Ability of *E. coli* Cyclic AMP Receptor Protein To Differentiate Cyclic Nucelotides: Effects of Single Site Mutations†

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ABSTRACT: *Escherichia coli* cyclic AMP receptor protein (CRP) is a global transcriptional regulator which controls the expression of many different genes. Although different cyclic nucleotides can bind to CRP with almost equal affinity, only in the presence of cAMP could wild-type CRP bind to specific DNA sequences. Molecular genetic studies have identified a class of mutants, CRP*, which either do not require exogenous cAMP for activation or can be activated by cGMP. Thus, these mutants might aid in identifying the structural elements that are involved in the modulation of CRP to correctly differentiate the messages embedded in cyclic nucleotides. In this in vitro study, five CRP* mutants, namely, D53H, S62F, G141Q, G141K, and L148R, were tested for their abilities to bind the *lac* promoter sequence and the effects of cyclic nucleotides in modulating DNA sequence recognition. For comparison, non-CRP* mutants K52N, T127L, H159L, and K52N/H159L were studied. cCMP and cGMP can replace cAMP as an allosteric effector in all of these CRP mutants except S62F and non-CRP* mutants. The D53H, G141Q, G141K, and L148R mutants exhibit significantly higher affinity for the *lac* promoter sequence than wild-type CRP while S62F and the non-CRP* mutants exhibit reduced affinity. To probe the pathway of communication, the energetics of subunit assembly in these mutants were monitored by sedimentation equilibrium, and the conformational states of these mutants were probed by proteolysis and accessibility of Cys178 to chemical modifications. Results from these studies imply that signals due to mutations are mostly transmitted through the subunit interface. Thus, residues in CRP outside of the cyclic nucleotide binding site modulate the ability of CRP to differentiate these three cyclic nucleotides through long-range communication. Furthermore, this study shows that CRP* mutations do not impart any unique properties to CRP except that the DNA binding constants are shifted to a regime of higher affinity.

Escherichia coli cyclic AMP receptor protein, CRP, is a global transcriptional regulator that controls the expression of many genes that are responsive to the fluctuation of the cellular concentration of cAMP during its cell cycle (*1*−*4*). The first step in the mechanism of CRP activity involves an allosteric activation of the protein by cAMP binding. In the absence of cAMP, wild-type CRP does not bind specific DNA. Although other cyclic nucleotides can bind to wild-type CRP, only cAMP can serve as an activator. Thus, CRP is designed with a capability to recognize and distinguish the various cyclic nucleotides. This pattern of behavior implies that CRP has the ability to interpret the messages imbedded in the cyclic nucleotides, to transmit the messages to the DNA binding site, and to implement the instruction brought by the messages by binding or not binding to specific DNA sequences.

cAMP is recognized by CRP as a consequence of an intricate network of interactions between cAMP and CRP residues such as Glu72, Arg82, Thr127, and Ser128 (*5*, *6*). The structural studies on the CRP−cAMP complex provide valuable information on the participation of these specific side chains in binding these ligands (*7*, *8*). It is not known if other structural elements would modulate this network of interaction. In addition, there is much less known about the structural elements in CRP that are responsible for transmitting the information from the cAMP binding domain to the DNA binding domain. In an effort to identify and elucidate the roles of structural elements in signal transmission, Cheng and Lee (*9*−*11*) studied a class of CRP mutants, designated as CRP*. In vivo studies indicate that these mutants assume the activated conformation without binding cAMP (*12*−*15*). Results of these in vitro studies imply that these sites of mutation might be involved in the allosteric activation processes which include recognition, interpretation, and transmission of signal. In vitro studies by Cheng and Lee (*10*, *11*) show that the G141Q CRP* mutant still requires the binding of cyclic nucleotide for activation, although there is an apparent relaxation of specificity for cAMP. Other cyclic nucleotides can behave as cAMP; namely, the CRP−cNMP complex binds specific DNA sequence in contrast to wild-type CRP which shows no binding. This result implicates residue 141 in modulating the functional properties of CRP and that this class of CRP mutants might aid in identifying the structural elements involved in differentiating the messages imbedded in cyclic nucleotides. In an effort to address the issues on the identity of structural elements

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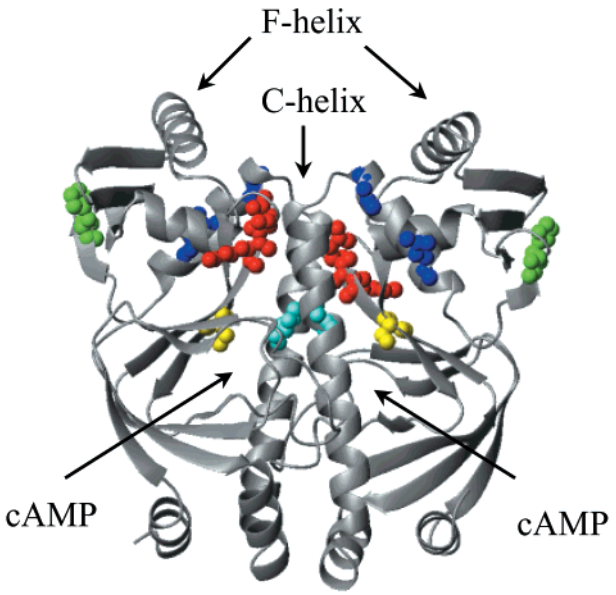

FIGURE 1: Structure of the CRP dimer. The locations of residues 52 and 53 are marked as red, 62 as yellow, 141 and 148 as blue, 159 as green, and 127 as cyan. The structure of protein was drawn using Molmol software (Institute für Molekularbiologie und Biophysik ETH−Hönggerbertz, CH-8093 Zürich) and atomic coordinates of CRP (1G6N) (*7*) from the RCSB Protein DataBank (http://www.rcsb.org/pdb/).

involved in differentiating messages and the roles of these structural components, other CRP* mutants were generated by site-directed mutagenesis, expressed, and purified. Five mutants, namely, D53H, S62F, G141Q, G141K, and L148R, were chosen because of the diversity of their locations in the CRP structure and also due to their in vivo characteristics. Residue 53 is located in a loop between sheets 4 and 5 while residue 62 resides in sheet 5. Both residues are in the cAMP binding N-terminal domain of CRP, while residues 141 and 148 are in the D-helix of the DNA binding C-terminal domain. Results from in vivo studies indicate that in this series of CRP* mutants, S62F CRP exhibits the weakest efficiency in activating gene expression (*16*). To probe the uniqueness of the structural elements identified by these CRP* mutants, non-CRP* mutants, namely, residues 52, 127, and 159, and the double mutant 52/159 were also studied. Residues 52 and 159 are located in sheets 4 and 9, respectively, and also in the cAMP and DNA binding domain, respectively. Residue 127 is located in the C-helix that is involved in intersubunit interactions. The locations of the sites of mutation are shown in Figure 1. The purified CRP mutants were tested for their abilities to bind specific DNA sequence and the effects of cyclic nucleotides in modulating DNA sequence recognition. Furthermore, the effects of these mutations on intersubunit communication were probed.

