as an intracellular message in both prokaryotes and eukaryotes by transmitting information through proteins such as protein kinase A (PKA), cyclic nucleotide-gated ion channels (CNGC), and cAMP receptor protein in Escherichia coli (CRP). These proteins are involved in a very diverse set of cellular functions such as signal transduction, excitability, and gene expression (1–6). These proteins of diverse functions all consist of a cAMP binding motif. The structural motif, which serves as cAMP receptor, is found to display a high degree of similarity. X-ray crystallography and homology modeling results show that, despite obvious divergence of sequence among the receptor domains and significantly different biological functions of these proteins, their CNB domains appear to share a common architecture, all consisting of an α-helix (helix A), an eight-stranded β-roll, and two more α-helices (helices B and C). The body of the CNB pocket is mainly located in the β-roll, with the C-helix forming the back of the binding pocket (2, 8). The superimposition of the structures of CNB domains from CRP and the regulatory subunits of PKA, as shown below in Fig. 1, indicates that the β-roll basically assumes the same structure with the exception of loops 3 and 4 between strands 4 and 5, and strands 6 and 7, respectively. In some cases, such as in CNGC and PKA, loop 3 is shortened whereas loop 4 is lengthened, as shown in Fig. 1. Only six residues (Gly-33, Gly-45, Gly-71, Glu-72, Arg-82, and Ala-84 using the CRP sequence as reference) are invariant among all members of the families. It has been suggested that the invariant residues play important and conserved roles in the folding and function of the CNB sites of these diverse proteins. Gly-33, Gly-45, and Gly-71 are involved in turns between strands of the β-roll; Arg-82 and Gly-72 contact the cyclic nucleotide, and the function of Ala-84 is uncertain (3). Despite large variation of primary sequences, the sizes of secondary structural elements of the CNB domain are much conserved among the family. For example, the alignment of CRP and CNGCs by keeping the six conserve residues at the same positions shows that the size differences in secondary structural elements are only located at two loops, e.g. loop 3 (between β4 and β5) of CNGCs is shorter than that in CRP by three residues, and loop 4 (between β6 and β7) of CNGCs is 5 residues longer than that of CRP (see Fig. 2) (6). Similarly, loops 3 and 4 among the various protein kinase isozymes also show heterogeneity in size, as shown in Fig. 2.

Based on the sequence alignment, it is interesting to note that loops 3 and 4 are the only structural elements that are different in size among these various sources of CNB domain. To elucidate the roles of these loops the cAMP receptor protein, CRP, from E. coli is employed as a model system for investigation. CRP of Escherichia coli, also referred to as the catabolite gene activator protein, is a 47,238-Da homodimer. Each subunit has two domains: the large N-terminal domain is a cyclic nucleotide binding domain, and the small C-terminal domain is a DNA binding domain. ApoCRP has very low affinity for DNA and cannot differentiate between specific and nonspecific DNA sequences, whereas holoCRP exhibits high affinity for specific DNA sequences. It is known that binding of cAMP allosterically induces CRP to assume conformations that exhibit high affinity for specific DNA sequences (9–13). It has been suggested that allostERIC conformational change, which includes subunit realignment and domain rearrangement, occurs upon the binding of cAMP to CRP. These changes are mediated by interactions involving the subunit and domain interfaces. The C-helix and the hinge region between the domains have been found to play...
key roles in transmitting the allosteric signal. Although earlier spectroscopic comparison between the homo- and apoCRP showed no apparent secondary structural changes (14–16), the results of protein footprinting experiments and recent NMR studies indicate that there are wide-ranging structural differences between apoCRP and holoCRP. Binding, while perturbing the β-roll that forms the cAMP binding pocket, has little effect on the secondary structure elements contained in either the N- or the C-terminal domains. There does, however, appear to be a significant difference around the C terminus of C-helix, the hinge region, and loop 3 (1, 17–20). These studies identified the location of structural changes induced by cAMP binding but do not provide information on the role of these structures in this β-roll motif in binding cyclic nucleotides.

