Published in final edited form as:

Biochemistry. 2020 February 04; 59(4): 460–470. doi:10.1021/acs.biochem.9b00895.

Structural Energy Landscapes and Plasticity of the Microstates of Apo *Escherichia coli* cAMP Receptor Protein

Rati Chkheidze,

Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1055, United States

Wilfredo Evangelista,

Centro de Espectroscopia de Resonancia Magnética Nuclear (CERMN), Departamento de Ciencias-Química, Pontificia Universidad Católica del Perú, Lima 32, Peru

Mark A. White,

Sealy Center for Structural Biology and Molecular Biophysics, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1055, United States

Y. Whitney Yin,

Department of Pharmacology and Toxicology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1055, United States

J. Ching Lee

Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1055, United States

Abstract

The theory for allostery has evolved to a modern energy landscape ensemble theory, the major feature of which is the existence of multiple microstates in equilibrium. The properties of microstates are not well defined due to their transient nature. Characterization of apo protein microstates is important because the specific complex of the ligand-bound microstate defines the biological function. The information needed to link biological function and structure is a quantitative correlation of the energy landscapes between the apo and holo protein states. We employed the *Escherichia coli* cAMP receptor protein (CRP) system to test the features embedded in the ensemble theory because multiple crystalline apo and holo structures are available. Small angle X-ray scattering data eliminated one of the three apo states but not the other two. We defined the underlying energy landscape differences among the apo microstates by employing the computation algorithm COREX/BEST. The same connectivity patterns among residues in apo CRP are retained upon binding of cAMP. The microstates of apo CRP differ from one another by minor structural perturbations, resulting in changes in the energy landscapes of the various domains of CRP. Using the differences in energy landscapes among these apo states, we computed

Corresponding Author Phone: (409) 772-2281; jlee@utmb.edu.

Author Contributions

R.C. and W.E. contributed equally to this work. W.E. conducted the computation analysis. R.C. conducted the SAXS studies. M.A.W. performed the bead modeling of the SAXS data. All authors contributed to writing the manuscript.

The authors declare no competing financial interest.

the cAMP binding energetics that were compared with solution biophysical results. Only one of the three apo microstates yielded data consistent with the solution data. The relative magnitude of changes in energy landscapes embedded in various apo microstates apparently defines the ultimate outcome of the cooperativity of binding.

Graphical Abstract

Allostery is a complex phenomenon that regulates and fine-tunes the activity of proteins to impart a wide range of functions and regulatory properties. It is a prevalent process of signal transmission, by which local energetic perturbation at one site is sensed throughout the molecule to functionally or structurally affect the remote functional site. The fluctuation of the macromolecule plays a crucial role in signal transduction and makes it possible to fine-tune its activity in response to various external stimuli or a change in environmental conditions.¹⁻⁷ Many elegant studies employed molecular dynamics simulation and/or other biophysical methods to experimentally monitor dynamic motions and potential pathways of signal transmission.⁸⁻¹¹ However, there is a dearth of knowledge to connect the fundamental engineering in the inactive apo state that provides the mechanics for signal transmission and how those are different from those of the holo state. The *Escherichia coli* cAMP receptor protein (CRP) can bind different types of cyclic mononucleotides with different modes of cooperativity, varying from negative to positive.¹²⁻¹⁴ CRP–cAMP complexes can bind to different DNA sequences with a conserved -TGTGA- half-site. Consequently, CRP is involved in modulating expression of many genes.¹⁵ Due to the apparent functional flexibility of CRP, the current hypothesis of the ensemble theory seems to be the best model for understanding CRP. The underlying features of the ensemble theory for CRP are summarized in Figure 1. There are multiple inactive states to one of which a cyclic nucleotide may preferentially bind forming the active state. Hence, the CRP system consists of all of the components for probing the significance of differences in inactive states because high-resolution structures are available in addition to the active state. We employed the structure-based algorithm COREX/BEST to derive the energetic landscapes and to correlate these landscapes to the thermodynamics defined by the functional landscapes. We elucidate (1) the differences in energy landscapes embedded in structures of apo CRP microstates, (2) the correlation between the structural differences and thermodynamics of CRP–ligand

interactions to elucidate structural changes that alter allosteric behavior, and (3) the correlation between conclusions derived from structural energy landscapes of microstates and the proposed pathway of signaling gleaned from a molecular dynamics study.¹⁶