EXPERIMENTAL PROCEDURES

All in vitro experiments were conducted in 50 mM Tris, 100 mM KCl, and 1 mM EDTA [TEK (100)][1] at pH 7.8 and 25 °C. The concentrations of protein, cyclic nucleotides, and fluorescence probes were determined by absorption spectroscopy using the following absorption coefficients: 40 800 M^{-1} cm^{-1} at 278 nm for CRP dimer; 14 650 M^{-1} cm^{-1} at 259 nm, 12 950 M^{-1} cm^{-1} at 254 nm, and 9100 M^{-1} cm^{-1} at 271 nm for cAMP, cGMP, and cCMP, respectively; 33 000 M^{-1} cm^{-1} at 385 nm for CPM.

Site-Directed Mutagenesis. The Promega Altered Site in vitro Mutagenesis System was used to introduce specific point mutations into the *crp* gene using a previously published procedure (*9*−*11*). The desired mutant was directly screened by DNA sequencing using the Sequenase Version 2.0 kit from United States Biochemical Corp.

lac Operon Activation in Vivo. To test the effects of the D53H, G141Q, G141K, and L148R mutation on *lac* operon expression in vivo, *E. coli* strain CA8445/pRK248cI^{ts} transformed with plasmid pPLC28 that contained the mutant *crp* genes was streaked on MacConkey lactose indicator plates in the absence or presence of 0.5 mM cAMP. The plates were incubated at 37 °C overnight. The fermentation responses of the mutants were scored according to the color of the colonies on the plates.

Expression of Mutants. The specific *E. coli* strains and procedures employed for induction were the same as previously published (*9*−*11*).

Protein Purification. Wild-type and mutant CRPs were purified from *E. coli* strain K12 ΔH1 and CA8445/pRK248cI^{ts}, respectively, using a previously described protocol (*9*). All purified CRP proteins are >99% homogeneous as judged by SDS−PAGE stained by 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. Purity and mass of proteins were further checked by mass spectrometry.

DNA Binding. Fluorescence anisotropy measurements, using the SLM 8000C spectrofluorometer, were employed for quantitative evaluation of the CRP−DNA interaction (*17*). The DNA binding site was the 26 bp fragment of the *lac PI* promoter with the sequence 5′-ATTAATGTGAGTTAGCTCACTCATTA-3′. The underlined sequence is the primary binding site for CRP. The reaction mixture of 1300−1350 μL contained 12 nM CPM-labeled 26 bp fragment of *lac* promoter DNA and 200 μM cyclic nucleotide. At 200 μM, the high-affinity sites for cyclic nucleotides are occupied in all CRP's employed in this study.[2] Small volumes of concentrated CRP, ranging from 0.5 to 200 μM, depending on the mutant under investigation, were titrated into the reaction mixture in 1 or 2 μL aliquots. The anisotropy of the CPM-labeled DNA was measured after each addition of protein. The excitation wavelength was 390 nm. An Oriel band-pass filter (model 59816) was placed in the excitation path to reduce second-order scatter. Oriel band-pass filters (model 59850) were placed in the emission paths to monitor the total emission between 470 and 570 nm. Experiments were performed in TEK (100) buffer, and data were fitted to the following equation by nonlinear least-squares (Sigmaplot window version 5.0) to determine the apparent

[1] Abbreviations: CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; TEK (100) buffer, 50 mM Tris, 0.1 M KCl, 1 mM EDTA at pH 7.8; DTNB, dithionitrobenzoic acid.

[2] Lin, S.-H., Kovac, L., and Lee, J. C., manuscript in preparation.

association constant for CRP−DNA interaction, K_x:

$$A = A_D + (A_{PD} - A_D) \times \frac{(K_xD_T + K_xP_T + 1 - \sqrt{(K_xD_T + K_xP_T + 1)^2 - 4K_x^2D_TP_T})}{2K_xD_T} \quad (1)$$

where A is the measured value of the anisotropy, A_D and A_{PD} are values of anisotropy associated with free DNA and CRP−DNA complex, respectively, D_T and P_T are the total molar concentrations of DNA and dimeric protein, respectively, and x is A, C, or G for cAMP, cCMP, or cGMP, respectively.

Subunit Association. The quaternary structure of CRP mutant was monitored by sedimentation equilibrium using the published procedure (*10*, *18*). In most cases, the subunit−subunit interaction of CRP is strong and cannot be estimated directly by sedimentation equilibrium. The CRP dimerization was weakened by increasing amounts of GuHCl, and the quaternary structure of CRP was monitored under each GuHCl concentration. Briefly, experiments were conducted in TEK (100) buffer at 20 °C using a Beckman XL-A analytical ultracentrifuge. 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 centerpiece. The high-speed, meniscus-depletion procedure was employed (*19*).

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

$$C = E + C_{1,r0}\exp\left[\frac{(1-\bar{v}\rho)\omega^2}{2RT}\cdot M(r^2 - r_0^2)\right] + C_{2,r_0}{}^2K_a\exp\left[\frac{(1-\bar{v}\rho)\omega^2}{2RT}2M(r^2 - r_0^2)\right] \quad (2)$$

where C is the observed CRP concentration in absorbance at radial position r, E is the baseline offset, $C_{1,r0}$ and $C_{2,r0}$ are the CRP concentrations of monomeric and dimeric CRP, respectively, at the meniscus r_0, $\bar{v}$ is the partial specific volume, ρ is the solvent density, ω 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_a is the apparent association constant. The value of $\bar{v}$ 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 (*20*). The apparent partial specific volume of CRP in 6 M GuHCl was calculated using the procedure of Lee and Timasheff (*21*). The corresponding values in lower concentrations of GuHCl were interpolated by assuming a linear relationship between GuHCl bound and denaturant concentration.

Once the values of K_a were estimated by eq 2, ΔG_a values, the free energy changes upon subunit assembly at different GuHCl concentrations, were calculated, and then were fitted by a linear least-squares program to eq 3:

$$-RT\,ln\,K_a = \Delta G_a = \Delta G_a{}^0 + m_a[\text{GuHCl}] \quad (3)$$

where R is the gas constant, T is the absolute temperature, $\Delta G_a{}^0$ is the extrapolated free energy changes of subunit assembly of CRP in the absence of denaturant, and m_a is the dependence of ΔG_a on GuHCl concentration.

Proteolytic Digestion. Susceptibility to proteolysis was employed as a tool to monitor structural changes in CRP. Reactions were conducted in 20 μL of a mixture which contained CRP at 0.25 mg/mL and chymotrypsin or subtilisin at 3 μg/mL and different concentrations of cAMP in TEK (100) buffer. The reactions were carried out at 25 °C for 80−240 min and stopped by adding 0.5 μL of 50 mM PMSF in 2-propanol. A 10 μL aliquot of the reaction mixture was withdrawn and mixed with 10 μL of sample buffer and loaded onto a 15% SDS−PAGE slab gel for analysis using a Bio-Rad Mini Protean II electrophoresis system. The gels were stained with Coomassie Blue R-250.