It was postulated that loop 3 in CRP is involved in both interdomain and intersubunit interactions, whereas loop 4 contacts the coiled-coil C-helices and forms part of the dimer interface (1), although the effects of these interactions in CRP function were not predictable by structural analysis alone. In recent studies, it was shown that a D53H mutation in loop 3 leads to enhancements of the magnitude of positive cooperativity in cAMP binding and affinity for specific DNA (21, 22). These solution biophysical data are consistent with the proposal that loop 3 plays a role in interdomain and intersubunit communications, although the specific nature of this role is unknown. In this study, three mutants of CRP are constructed according to the difference in sequence alignment between CRP and CNGC, namely, a deletion of residues 54–56 in loop 3 and insertion of 5 residues between residues 78 and 79, respectively. The choices for specific sequences for deletion and insertion are based on the availability of information on CNGC. Consequently, it is possible to compare and contrast the data acquired in this study with the literature. These mutants are the subjects of investigation to elucidate the roles of these loops in the normal function of CRP.

**Site-directed Mutagenesis**—The sites and nature of mutations in loops 3 and 4 are based on the sequence differences between CRP and CNGC, namely, a deletion of Glu-54, Glu-55, and Gly-56 in loop 3 and an insertion of the sequence KGSKM between Gly-78 and Gly-79 in loop 4, as shown in Fig. 2. An overlap extension PCR method was used (23). The outer pairs of primers include an NdeI site and an HindIII site, respectively. The sequences were: TAA CCG CATATG TTG CGT GG and CCTT AA CAG CTC CAG C CAA CTT AA CGA GGG CCC TTG G. The sequences of mutagenesis primers for insertion were ACG TTC CTG GCC CAT CTT AGA GCC CTT CTC TAA AAA CAG GCC CAG and GTT TTA AGA G AAG GCC TCT AAG ATG GGC CAG GAA CTT AGC AGC GCA; those for deletion were AGG AGA GGA TCA TTT CTT GT GTC TTT GAT CAG CAC TGC C and GGG AGT GCT GAT CAA GAA CAA ATG ATC CAG TGC T. The products of the second round of amplification were inserted into the pET30a plasmid, and the constructs were sequenced after cloning.

**Protein Purification**—Wild-type and mutant CRPs were purified from *E. coli* strain HMS174(DE3) using a previously described protocol (21, 24). All purified CRP proteins were >99% homogeneous as judged by SDS-PAGE stained with Coomassie Blue; 50–60 μg of CRP was routinely loaded onto each lane. Furthermore, the ratios of the absorbance at 280 nm to that at 260 nm were >1.86, indicating the absence of nucleic acid contamination. The mass of proteins was further checked by mass spectrometry.

**Analytical Ultracentrifugation**—Experiments were conducted at appropriate speeds in a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics and an An60Ti rotor. Sedimentation velocity experiments were performed at 42K rpm. Velocity data were collected at 280 nm at a spacing of 0.002 cm with no averaging in a continuous scan mode and were analyzed using DCDT version 1.12. The reported weight-average sedimentation coefficient values (s_{w,av}) obtained from DCDT + were calculated by a weighted integration over the entire range of sedimentation coefficients covered by the g(s) distribution and corrected for the solution density and viscosity.

The apparent weight-average molecular weights were obtained by fitting the sedimentation equilibrium data with the following equation,

\[
C = E + C_1 \exp \left( \frac{1 - \gamma_1 \rho_{u} \upsilon^2}{2RT} \right)^{-1} M^{(a - r)^2} + c_2 \exp \left( \frac{1 - \gamma_2 \rho_{w} \upsilon^2}{2RT} \right)^{-1} 2M^{(a - r)^2}  
\]

where \( C \) is the observed CRP concentration in absorbance at radial position \( r \), \( E \) is the baseline offset, \( C_1 \) and \( C_2 \) are the CRP concentrations of monomeric and dimeric CRP, respectively, at the meniscus, \( \upsilon \) is the partial specific volume, \( \rho \) is the solvent density, \( \omega \) is the angular velocity, \( M \) is the apparent weight-average molecular weight, and \( R \) and \( T \) are the gas constant and temperature in degrees kelvin, respectively. \( K_p \) is the apparent association constant. The value of \( \upsilon \) of CRP in Tris buffer is 0.744 and was derived from the amino acid composition of CRP using the method of Cohn and Edsall (25). The apparent partial specific volumes of wild-type and mutant CRPs in 6 M GdnHCl were calculated using the procedure of Lee and Timasheff (26). The corresponding values in lower concentrations of GdnHCl were interpolated by assuming a linear relationship between GdnHCl bound and denaturant concentration.