CRP is a homodimeric protein composed of two chemically identical subunits that each have 209 amino acid residues. Each subunit contains two domains: a larger amino-terminal domain consisting of residues V1–L137 (NBD), which contains the high-affinity cAMP binding site, and a smaller carboxyl-terminal DNA binding domain that includes residues D138–R209 (DBD). The two functional domains are connected covalently by a stretch of polypeptides (F135–T140) denoted as the hinge region.^{17–20} There are two high-affinity nucleotide binding sites in the CRP molecule that binds cAMPs cooperatively. The magnitude and nature of cAMP binding can be modulated by experimental parameters or mutations.¹⁷ There is a linear correlation between the protein dynamics and cooperativity of cAMP binding.^{18–20} Hence, there is ample experimental evidence to link protein dynamics, which in turn can be expressed in terms of energetics, to functional thermodynamics. There have been reports of apo CRP structures obtained by two different techniques: nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction.^{21–24} The thermodynamics of this binding reaction is pH-dependent.²⁵ Hence, in this study the apo CRP structures derived from X-ray crystallography were studied (Figure 2a-c). These microstates were defined in a single crystal at the same pH (7.5) at which the cAMP binding studies were conducted, allowing us to attempt a quantitative correlation between the energetics of the structural and functional landscapes. However, the NMR study was conducted at pH 6.0; thus, they are not included in this study. The basic quaternary structural difference among these high-resolution structures of apo CRP is the orientation of the DNA binding domains with respect to the cAMP binding domains. To elucidate the potential dominant apo states in solution, we used small angle X-ray scattering (SAXS) to evaluate the shape of the apo CRP in solution. We compared the SAXS information to the simulated scattering data generated from the published X-ray information using CRY SOL software and identified the existence of one predominant microstate in solution that would exhibit similar thermodynamic parameters for cAMP binding.

MATERIALS AND METHODS

SAXS Experiment.

For each apo CRP SAXS scan experiment, protein from the storage solution was dialyzed against two changes of 1 L of TEK100 buffer (50 mM Tris, 1 mM EDTA, and 100 mM KCl) at pH 7.5 for at least 8 h in each change in fresh buffer. The protein solution was concentrated in Centricon centrifugal filter tubes with 10000 molecular weight filter membranes, to achieve the desired concentration. The filtered solution was used to establish a baseline (background) scattering.

SAXS scans were conducted with the Rigaku BioSAXS-1000 device. Each experiment consisted of seven 1 h filtered buffer (background) scans and seven 1 h protein solution scans. As there was a concern about radiation damage of the protein, the protein solution was changed every other scan.

Software Used for SAXS Data Analysis.

SAXSLab 2.0.0b23 software from Rigaku was used for data acquisition, scan averaging, and subtraction of background scattering from protein solution scattering. For each experimental condition, the protein and background solutions were scanned seven times each to obtain and average the scattering curves. To extract the protein scattering curve, the averaged background scattering was subtracted from the averaged protein solution scattering. Low angle scattering intensities ($q = 0.004\text{--}0.016 \text{ \AA}^{-1}$) were ignored assuming that they were due to high noise levels. Primus QT from the EMBL Hamburg group was used for data comparison, scaling, and radius of gyration calculation.²⁶ The radius of gyration was used to assess the degree of aggregation in experimental samples and obtained by the Guinier approximation:

$$\ln[I(q)] = \ln[I(0)] - R_g^2 q^2 / 3 \quad (1)$$

where I is the scattering intensity and R_g is the radius of gyration. To obtain the theoretical scattering curves from coordinates available for the crystal structures, we used CRY SOL software²⁶ with the following parameters: maximum order of harmonics of 50, order of Fibonacci grid of 18, and 256 points in the curve. The rest of the parameters were set to default. The SAXS scattering intensity is proportional to the protein concentration, so even a very small difference in protein concentration may change the intensity. Hence, to overlay and compare scattering curves, from different samples, it was necessary to scale the data. The shapes of the scattering curves were shown to be sensitive to aggregation in the low q region ($0.075 \text{ \AA}^{-1}$) while almost not sensitive in the q range of $0.075\text{--}0.085 \text{ \AA}^{-1}$, so for comparison, all of the scattering data, including scattering curves, were scaled using the data points in the q range of $0.075\text{--}0.085 \text{ \AA}^{-1}$.

For additional modeling, the DAMMIF consensus bead model was aligned with the Protein Data Bank (PDB) models with SUPCOMB. The simulated curves were expressed as a logarithmic Guinier plot: $\log I(q)$ versus q , where I is the scattering intensity, $q = 4\pi \sin \theta / \lambda$ and $2 \sin \theta$ is the scattering angle and λ is the wavelength. The curves were also expressed as a Kratky plot: Ixq^2 versus q .

Computation Analysis of the Apo and Holo CRP Conformational Ensemble.