Kinetics of Cys178 Modification. The changes in reactivity of Cys178 in response to cAMP binding were studied by monitoring the rate of modification by DTNB. Equal volumes of 600 μM DTNB and 4−6 μM CRP mutant solutions were mixed by the SFA-II Rapid Kinetics Accessory. The change in absorbance at 412 nm was monitored as a function of time by an AVIV Spectrophometer 14 DS. The rate constants for the reaction were obtained by fitting the experimental data to the equation for a psendo-first-order reaction by a nonlinear least-squares procedure.

Circular Dichroism Spectroscopy. CD measurements were performed on an AVIV model 60 DS spectropolarimeter. CD spectra were measured over the range of 200−340 nm using 0.1 or 1.0 cm path length cells. Each spectrum was recorded in 0.5 nm wavelength increments, and signal was acquired for 1 s at each wavelength.

RESULTS

Requirements of Cyclic Nucleotides for Activation. The phenotype of CRP mutants can be tested by using MacConkey agar indicator plates. When a mutant *crp* allele is expressed in a crp$^-$/cya$^-$ strain, a CRP* phenotype is defined as showing purple colonies on a MacConkey lactose plate in the absence of exogenous cAMP or in the presence of cGMP. A CRP$^+$ phenotype displays purple colonies only in the presence of cAMP, while a CRP$^-$ phenotype is a mutant that always shows white colonies. D53H, G141Q, G141K, and L148R CRP mutants displayed purple colonies in the MacConkey plates in the absence of exogenous cAMP. Thus, these mutants do exhibit the expected CRP* phenotype.

The structures of CRP mutants were monitored by CD. The spectra of D53H and L148R CRP were presented as typical results, as shown in Figure 2. Both the near- and far-UV spectra are essentially identical to each other and to the wild-type CRP. Thus, mutations of CRP at these sites do not elicit any observable differences in the secondary and tertiary structures of CRP.

In an earlier study of the G141Q CRP mutant, it was shown that it requires cAMP for activation (*9*). Thus, the requirement of cAMP for the allosteric activation of these CRP* mutants was tested. Binding of these CRP mutants to the 26 bp *lac* CRP binding sequence was monitored by the fluorescence anisotropy method (*17*). Results of the L148R mutant are employed to illustrate the behavior of CRP* mutants. In the absence of cAMP, there was no observable increase in anisotropy up to 10^{-8} M L148R CRP* mutant, as shown in Figure 3A. Only at protein concentrations

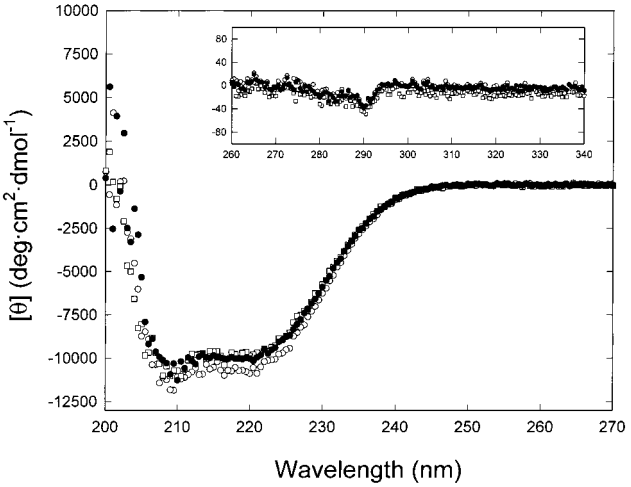

FIGURE 2: CD spectra of the wild type (●) and D53H (○) and L148R (□) mutants of CRP in TEK (100) buffer. Protein concentrations used for the measurement were 0.81, 0.64, and 0.62 mg/mL for wild-type, D53H, and L148R CRP, respectively. For each sample, three repetitive scans were acquired and averaged. Inset: CD signals of the wild-type and mutants in the near-UV region. The axes of the insert are the same as the main figure.

approaching 10^{-7} M was a measurable increase in anisotropy observed. However, in the presence of 2×10^{-4} M cAMP, an increase in anisotropy was observed even at 10^{-9} M concentrations of protein, and the observed increase in anisotropy approaches a plateau before reaching a protein concentration of 10^{-7} M. These results imply that the L148R CRP* mutant binds to specific DNA sequence in the absence of cAMP although the binding affinity is weak. The L148R CRP* mutant is activated by cAMP binding, and its ability to distinguish between specific and random sequences remains intact.[3,4]

Similar studies were conducted with the other mutants. Within the same protein concentration range, there was no observable increase in anisotropy in the absence of cAMP, although a significant amount of binding to the 26 bp *lac* CRP binding sequence was observed in all mutant forms in the presence of 2×10^{-4} M cAMP.

Specific DNA sequence recognition by wild-type CRP is modulated by cAMP and cAMP alone, although other cyclic nucleotides can bind to the protein. Yet, it was reported that the G141Q CRP* mutant exhibits a relaxation in its selectivity toward cAMP (*9*). The G141Q mutant was shown to bind specific DNA sequence in the presence of other cyclic nucleotides. The ability of all of the CRP mutants to bind *lac* in the presence of cGMP and cCMP was tested. Figure 3B shows the results of L148R mutant binding to the *lac* fragment in the presence of 2×10^{-4} M cGMP and cCMP. It is evident that both cyclic nucleotides can bind to this mutant CRP. These CRP−cNMP complexes bind to specific DNA sequence, although the affinity for the same DNA sequence was modulated by the specific cyclic nucleotide present. The binding isotherms were fitted to eq 1 to determine the binding constants. Although there were no obvious plateau values of anisotropy at saturation, a data set which reaches 90% saturation would lead to only 5−10%