The quaternary structure of CRP mutant was monitored by sedimentation equilibrium using a published procedure (27). In most cases, the subunit-subunit interaction of CRP was strong and could not be estimated directly by sedimentation equilibrium. The CRP dimerization was weakened by increasing amounts of GdnHCl, and the quaternary structure of CRP was monitored under each GdnHCl concentration. The loading CRP concentrations were between 0.2 and 0.4 mg/ml. Usually, 200-μl samples were loaded in a 12-mm Epon charcoal-filled center-piece. The high speed, meniscus depletion procedure was employed (28).

Having determined the value of \( K_d \) by Equation 1, \( \Delta G \), values, the free energy changes for subunit assembly at different concentrations of GdnHCl, were calculated and fitted by a linear least-squares analysis to Equation 2,

\[
-RT \ln K_d = \Delta G \times m_0 + \Delta G_d \times m_0 \times [\text{GdnHCl}] 
\]

where \( R \) and \( T \) are the gas constant and absolute temperatures, respectively. \( \Delta G \) is the extrapolated free energy changes of subunit assembly of CRP in buffer, and \( m_0 \) is the dependence of \( \Delta G \) on denaturant concentration.

**Circular Dichroism Data Acquisition and Analysis**—CD measurements were performed on an AVIV 62DS CD spectropolarimeter using
**Loops in Cyclic Nucleotide Binding Domain**

Fig. 2. **Amino acid sequence alignment of CRP and other cyclic nucleotide binding domains.** See Refs. 6 and 8 for citations of specific protein sequences.

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![Diagram](https://via.placeholder.com/150)

A 0.1-cm (for far-UV region) or 1.0-cm (for near-UV region) path length microcuvette (200-μl capacity). The protein concentration was used 7 μM. CD spectra were measured over the range of 200–320 nm. Each spectrum was recorded in 0.5-nm wavelength increments, and signal was acquired for 1 s at each wavelength. Each measurement was performed in triplicate. Deviations between scans were negligible. Baseline subtraction and smoothing of spectra curves were performed using the AVIV CDS program.

**Fluorescence Data Acquisition and Analysis**—Fluorescence measurements were carried out in 1-cm quartz cuvettes at 22.5°C using a PerkinElmer Life Sciences LS50B luminescence spectrometer. Protein concentration was 5 μM. Samples were excited at 295 nm, and tryptophan emission was monitored from 310 to 400 nm. Acrylamide quenching measurements were carried out on samples containing acrylamide (0–0.7 μM), either without or with 200 μM cyclic nucleotide. At 230–240°C, the underlined sequence is the primary DNA binding site, and the secondary sequence is the primary protein binding site.

**DNA Binding**—Fluorescence anisotropy measurements, by the SLM 8000C spectrophorimeter, were employed for quantitative evaluation of the CRP-DNA interaction. The DNA binding site was the 26-bp lac PI promoter with the sequence 5′-ATTAAAGTT-GAGTTAGCCCTACTCATTA-3′. The underlined sequence is the primary binding site for CRP. The reaction mixture of 1300–1350 μM contained 12 μM of the CPM-labeled 28-bp fragment of lac PI promoter and DNA and 200 μM cyclic nucleotide. At 230 μM, the high affinity sites for cyclic nucleotides are occupied in all CRPs employed in this study (22). The detailed experimental and data analysis protocols have been previously described (21). Briefly, the data were fitted to the following equation by non-linear least-squares to determine the apparent association constant for CRP-DNA interaction, K.

\[
F_{obs} = \sum_{i=1}^{n} X_i F_i; \quad X_i = \alpha_i / \sum_{i=0}^{\infty} \alpha_i
\]  
(Eq. 5)

where \(F_{obs}\) is the normalized value of observed fluorescence intensity at 480 nm, \(n\) is the total number of DNA molecules bound to a CRP molecule, \(i\) is the number of bound DNA molecules, and \(X_i\) and \(F_i\) are the fractions and fluorescence property, respectively. \(X_i\) is related to the fraction distribution parameter, \(\alpha_i\), which corresponds to the number of bound DNA molecules. DNA binding constant is the association constant for CRP-DNA interaction, \(K\).