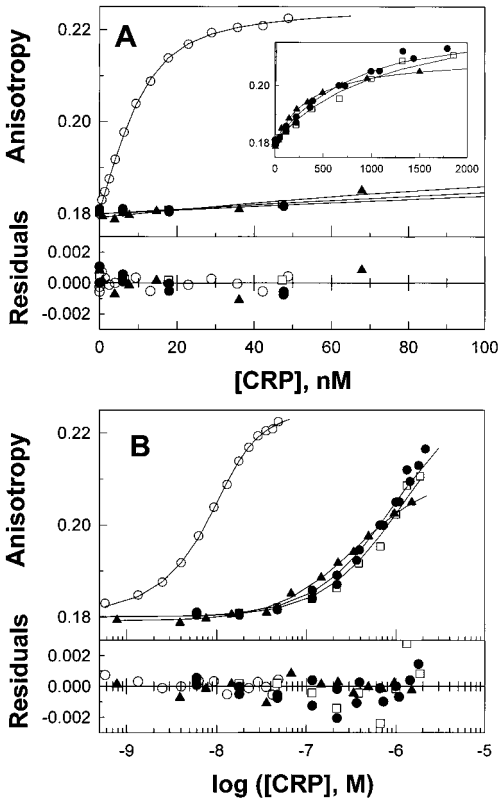

FIGURE 3: (A) Typical binding isotherms of L148R CRP binding to *lac26* DNA in TEK (100) buffer at pH 7.8 and 25 °C. Inset: Plot of data at high CRP concentration. (B) Binding isotherms in the format of a semilog plot. The anisotropy of CPM-labeled DNA was normalized to 0.1800. The symbols and corresponding cyclic nucleotides are the following: (○) 200 μM cAMP, (□) 200 μM cGMP, (▲) 200 μM cCMP, and (●) no cyclic nucleotide. The solid line represents the best fit of the data to eq 1. When the isothermal titration failed to reach a saturation level, e.g., cGMP and no cNMP, the fittings were done with a constraint of $A_{PD} - A_D = 0.042$, a value recovered from the fitting of data with cAMP. This same value for ΔA has consistently been returned in fitting in all experiments using this DNA sequence. At least two binding curves were measured for each condition, although single titration is presented. The goodness of fit is shown by the random distribution of residuals about zero.

Table 1: Affinity of CRP−*lac26* DNA Complex Formation, with 200 μM cNMP

	$K_{app} \times 10^{-6}$ M^{-1}			
	cAMP	cGMP	cCMP	ratio[d]
WT	49.0 ± 6.4	nm[b]	(0.086−0.186)[c]	350
K52N	6.7 ± 0.4	nm	(0.32−0.6)	13
D53H[a]	731 ± 122	0.12 ± 0.01	2.6 ± 0.1	281
S62F[a]	0.54 ± 0.03	nm	0.12 ± 0.02	5
T127L	0.12 ± 0.01	0.06 ± 0.01	(0.012−0.11)[c]	10
G141K[a]	181 ± 12	2.1 ± 0.5	9.7 ± 2	19
G141Q[a]	63.3 ± 6.6	1.7 ± 0.2	5.3 ± 0.4	12
L148R[a]	330 ± 15	0.6 ± 0.1	2.4 ± 0.2	138
H159L	38.9 ± 4.3	nm	(0.58−0.17)[c]	65
K52N/H159L	3.2 ± 0.2	nm	(0.04−0.06)[c]	50

[a] Denotes CRP* mutants. [b] nm: affinity too low to be measured with accuracy. [c] Due to the low affinity, a range of binding constant was reported. [d] Ratio of K_{app} in the presence of cAMP and that of cCMP and is expressed as 'modulation index'.

uncertainty in the K_{app} value. Thus, the fitted values of K_{app} are valid. Results are summarized in Table 1. At the same concentration of cyclic nucleotide, the apparent DNA binding affinity increases in the order of cGMP, cCMP, cAMP.

[3] Lin, S.-H., and Lee, J. C., manuscript in preparation.
[4] Li, J., Lin, S.-H., and Lee, J. C., manuscript in preparation.

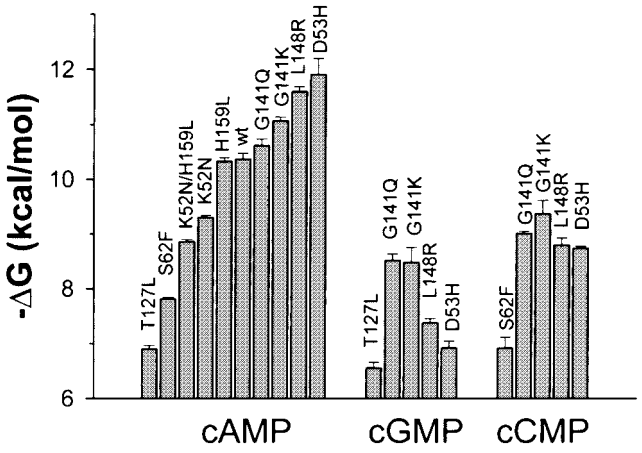

FIGURE 4: Changes in free energy of CRP−DNA complex formation as a function of cNMP. Typical anisotropy titrations as shown in Figure 3 were performed with 200 μM cNMP to obtain K_{app}. The association constants for the CRP−DNA complex are summarized in Table 1.

Table 2: Apparent DNA Binding Affinity of G141Q and G141K CRP Mutants in the Presence of Different Cyclic Nucleotides[a]

cyclic nucleotides	G141Q (M^{-1})	G141K (M^{-1})
cAMP	$3.2(2.8-3.7) \times 10^8$	$9.2(6.2-12.2) \times 10^8$
N^6-monobutryl-cAMP	$5.0(2.9-7.1) \times 10^7$	$4.1(0.7-7.5) \times 10^8$
CCMP	$2.0(1.6-2.5) \times 10^7$	$9.7(6.1-13.4) \times 10^7$
2′-deoxy-cAMP	$5.7(4.3-7.1) \times 10^6$	$2.3(1.2-3.4) \times 10^7$
CGMP	$4.0(3.1-5.0) \times 10^7$	$2.1(1.2-3.0) \times 10^7$
CUMP	$1.7(1.0-2.4) \times 10^6$	$1.3(0.7-2.0) \times 10^7$

[a] The values in parentheses are limits within a 75% confidence level, and the cyclic nucleotide concentrations were 200 μM.

The binding affinities of the other CRP mutants for the 26 bp *lac* sequence in the presence of a 2×10^{-4} M sample of different cyclic nucleotides were monitored. Results are summarized in Table 1. Figure 4 shows the ΔG values for DNA binding of all the mutants as a function of cyclic nucleotides. In the presence of 2×10^{-4} M cAMP, CRP* mutants (G141Q, G141K, L148R, and D53H) exhibit stronger affinity for the *lac* sequence than wild-type CRP. However, all the other mutants exhibit the opposite trend, namely, weaker affinity. It is most interesting to note that CRP* mutants are the only mutants, with the exception of S62F CRP, that exhibit measurable binding to the *lac* sequence in the presence of ALL of these cyclic nucleotides. Although the T127L and S62F mutants exhibit a relaxation in their requirement for cAMP, they do not exhibit the same behavior as the CRP* mutants, namely, a measurable binding to specific DNA sequence in the presence of all these cyclic nucleotides. Another intriguing observation is that in the presence of cGMP the rank order of ΔG's for the CRP* mutants is the opposite of that in the presence of cAMP.

The effect of amino acid side chain was tested with the G141Q and G141K CRP* mutants. The binding affinity of these mutants to a 40 bp *lac PI* sequence was determined as a function of various cyclic nucleotides, and the results are summarized in Table 2. It is interesting to note that all of the binding affinities associated with the G141K mutant are higher than those of the G141Q mutant, with the exception of that in the presence of cGMP. These results imply that both cyclic nucleotides and amino acid side chains contain information which combine to modulate the binding affinity for specific DNA sequence.

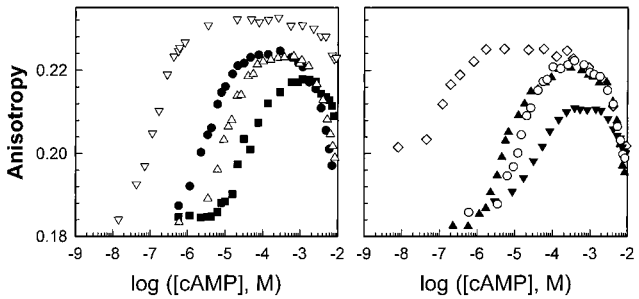

FIGURE 5: Interaction of CRPs with *lac26* DNA as monitored by fluorescence anisotropy as a function of cAMP. The experiment was done by titrating concentrated cAMP into a reaction mixture containing 12.9 nM DNA and wild-type or mutant CPR. The symbols and protein concentrations are as follows: (●) wild-type, 250 nM; (▽) D53H, 300 nM; (△) K52N, 2.18 μM; (■) S62F, 3.70 μM; (◇) L148R, 740 nM; (▲) H159L, 250 nM; (○) K52N/H159L, 3.0 μM; and (▼) T127L, 10.69 μM. The concentration of CRP was set at the level so that >97% of DNA was bound with protein at 200 μM cAMP.[3]

DNA Binding as a Function of Cyclic Nucleotide Concentration. The relationship between DNA binding affinity and cAMP concentrations is biphasic regardless of DNA sequence (*22*, *23*). An initial increase in the value of binding constant is followed by a decrease with further increasing cAMP concentration. This biphasic behavior is a direct consequence of weaker affinity for specific DNA sequence when CRP is loaded with multiple cAMP molecules (*22*, *23*). It is important to evaluate if the mutations have affected this basic behavior of CRP. Hence, the binding of these CRP mutants to *lac*-26 DNA was monitored as a function of cAMP concentration. It is evident from the results shown in Figure 5 that this characteristic biphasic behavior is maintained in all mutants studied, although the concentration range of cAMP under which this biphasic behavior is manifested is a characteristic of the specific mutant. For example, in the case of D53H and L148R mutants, a significant amount of CRP−cAMP−DNA complex was found at cAMP concentrations less than 10^{-6} M, a concentration at which the wild-type CRP only began to form a measurable amount of DNA−protein complex. All the other mutants required higher concentrations of cAMP. The data shown in Figure 5 are not quantitative reflections of DNA binding constants as a function of cAMP concentration. However, the biphasic nature of the data does reflect a weaker binding affinity when CRP is loaded with cAMP at the weak site. Hence, mutations in these residues do not lead to any qualitative alterations in the basic mode of DNA recognition by CRP.

Subunit Association. 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 each of the mutants at different concentrations of GuHCl were conducted to determine the apparent association constant. The sedimentation experiments were conducted at 25 000 rpm in different GuHCl concentrations. It is essential to conduct these experiments in the presence of denaturant because the association is strong and the presence of denaturant would weaken the subunit assembly, enabling the association−disassociation reaction to be monitored. However, the presence of denaturant leads to a complication, namely, protein unfolding coupled to subunit dissociation. The subunit

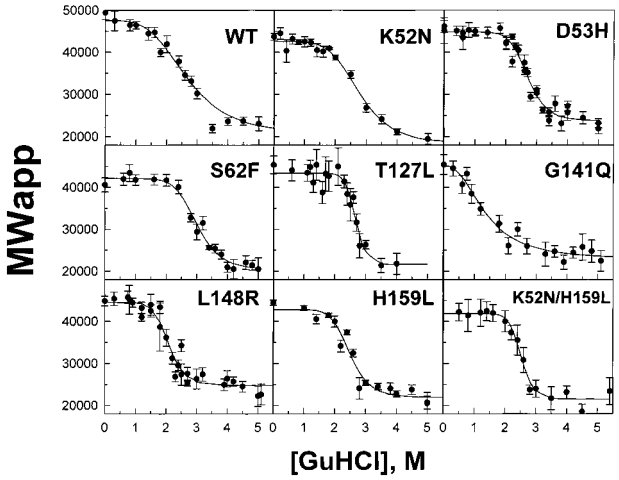

FIGURE 6: Apparent weight-average molecular weight of CRP as a function of GuHCl concentration. The lines were drawn to indicate the trend of the data.

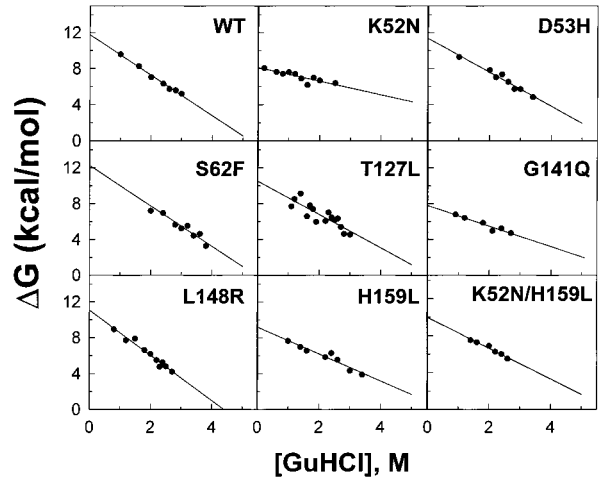

FIGURE 7: Free energy changes for dimer association of CRP as a function of GuHCl concentration. The observed ΔG_a values at different GuHCl concentrations were fitted by a linear least-squares program to eq 3. The recovered parameters are listed in Table 3.

dissociation monitored by sedimentation is an apparent parameter. Nevertheless, a study of subunit association as a function of denaturant concentration should provide data for extrapolation to infinite dilution of denaturant to yield the energetics of subunit association in buffer. Figure 6 shows the results of the sedimentation data. The concentration of GuHCl at which the MWapp value indicates a 50% mixture of monomer and dimer is a function of the specific mutation. This is most noticeable with the G141Q mutation. In addition, the shape of the curve can also be perturbed by mutation; e.g., the transitions for wild-type, K52N, and G141Q CRP are apparently less cooperative than the rest. The sedimentation data were further analyzed to estimate the apparent dimer association constant, K_a, at each individual GuHCl concentration. These K_a values were converted to ΔG_a, and a plot of ΔG_a versus GuHCl concentration displays an apparent linear relationship. Extrapolation of the data to zero GuHCl concentration yields values of ΔG_a^0 in buffer, as shown in Figure 7. Table 3 summarizes the results on subunit assembly of all the mutants and wild-type CRP. The most surprising observation is the difference in the energetics of subunit assembly among the CRP* mutants. These results indicate that the consequences of mutations, maybe with the exception

Table 3: Free Energy Change of Dimer Association

CRP	$-\Delta G_a^0$ (kcal/mol)	m_a
WT	11.8 ± 0.2	−2.3 ± 0.1
K52N	8.1 ± 0.1	−0.07 ± 0.07
D53H[a]	10.9 ± 0.4	−1.9 ± 0.2
S62F[a]	12.3 ± 1.1	−2.3 ± 0.4
T127L	10.5 ± 0.7	−1.9 ± 0.3
G141Q[a]	7.8 ± 0.0.3	−1.5 ± 0.2
G141K[a]	7.6 ± 0.3	−2.3 ± 0.2
L148R[a]	11.1 ± 0.4	−2.5 ± 0.2
H159L	9.2 ± 0.2	−1.5 ± 0.1
K52N/H159L	10.0 ± 0.3	−1.7 ± 0.2

[a] Denotes CRP* mutant.