\[
A = \Delta A_0 + (\Delta A_2 - \Delta A_0) \times (KD_2 + KP_2 + 1) / (KD_1 + KP_1 + 1)^2 - 4K_2 DP_2 / 2KD_2
\]  
(Eq. 6)

where \(A\) is the measured value of the anisotropy, \(\Delta A_0\) and \(\Delta A_2\) values are anisotropy with free DNA and CRP-DNA complex, respectively, \(D_1\) and \(D_2\) are the total molar concentrations of DNA and dimeric protein, respectively.
GdnHCl Denaturation—Stock solutions of 6.9 M GdnHCl were prepared in TEK (100), and the concentrations were determined with a Mettler-Paar Precision density meter. Proteins at 5 μM were unfolded in various concentrations of GdnHCl for 1 h at room temperature. Protein unfolding was monitored by CD, and the data were expressed as 

\[ S_D \]  

the measured CD signal was normalized to 

\[ S_D \] at 0 M GdnHCl.

RESULTS

Quaternary Structure and Hydrodynamic Properties—The mass of the purified protein was determined by mass spectrometry. In general, the observed mass did not deviate more than 5 Da from the calculated mass. Thus, the identities of these proteins were confirmed by both mass and DNA sequence. The molecular weight of the proteins in TEK (100) was monitored by sedimentation equilibrium. A set of typical data is shown in Fig. 3A. The molecular weights calculated from fitting a single-species model to the sedimentation equilibrium data were 4.5 ± 0.9 × 10^4, 4.4 ± 0.7 × 10^4, 4.7 ± 0.9 × 10^4, and 4.5 ± 0.7 × 10^4 for wild-type, deletion, insertion, and double-mutant CRP, respectively. The observed molecular weights correspond well to twice the calculated molecular weights of polypeptides, indicating that there was no change in the state of oligomerization, i.e., the smallest kinetic units in solution of all these CRPs were dimers. The hydrodynamic property of these proteins was monitored by sedimentation velocity. The weight-average sedimentation coefficient (\( S_{20,w} \)) was determined by fitting the sedimentation velocity data to a single species with the DCDT+ analysis, as shown in Fig. 3B. The results show that the data fit well to a single species, and the values for \( S_{20,w} \) were 3.67 (3.65, 3.70), 3.51 (3.48, 3.55), 3.67 (3.65, 3.71), and 3.48 (3.45, 3.52) for wild-type, deletion, insertion, and double-mutant CRP, respectively. The numbers in parentheses represent the distribution of a 68% confidence interval. The decrease in \( S_{20,w} \) for deletion and double mutants indicates either an increase in asymmetry or that these molecules assume a less compact conformation.

Secondary and Tertiary Structures—The secondary structures of the mutant CRP were monitored by CD, as shown in Fig. 4A. All mutants have far-UV CD spectra similar to that of wild-type CRP, so it can be concluded that these loop mutations do not significantly change the secondary structural content of

Fig. 3. Analytical ultracentrifugation data of CRPs in TEK (100) buffer at pH 7.8 and 22.5 ºC. The loading protein concentrations were 15 μM for wild-type (●), 9 μM for deletion (○), 13 μM for insertion (▼), and 16 μM for double mutant (▼), respectively. Dots represent data, and the solid lines represent the best fits of the data. A, sedimentation equilibrium; B, sedimentation velocity.

Fig. 4. CD spectra of CRPs in TEK (100) buffer at pH 7.8 and 22.5 ºC. Protein concentrations were 5 μM (●) wild-type, (○) deletion, (▼) insertion, (▼) double mutant. A, far-UV region; B, near-UV region.
CRP. Significant differences, however, are observed in the near-UV CD spectra, as shown in Fig. 4B. The spectra for all samples showed fine features between 250 and 300 nm, although the magnitudes of ellipticity for mutants were greater than that of the wild-type CRP. The 255- and 265-nm peaks are most likely reflective of Phe residues; the 275-nm peak of Tyr residues and the 285- and 295-nm peaks of Trp residues. These results indicate that these loops mutations lead to a perturbation of the microenvironments of the aromatic residues of CRP.

Because all aromatic residues (5 Phe, 6 Tyr, and 2 Trp per CRP subunit) with the exception of Tyr-206 and Phe-136 are located in or near the CNB domain, these CD results indicate that the local environments of the CNB domain of CRP are perturbed by these mutations without significantly altering the net content of the secondary structures.