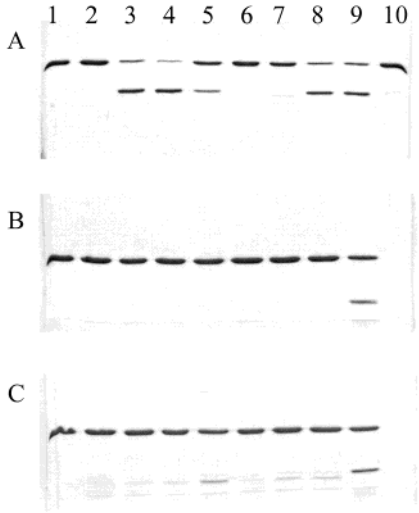

FIGURE 8: (A) Proteolytic digestion of D53H (lanes 1−5) and L148R mutant of CRP (lanes 6−10) by chymotrypsin at different cAMP concentrations. Lanes 1 and 6 are D53H and L148R alone. The rest of them contain protease and cAMP as follows: lanes 2 and 7, 0 cAMP; lanes 3 and 8, 20 μM cAMP; lanes 4 and 9, 200 μM cAMP; lanes 5 and 10, 10 mM cAMP. (B) Proteolytic digestion of the D53H mutant of CRP by chymotrypsin at different cyclic nucleotide concentrations. Lane 1 is D53H alone, and lanes 2−9 contain protease and different concentrations of cyclic nucleotide: 2, 0; 3, 20 μM cGMP; 4, 150 μM cGMP; 5, 10 mM cGMP; 6, 20 μM cCMP; 7, 150 μM cCMP; 8, 10 mM cCMP; 9, 10 μM cAMP. (C) Proteolytic digestion of the L148R mutant of CRP by chymotrypsin at different cyclic nucleotide concentrations. Lane 1 is L148R alone, and lanes 2−9 contain protease and different concentrations of cyclic nucleotide: 2, 0; 3, 20 μM cGMP; 4, 150 μM cGMP; 5, 10 mM cGMP; 6, 20 μM cCMP; 7, 150 μM cCMP; 8, 10 mM cCMP; 9, 15 μM cAMP.

of D53H, S62F, T127L, and L148R, are communicated through the subunit interface.

Proteolytic Digestion. The exposure of proteolytic sites in the C-helix has been employed as a probe of conformational changes in this structural element. This approach was employed to assess the effect of mutations on the C-helix, which is apparently involved in intersubunit interaction. In this particular study, mutants which exhibit apparently similar values of ΔG_a^0 for subunit assembly, namely, D53H, S62F, T127L, and L148R mutants, were studied. Figure 8A shows the results of exposure of D53H and L148R CRP to chymotrypsin as a function of cAMP concentration. It is evident that L148R CRP was sensitive to chymotrypsin even in the absence of cAMP while D53H CRP behaved like wild-type CRP, which was not susceptible to proteolytic digestion in the absence of cAMP. In the presence of increasing concentrations of cAMP, both mutants were more susceptible

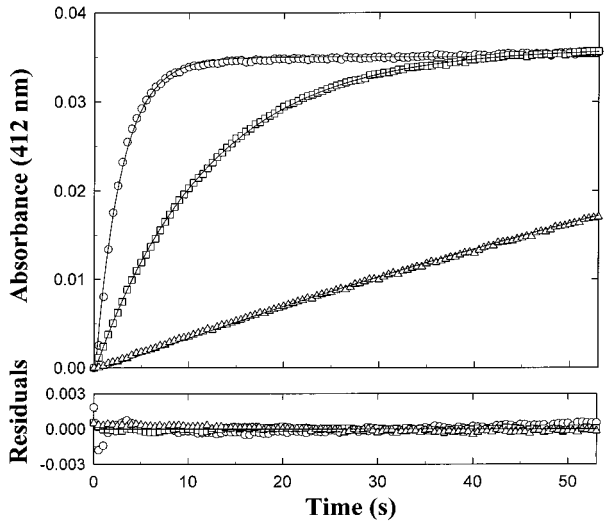

FIGURE 9: Rate of Cys178 modification of G141Q mutant by DTNB in the absence of cAMP (○) and the presence of 150 μM cAMP (□) and 5 mM cAMP (△).

Table 4: Kinetics of Cys178 Modification[a]

	cAMP concentration		
	0 μM k_{obs} (s^{-1})	150 μM k_{obs} (s^{-1})	5 mM k_{obs} (s^{-1})
WT	0.0946 (0.0938−0.0954)	0.181 (0.176−0.185)	0.0722 (0.0714−0.0729)
G141Q	0.37 (0.35−0.38)	0.0844 (0.0837−0.0852)	0.0067 (0.0066−0.0067)
D53H	0.30 (0.28−0.33)	0.20 (0.18−0.21)	0.10 (0.09−0.12)
L148R	0.33 (0.32−0.34)	0.134 (0.132−0.135)	0.022 (0.020−0.024)

[a] k_{obs}: rate constant of Cys178 reaction with DTNB. Values in parentheses are limits within a 75% confidence level.

to digestion but became less at millimolar levels of cAMP, an observation similar to that of wild-type CRP. Figure 8B shows that D53H CRP was not digested in the presence of cCMP or cGMP whereas L148R CRP was, as shown in Figure 8C. Thus, D53H CRP exhibited behavior similar to that of wild-type CRP while L148R CRP was quite different. On the other hand, the S62F and T127L mutants behaved in a manner very similar to wild-type CRP. Thus, the proteolytic digestion data indicate that the mutations in residues 62 and 127 do not significantly perturb the C-helix and its response to cyclic nucleotide binding.

Cys178 Modification. In wild-type CRP, Cys178, which is located in the C-terminal DNA binding domain, becomes accessible to chemical modification upon binding of cAMP to the strong site but becomes less exposed upon occupancy of the weak site (*23*). Thus, the kinetics of Cys178 modification were employed as a probe of interdomain communication. The kinetic data of G141Q CRP Cys178 modification are shown in Figure 9. The apparent rate of modification was most rapid in the absence of cAMP with decreasing rate in the presence of increasing concentrations of cAMP. The data were analyzed by the pseudo-first-order rate equation, and the results are summarized in Table 4. The fits were good as shown by the randomness of the residuals. There was a 4-fold decrease in rate when the cAMP concentration was increased from 0 to 1.5×10^{-4} M. With further increase to 5×10^{-3} M, there was an approximately 10-fold decrease in rate. In the absence of cAMP, a G→Q mutation in residue 141 led to a significant increase in the accessibility of Cys178 as evidenced by the approximately 4-fold increase in k_{obs}, as shown in Table 4. This result implies that there is an intradomain interaction between residues 141 and 178 within the C-terminal DNA binding domain. Furthermore, binding of cAMP either to the strong or to the weak site does not restore the local environments of Cys178 in G141Q CRP to the equivalent complexes in wild-type CRP since the k_{obs}'s are different.

To test if the perturbation of the microenvironment of Cys178 is related to the site of mutation, the same set of experiments were conducted on the D53H and L148R mutants. The results are similar, as summarized in Table 4. In both cases, the mutations led to an increase in accessibility of Cys178 in the absence of cAMP. This result implies that the D53H mutation leads to alteration in the interdomain interaction since residues 53 and 178 are located in different domains, although the proximity of these residues (approximately 15 Å apart) might imply that the interaction is regional and short-range.

DISCUSSION

In wild-type CRP, cAMP provides the message for specific DNA sequence recognition. In the absence of cAMP, CRP does not bind or binds with very low affinity to specific DNA sequence. Although CRP binds other cyclic nucleotides, cAMP is the only natural cyclic nucleotide that fulfills this function as an allosteric effector. However, Cheng and Lee reported that the G141Q CRP* mutant exhibits an apparent relaxation in the effector specificity since other cyclic nucleotides can serve as allosteric effectors also. The binding affinity of the G141Q mutant for the *lac* sequence is a function of the specific cyclic nucleotide bound. The apparent DNA binding affinity ranges from 2×10^6 to 3×10^8 M^{-1} with cAMP being the ligand which induces G141Q CRP to bind with a greater affinity (*9*). Based on these results, it was proposed that the effect of mutation in the CRP* mutants did not alter the requirement of cyclic nucleotide as an allosteric effector but apparently the strict specificity exhibited for cAMP has been relaxed. Each cyclic nucleotide contains its own message which is manifested as differences in binding affinity for *lac PI* sequence (*9*). The report by Cheng and Lee raises the issue on the mechanism of CRP* mutation. Do these mutations alter the ability of CRP to differentiate cyclic nucleotides? Or, do they only elicit a quantitative shift in the basic mode of reaction in wild-type CRP? In this study, other CRP* mutants are chosen to provide clues to address these possible mechanisms.

The basic mode of reaction of wild-type CRP with respect to cAMP concentration is biphasic (*22*, *23*). Occupancy of the strong cAMP binding sites leads to a high affinity to specific DNA sites while the subsequent occupancy of the weaker cAMP binding sites elicits a weaker affinity. This biphasic mode of reaction is retained by all mutants, not just the CRP* mutants. A closer observation of the data shown in Figure 5 in this work and in Figure 2 of Cheng and Lee (*9*) shows that the leading edges of the biphasic curves have shifted to the left and right of the wild-type CRP in D53H, L148R, and the other CRP mutants, respectively. This shift implies that D53H and T127L have the highest and lowest affinity for DNA, respectively. Further inspection indicates

Scheme 1

$$\begin{array}{ccccccc} P & + L & \leftrightarrow & PL_1 + L & \leftrightarrow & PL_2 + L & \leftrightarrow & PL_n \\ + & & & + & & + & & + \\ D & & & D & & D & & D \\ \updownarrow & & & \updownarrow & & \updownarrow & & \updownarrow \\ PD & + L & \leftrightarrow & PLD + L & \leftrightarrow & PL_2D + L & \leftrightarrow & PL_nD \end{array}$$

that the intervals between the leading and trailing edges of the curves for CRP* mutants are wider than those of the wild type and other mutants. These observations imply that the various binding constants corresponding to the different liganded states of CRP, as shown in reaction Scheme 1, are significantly perturbed by CRP* mutations.

The observed changes are very similar to those simulated by altering only the binding constants without a change in the mechanism (*22*). The specific nature and magnitude of changes await results of a detailed study on the binding affinities of cAMP and the corresponding DNA binding affinity of the various liganded states of CRP.[2]

All the CRP* mutations enhance the affinity for specific DNA sequence with the exception of the S62F mutant. Non-CRP* mutations lead to a decrease in affinity. In all cases, there are no apparent alterations in the biphasic mode of behavior with respect to cAMP concentration. Hence, it might be proposed that CRP* mutation does not render CRP to adopt new properties that the wild-type CRP does not exhibit. The effect of CRP* mutation is to shift the DNA binding constants to a regime that can be monitored by these in vitro assays. The non-CRP* mutation is not different from the CRP* mutation except that the DNA binding constants are shifted to a regime that is even more difficult to measure. Thus, one may categorize these mutants into two classes, namely, D53H, G141Q, G141K, and L148R as positive mutations while K52N, S62F, T127L, H159L, and K52N/H159L as negative mutations. These two classes enhance and suppress the ability to bind specific DNA sequences, using the wild-type CRP as the standard state.

Mutations at residues 53, 141, and 148 of CRP lead to the only CRP* mutants that exhibit measurable affinities in the presence of all three cyclic nucleotides tested in this study. cAMP is still the most efficient effector as indicated by the high DNA binding constants in the presence of cAMP. Thus, the message imbedded in cAMP is still differentiated by these CRP mutants in the same way. In all cases, cGMP carries the least efficient message since the DNA binding constants are the lowest in each of the CRP mutants under investigation. The observed DNA binding constants, in the presence of cGMP, range from 6×10^4 to 2×10^6 M^{-1}. These values should be viewed as approximations due to difficulties in defining a complete binding isotherm with such low affinities. A closer evaluation of the DNA binding constants reveals a very interesting pattern. In the presence of cAMP, the binding constant increases in the order of G141Q, G141K, L148R, D53H, as shown in Figure 4. The order is reversed in the presence of cGMP and shows D53H, L148R, G141K, G141Q. cCMP elicits a different order of increasing affinity. Thus, the specific nature of cyclic nucleotide has a significant effect on the DNA binding constants. This effect can be better demonstrated by examining the ratio of DNA binding affinity in the presence of different cyclic nucleotides. A 'modulation index' will be employed to facilitate discussion, and it is defined as the ratio of binding constant in cAMP to that in cCMP, as summarized in Table 1. It ranges from about 350- to 5-fold. The choice of using K_{app} in cCMP instead of cGMP is simply due to the greater accuracy in these parameters. Let us examine the effects of mutations and their linkage with cyclic nucleotides to modulate the binding affinity of these mutant CRPs to *lac*-26 sequence. In wild-type CRP, the modulation index is about 300−400. In the D53H CRP mutant, the index is maintained at about 300. However, the index for the L148R CRP mutant is reduced to about 150. Most intriguing is the index for the G141Q CRP mutant, which exhibits a value of only about 10. The low modulation index implies that, in comparison, a mutation at residue 141 compromises CRP's ability to differentiate the different cyclic nucleotides. The differences in modulation indices indicate that mutation at different locations in CRP compromises the capability of CRP to interpret the effector message to different extents. There is no outstanding feature in the group of CRP* mutants that is qualitatively different from wild-type and other CRP mutants. Some maintain a high modulation index, and some do not. Another interesting observation is that there is only a marginal difference in the DNA binding affinities among this series of CRP mutants when cCMP replaces cAMP, the observed differences being no more than 20-fold. In addition, there are differences in the binding constants for the *lac* sequence exhibited by the G141Q and G141K mutants in the presence of various cyclic nucleotides. These results all point to a CRP molecule that is designed with a high degree of plasticity in those residues which impart characteristics to CRP to receive and interpret external messages with high sensitivity. The external messages in the various cyclic nucleotides are received intact as indicated by the same rank order of DNA binding affinity within each mutant; namely, cAMP and cGMP lead to the highest and lowest affinity, respectively.