**Solvent Accessibility of Trp Residues**—Each CRP subunit has 2 Trp residues, both of which are located in the CNB domain. Trp 85 is located inside the β-roll and close to the dimer interface. Thus, intrinsic Trp fluorescence is a useful probe of the microenvironments of the CNB domain. The fluorescence emission spectra of the deletion and double mutants show a red-shift as compared with that of wild-type CRP, as shown in Fig. 5A. The intensities of the emission spectra of the deletion and double mutants were significantly higher than that of wild-type CRP. The difference between the insertion mutant and wild-type was much smaller (Fig. 5A). These results indicate that deletion of loop 3 leads to a more polar environment around the Trp residues. Insertion of extra residues in loop 4 had no apparent effect and did not compensate for the effect of deletion of loop 3. In the presence of cAMP, significant fluorescence blue-shifts and quenching occurred in the emission spectra of the deletion and double mutants, as shown in Fig. 5B. But the spectra of the insertion mutant and wild-type CRP did not change much. The blue shifts in spectra indicate that cAMP binding induces a change in the microenvironments of Trp so that they are less polar in deletion and double mutants, whereas there are no such detectable changes in the insertion mutant and wild-type CRP. These differences indicate that cAMP binding induces much more significant conformational changes in the CNB domains due to the deletion at loop 3.

The accessibility of Trp residues is quantitatively assessed using fluorescence collisional quenching by acrylamide. Results of these measurements are shown in Fig. 6. The data were fitted to Equation 3, and the parameters are summarized in Table I. In the apo-state, the magnitude of collisional quenching constants ($K_{q}$) was in the increasing order of insertion $<$ wild-type $<$ double $<$ deletion. These results imply that the Trp residues of the mutants that consist of a deletion in loop 3 are
were monitored by sedimentation equilibrium. The data were determined by the linear extrapolation method, as shown in Fig. 7. The extrapolated values of $\Delta G^o$ are summarized in Table I. These results show that mutations in these loops induce a destabilization of the formation of CRP dimers. Interestingly, simultaneous mutations in both loops did not further decrease the stability of intersubunit interface. In fact, the effect of deletion seemed to be dominant.

**Protein Stability**—Unfolding of wild-type CRP and mutants was monitored by CD in the presence of increasing concentrations of GdnHCl. Ellipticity at 222 nm was employed as the parameter to reflect on the changes in secondary structure. The unfolding curves of wild-type and mutant CRP are shown in Fig. 8. The detectable differences in the unfolding curves are localized between 0 to 3 M GdnHCl, after which the curves seem to merge into the same curve. The similarity among curves at similar for all the mutants, the spectroscopically detected unfolding curves at low GdnHCl concentrations are not identical for these mutants, as shown in Fig. 8. This implies that there are likely differences in structural stability among wild-type, insertion, and deletion mutants while there is no observable differences between the deletion and double mutants. The specific nature of the differences in stability has yet to be defined.

**Cyclic Nucleotide Binding**—Each cAMP molecule interacts with one $\beta$-roll and both C-helices from adjacent subunits (1). It is not surprising that the affinity to cAMP and the cooperativity of cAMP binding are related to arrangements both within a CNB domain and between the two CNB domains. The binding of cyclic nucleotide to CRP was monitored by fluorescence, and the results are shown in Fig. 9. The binding isotherm for wild-type CRP exhibits the biphasic behavior as reported before (22), representing the binding of cAMP to the high and low affinity sites. The binding isotherms for the deletion and double mutants are almost identical but significantly different from that of wild-type CRP. They show a significant degree of positive cooperativity, and the second binding phase seems to be absent. The binding isotherm for the insertion mutant coincides with the initial phase of that of the wild-type, but there is no evidence of another binding event at high concentrations of cAMP. These results imply that the mutants have either lost their ability to bind cAMP at the low affinity site or that the surfaces of these mutants have been altered so significantly that the binding is not reflected by ANS fluorescence. The binding of cAMP to the high affinity sites in the insertion mutant exhibits similar affinity as that of wild-type CRP. The difference is that wild-type CRP shows moderate positive cooperativity of cAMP binding, whereas the insertion mutant shows very weak negative cooperativity. On the contrary, the deletion and double mutants show very strong positive cooperativity of cAMP binding. The degree of positive cooperativity is in the following decreasing order: deletion > double > wild-type > insertion, as reflected by the value of $k_2/k_1$ summarized in Table II.

**Energetics of Subunit Assembly**—The effect of mutations on the energetics of subunit assembly was probed by monitoring the dissociation of the dimeric protein in the presence of GdnHCl. The apparent molecular weights of these proteins in buffer, $M_a$, were determined by the linear extrapolation method, as shown in Fig. 7. The extrapolated values of $M_a$ are summarized in Table I. These results are consistent with those of fluorescence experiments, which monitored the emission spectra of CRP, confirming that the deletion apparently exposes the Trp residues of the Trp residues to solvent, and, on the contrary, the insertion slightly buries the Trp residues into the protein matrix. Furthermore, two more important points can be inferred from the environmental change of the Trp residues accompanying cAMP binding. First, the increased accessibility of Trp induced by deletion can be reversed by cAMP binding. Second, the insertion mutational change of the Trp residues to solvent, and, on the contrary, the insertion slightly buries the Trp residues into the protein matrix. Furthermore, two more important points can be inferred from the environmental change of the Trp residues accompanying cAMP binding. First, the increased accessibility of Trp induced by deletion can be reversed by cAMP binding. Second, the insertion mutation modulates this conformational change.

**Summary of structural data**

| CRP   | cAMP  | $K_w$  | $V$  | $\Delta G^o$ | $m_a$  |
|-------|-------|--------|------|--------------|--------|
| WT    | -     | 6.2 ± 0.2 | 0.07 ± 0.03 | 3.67 (3.65, 3.70) | 11.5 ± 0.8 | 2.2 ± 0.3 |
| Deletion | +     | 7.2 ± 0.1 | 0.06 ± 0.05 | 3.51 (3.48, 3.55) | 9.6 ± 0.4 | 1.5 ± 0.2 |
| Insertion | -     | 5.4 ± 0.1 | 0.26 ± 0.03 | 3.67 (3.65, 3.71) | 7.9 ± 0.3 | 1.0 ± 0.1 |
| Double | +     | 5.4 ± 0.2 | 0.28 ± 0.05 | 3.67 (3.45, 3.52) | 9.3 ± 0.4 | 1.4 ± 0.2 |

* With and without 230 μM of cAMP.

* Numbers in parentheses are the distributions of a 68% confidence interval.

**Fig. 7. Free energy changes for dimer association of CRP as a function of GdnHCl concentration.** The observed $\Delta G^o$ values at different GdnHCl concentrations were fitted by linear least squares analysis in accordance to Equation 2. The recovered parameters are listed in Table II. The symbols are the same as in previous figures.
DNA Binding Assay—Under the described experimental condition, in the apo-state or in the presence of 230 μM cGMP, all mutants and wild-type CRP showed only weak affinities for specific DNA: the observed binding constants $K_{\text{app}}$ were $<1 \times 10^3 \text{M}^{-1}$ for wild-type CRP and insertion mutant and between $1 \times 10^4$ and $1 \times 10^5 \text{M}^{-1}$ for the deletion and double mutants. In the presence of 230 μM cAMP, all these proteins exhibited a large increase in affinities for specific DNA; the binding constants ($K_{\text{app}}$, $1 \times 10^{-7} \text{M}^{-3}$) are $5.6 \pm 0.3$ for wild-type CRP, $2.0 \pm 0.1$ for deletion, $3.5 \pm 0.1$ for insertion, and $2.4 \pm 0.1$ for double mutants, as shown in Fig. 10 and summarized in Table II. The affinity of wild-type CRP for DNA is about 2- to 3-fold higher than all of the mutants. These results indicate that these mutations do not change the specific requirement of activation by cAMP. The mutations do not qualitatively but quantitatively change the cAMP-dependent DNA affinity of CRP, which responds only specifically to cAMP and not to cGMP. In other words, these results imply that these mutations perturb but do not change the mechanism of allosteric communication between the CNB and DNA binding domains.