In this study, all mutants exert an effect on the CRP molecule as indicated by the changes in the modulation index (Table 1). The effect is observed regardless of the classification of the mutant, be it CRP* or non-CRP*, and the location of these mutations. The observed effect must be the consequence of a perturbation of one or more communication networks which most likely include the subunit interface. The observed differences in the association constants of the intersubunit interaction in these CRP mutants may provide a hint on the mechanism. The relation between the modulation index and subunit association constant in terms of ΔG's is shown in Figure 10. With the exception of the data points for the T127L and S62F mutants, as an approximation, there is a linear correlation between these parameters; namely, there is a decrease in the modulation index with a decrease in subunit−subunit affinity. This correlation may have its origin in the ability of these CRP species to undergo conformational changes. Assuming wild-type CRP exists as an ensemble of dynamic states, binding of cAMP may alter the distribution of this ensemble so that the F-helices in the two CRP subunits can interact with the specific DNA binding sequence effectively. A strong subunit−subunit interaction, such as that in wild-type and D53H CRP, indicates a quaternary constraint so that only a specific distribution of ensemble of states is allowed. This constraint leads to a high modulation index. A weakening of subunit interaction, observed in the G141K and G141Q CRP* mutants, implies

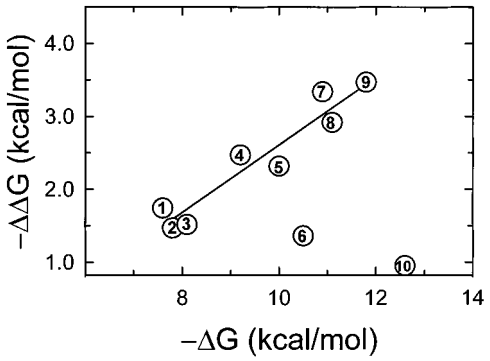

FIGURE 10: Relation between the modulation index and subunit association constant. Values of modulation index are expressed as $\Delta\Delta G$, the difference in DNA binding in the presence of cAMP and cCMP. DNA binding constants and subunit association constants are listed in Tables 1 and 3, respectively. Mutants and symbols are as follows: 1, 141K; 2, G141Q; 3, K52N; 4, H159L; 5, K52N/H159L; 6, T127L; 7, D53H; 8, L148R; 9, WT; 10, S62F.

that the quaternary constraint is lost to different extents as revealed by the different values of K_a. The loss of constraint leads to an increase in the dynamic states in the ensemble. This increase in the number of dynamic states can interact with various cyclic nucleotides, which exhibit different propensity in altering the distribution of this ensemble. cAMP is the most effective. Depending on the location of the residue and the nature of the side chain of the residue, the distribution of dynamic states can vary. Consequently, the alteration in dynamic states depends on the specific mutant. In return, these variabilities in the distribution of dynamic states and the effects of specific cyclic nucleotides on the distribution lead to the observed differences in modulation index reported in this study. The exceptions observed for T127L and S62F are intriguing. In the case of the T127L mutant, it might be due to the fact that this residue is involved in the binding of cAMP. It might be involved in more than subunit constraints (*24*, *25*). At present, there is no rationale for the behavior of the S62F mutant. Recently, Baker et al. (*27*) reported that a mutation of Y99 affects the dimer stability. It would be most interesting to test if these Y99 mutants exhibit any perturbation in their modulation indices.

The similarities and differences observed in the D53H and L148R mutants deserve further scrutiny. Residues 53 and 148 are not located in the same structural element, yet mutation in these residues lead to CRPs that exhibit similar behavior: e.g., an increase in affinity for DNA (Figure 5); similarity in energetics of subunit assembly (Table 3); maintenance of a reasonable modulation index (Table 1). Based on these observations, it might be tempting to conclude that they share a mechanism in eliciting these effects. However, a closer examination shows the differences. Although the energetics of subunit assembly remain similar to those of wild-type CRP, the L148R mutant is susceptible to proteolysis in the absence of cAMP. Thus, the proteolytic data indicate that the L148R mutation does alter the communication between subunits without significantly perturbing the energetics of intersubunit interaction. Furthermore, the L148R mutation also increases the accessibility of Cys178 in the absence of cAMP. In wild-type CRP, an increase in the accessibility of Cys178 is only observed in the presence of cAMP, an observation cited as evidence for CRP in an activated conformation (*23*, *26*). Hence, it may be concluded that the L148R mutation leads to subtle global conformational perturbations in CRP. In contrast, the D53H mutation yields a CRP molecule with many structural properties that are similar to wild-type CRP, namely, similar energetics of subunit assembly; susceptibility of the C-helix to proteolysis only in the presence of cAMP. However, there is an interesting difference in the accessibility of Cys178, which is accessible to chemical modification in the D53H mutant but not in wild-type CRP in the absence of cAMP. This result implies that a D53H mutation does perturb the structure of the DNA binding domain without significantly affecting the intersubunit interaction. Although residue 53 is located in the N-terminal, cAMP binding domain, it is situated in about equidistance as residue 148 from Cys178, approximately 15 Å. Thus, it is conceivable that residues 53 and 148 elicit their effects by shifting the CRP structural ensemble toward the activated form. The D53H mutation may elicit its effect via a local, regional perturbation while the L148R perturbation is more global in nature. Regardless of the mechanism of perturbation, both mutations influence the shift of CRP toward the activated form. Such an interpretation is consistent with the observation in Figure 5, showing a significantly greater population of CRP−DNA complex at low cAMP concentration.

The challenge for the future is to provide the evidence to correlate protein dynamics and function in CRP. Furthermore, one needs to define the effect of these proposed ensemble of states on transcription, which involves the interaction of RNA polymerase with this CRP−DNA complex.

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