**DISCUSSION**

Some loops in proteins seem to serve as no more than connectors between secondary structural elements, others play much more important functional roles, such as in defining stability (29, 30). On the basis of structural information and results of mutagenesis analyses, it was proposed that loop 3 in CRP is involved in both interdomain and intersubunit interactions, whereas loop 4 contacts the C-helices and is proposed to form part of the dimer interface (1). Some point mutations, either within or just outside of these loops, have been reported to significantly affect the function of CRP, e.g. K52N, D53H, and S62F (beside loop 3) and E72A, K82Q, and S83K (beside loop 4) (21, 22, 31–34). Results from protein footprinting and NMR experiments indicate that the regions, including these loops exhibit significant environmental changes upon cAMP binding (18, 19). All these results imply that these loops are involved in the functioning of CRP. This conjecture on structure-function correlation is further supported by an alignment analysis of the protein sequence of CNB (Fig. 2) that reveals an intriguing pattern. The sizes of loops 3 and 4 are the only ones of the loop structures that are varied. In cyclic nucleotide-gated channels there are deletion and extension in the sequences of loop 3 and 4, respectively. In cyclic nucleotide-dependent protein kinases, the sequence variation mostly resides in loop 3. Thus, it is of interest to probe for the roles of these loops in CRP.

CRP is a transcription factor that exhibits allosteric behavior. There is homotropic effects in the binding of cAMP and heterotropic effect between cAMP and DNA bindings. The deletion of residues 54–56 (EEG) in loop 3 leads to the most significant perturbations in the normal functional properties of CRP. The binding affinity of the first cAMP molecule is significantly weaker than that of the wild-type CRP, however, the mutation also enhances the binding affinity for the second cAMP molecule, leading to a significant enhancement of positive cooperativity as indicated by the steeper binding isotherm (Fig. 10). This mutation also lowers the energetics of subunit assembly. Thus, a perturbation of this loop amplifies its impact on functional sites that are located at different parts of CRP and spatially quite a few angstroms away. The specific nature of
Loops in Cyclic Nucleotide Binding Domain

**Table II. Summary of functional data**

| CRP                  | $k_1$     | $k_2$   | $k_3$  | $k_4/k_1$ | DNA binding$^b$ |
|---------------------|-----------|---------|--------|-----------|-----------------|
| Wild-type           | 1.5 ± 0.3 | 2.4 ± 1.5 | 1.7 ± 1.7 | 1.6       | 5.6 ± 0.3       |
| Deletion            | 2.9 ± 0.7 | 4.8 ± 1.0 | 170     | 2.0 ± 0.1 | 3.5 ± 0.1       |
| Insertion           | 2.3 ± 0.8 | 1.4 ± 0.3 | 0.6     | 3.5 ± 0.1 | 2.4 ± 0.1       |
| Double              | 4.6 ± 0.4 | 3.4 ± 0.3 | 74      |           |                 |

$^a$ Based on Ref. 1, 13, 20, CRP has 1–2 additional cAMP binding sites in its DNA binding domain. This model is fitted best for wild-type CRP, but not for mutants.

$^b$ In the presence of 230 μM cAMP.

**Fig. 10. Binding of CRP to lac26 DNA in TEK (100) buffer at pH 7.8 and 22.5 °C.** (●) wild-type, (○) deletion, (▲) insertion, (□) double mutant. The reaction mixture contained 18 nM DNA and 230 μM cAMP. The solid lines represent the best fits of the data in accordance to Equation 6.

Functional impacts by loop 3 mutation on CRP is apparently also observed in the various isozymes of protein kinases. As shown in Figs. 1 and 2, the sequence differences between sites A and B of the regulatory subunit is often localized in loop 3, namely, in general there is a deletion of a few residues in loop 3 of site A as compared with site B. It has long been established that the binding affinity of site A is lower than site B, an observation parallel that of the deletion and wild-type CRP, respectively. It is not surprising that cooperativity of ligand binding is different between these two types of sites (35–42).

One might speculate on the mechanism that enables loop 3 to exert these homotropic and heterotropic effects. Residue 136 of the adjacent subunit forms a complex with loop 3. This might be one of the paths of intersubunit and interdomain interactions. Any mutation that leads to a perturbation of this interaction could be expected to affect the allosteric behavior, as shown in this study. Furthermore, the nature of the perturbation could be manifested to yield different functional consequences. For example, mutations at residues 52 and 62 lead to decreases in both homotropic and heterotropic effects, but mutation at residues 53 leads to an opposite effect (21, 22). Thus, apparently, perturbations of residues in loop 3 can modulate the allosteric effects either positively or negatively. Therefore, the role of loop 3 is a modulator in the true sense.

The insertion of five residues in loop 4 seems to have only marginal effects on the functional properties of CRP. There is no significant perturbation in cAMP binding, and the effect on DNA binding is small, although the energetics of subunit assembly is reduced. Thus, these results are consistent with the proposal that loop 4 forms part of the dimer interface (1).

Results of the double mutant show that the functional impact of loop 3 is dominant over that of loop 4, which only modulates marginally the effects exerted by the deletion of loop 3.

Results of the double mutant of CRP can be compared with the report on the chimeric protein that was constructed by linking the CNB domain from Brø CNGC and the DNA binding domain from CRP (43). Although there are significant differences in the primary sequences between the CNB domains, the chimera reveals structure similar to that of wild-type CRP. Loop 3 and loop 4 of the chimera are of the same sizes as the double mutant of this study, thus it is valid to compare and contrast these proteins. The affinity of the chimera to DNA is about 2-fold lower than that of wild-type CRP (43) and is of the same level as the double mutant of this study. This similarity strongly implies that it is the two loops rather than the primary sequences that fine-tune the DNA affinity of CRP. Based on the crystal structure of the CRP–cAMP complex, there are two possible pathways through which these loops exert their functional roles. Pathway one: they join in the pathway of allosteric signal communication and are much involved in interfacial interactions between domains and subunits as it has been postulated (1, 9); pathway two: they are involved in ligand binding due to their proximity to the ligand binding pocket (1, 5). Although the non-conserved sequences of these loops within the CNB protein family suggest that they are unimportant to the basic structure and function of the CNB domain, results of this study indicate that these loops do play a significant role in defining the affinity and cooperativity of cAMP binding. However, the mutations in loop size quantitatively change the cAMP-dependent DNA affinity of CRP without changing the specificity of requiring cAMP but not cGMP for activation. Thus, it is clear that these loops do not alter the basic mechanism of activity but only modulate these activities. They may exert their effects via regulating interfacial interactions and consequently regulating the subunit realignment, such as that observed in this study.

The deletion of three residues in loop 3 not only affects the functional properties of CRP, it significantly perturbs the structure. The sedimentation velocity data shows that deletion causes a decrease of 0.2 S, a change that is greater than the distribution for a 68% confidence interval. Thus, this change indicates a perturbation in the hydrodynamic properties of CRP. Thus, the deletion causes the CRP dimer to either assume a more asymmetric or less compact conformation. The change in the global conformation is associated with a decrease in the energetics of subunit assembly and perturbations of the local environments of aromatic residues as indicated by the spectroscopic data. Results of the fluorescence studies indicate that the accessibility of Trp residues is increased by the deletion mutation. It was reported that Trp-13 is substantially solvent-exposed and contributes to ~80% of the Trp fluorescence,
whereas Trp-85 is buried in the matrix of CRP and contributes to roughly 20% of the Trp fluorescence (44). Results obtained using single-tryptophan-containing CRP mutants indicate that Trp-85 is accountable for the total change observed of the intrinsic Trp fluorescence in wild-type CRP (45). The above cited reports all strongly suggest that the significant changes on the tryptophan fluorescence in the deletion and the double mutants are most likely attributable to Trp-85. Because Trp-85 is sterically close to the dimer interface, the increased Trp solvent exposure is consistent with the fact that the dimer interfacial interaction is perturbed. The widening of the inter-subunit contact might lead to a change in the subunit realignment by cAMP binding, as indicated by the large shifts in emission spectra in the presence and absence of cAMP. In contrast, the changes in the Trp fluorescence emission spectra are small in wild-type CRP.

It has been reported that the strong positive cooperativity and the “functional polarity” are essential properties of the CNB domain of CNGC. “Functional polarity” is a term employed by these investigators to describe the phenomenon that the β-roll stabilizes the ligand in a state-independent manner, whereas the C-helix selectively stabilizes the ligand in the open state of the channel (46, 47). Just like the cooperativity of ligand binding in CRP, as was discussed above, the functional polarity is also the result of the subunit realignment. Recently, the subunit realignment involved in the cAMP modulation of HCN channels has been reported (48). Therefore, it is reasonable to postulate that the loosening of the inter-subunit contact caused by the truncated loop 3, which facilitates the subunit realignment, is important for the modulation of CNGC.

In summary, mutations of loops 3, and to a smaller extent in loop 4, lead to global structural perturbations such as subunit alignment. As a consequence, the functional properties are quantitatively altered. This global effect is consistent with the realignment. As a consequence, the functional properties are quantitatively altered. This global effect is consistent with the realignment.

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