To investigate the thermodynamic properties of protein fluctuations in the apo or holo CRP structures, we used the COREX/BEST algorithm.^{27,28} Briefly, the algorithm generates a native state ensemble from the target protein structure (PDB entries 3HIF and 1G6N) through the combined unfolding of adjacent groups of residues defined as folding units. In this case of CRP, the folding unit is 10 residues. They are treated as nativelylike or as unfolded peptides. Within this ensemble, the free energy of each conformational state, G_c , is calculated with a surface area parametrization that has been validated experimentally.^{27,28} By setting an equilibrium constant (K_c) between conformer c and the folded state $\{K_c = \exp[-G_c/(RT)]\}$, we define the probability (P_c) of each conformer as

$$P_c = K_c / \sum K_c = K_c / Q \quad (2)$$

where Q is the sum of all possible states in the ensemble (partition function). Moreover, G_c can be resolved into the energetic contributions from each residue in the protein via the equation $G_c = \sum_{i=1}^{N_{aa}} G_i$, which allows the investigation of thermodynamic properties at the residue level.⁵

Residue Stability in the Ensemble of the Apo and Holo CRPs.—Within the apo and holo CRP ensembles, we estimated the relative stability of each residue, $G_{f,i}$, as the ratio of the probability that residue i is in the folded state, $P_{f,i}$, to the probability that the same residue is in the unfolded state, $P_{u,i}$:

$$\Delta G_{f,i} = -RT \ln K_{f,i} = -RT \ln(P_{f,i} / P_{u,i}) \quad (3)$$

From eq 3, residues with high stability are mostly in the folded state in the ensemble, whereas residues with low stability are mostly in the unfolded state.

Characterization of the Long-Range Interactions in the Apo and Holo CRP

Ensemble.—The pairwise residue interactions in the apo and holo CRPs are described as the thermodynamic coupling between residues i and j .^{29,30} To obtain a quantitative value of the thermodynamic coupling, we evaluated the effect of an energetic perturbation on residue j , $g^{\text{pert},j}$, over the stability of residue i . This is accomplished by recasting eq 3 to consider the folding state of a second residue j . The effect of $g^{\text{pert},j}$ over the stability constant of residue i is

$$\Delta G_{f,i}^{\text{pert},j} = -RT \ln K_{f,i}^{\text{pert},j} = -RT \ln[(\phi^{\text{pert},j} P_{f,i|f,j} + P_{u,i|f,j}) / (\phi^{\text{pert},j} P_{u,i|f,j} + P_{u,i|u,j})] \quad (4)$$

In eq 3, the modified residue stability constant $G_{f,i}^{\text{pert},j}$ describes the probabilities of states where residues i and j are both folded ($P_{f,i|f,j}$) or both unfolded ($P_{u,i|u,j}$) or one in each state (either $P_{f,i|u,j}$ or $P_{u,i|f,j}$). Thus, an energetic perturbation on residue j ($\phi^{\text{pert},j} = \exp[-g^{\text{pert},j}/(RT)]$) can have a stabilizing, a destabilizing, or no effect over residue i . This thermodynamic effect can be quantified by subtracting eq 4 from eq 3:

$$\Delta \Delta G_{f,i}^{\text{perk},j} = \Delta G_{f,i} - \Delta G_{f,i}^{\text{perk},j} \quad (5)$$

Importantly, this analysis does not require residues i and j to be in the proximity of the primary sequence or in the tertiary structure of the protein. Thus, the value of $G_{f,i}^{\text{pert},j}$ from eq 5 characterizes long-range effects of residue j over residue i .

Given that a long-range effect is not a bidirectional phenomenon per se,²⁹ in this study we define thermodynamic coupling as a bidirectional long-range effect. In other words, thermodynamic coupling is the sum of the influence of a perturbation on residue j over residue i ($G_{f,i}^{\text{pert},j}$) and the reciprocal effect, namely, the influence of a perturbation on residue i over residue j ($G_{f,j}^{\text{pert},i}$):

$$\Delta\Delta G_{ij} = \Delta\Delta G_{f,i}^{\text{perk},j} + \Delta\Delta G_{f,j}^{\text{perk},i} \quad (6)$$

Thermodynamic coupling between two residues can be manifested in three ways: positive ($G_{ij} > 0$), negative ($G_{ij} < 0$), and neutral ($G_{ij} = 0$).²⁸ Positive coupling occurs when stabilization or destabilization of residue i stabilizes or destabilizes, respectively, residue j . For negative coupling, the principles are the same, but the effect is opposite. Namely, stabilization of residue i results in the destabilization of residue j , and vice versa. Neutral coupling indicates that the two residues are not thermodynamically coupled.

Equation 6 provides a quantitative descriptor for long-range interactions between residues in the apo CRPs. With this analysis, we can investigate the long-range effects of a mutation by calculating the thermodynamic coupling between residues of mutant proteins (G_{ij}^{mut}).

Residue-Specific Connectivity.—In a graphic presentation of the coupling between residues i and j , we introduce the parameter residue-specific connectivity (RSC).²⁷ In the context of the Monte Carlo sampling method, we use the correlation function to define the RSC as

$$\text{RSC}_{i,j} = \frac{\langle (S_i - \langle S_i \rangle)(S_j - \langle S_j \rangle) \rangle}{[\langle (S_i - \langle S_i \rangle)^2 \rangle \langle (S_j - \langle S_j \rangle)^2 \rangle]^{1/2}} \quad (7)$$

where S_i and S_j denote the folding state of residues i and j ($S = +1$ and -1), respectively, if a residue is always folded or unfolded in a particular state and $\langle S_i \rangle$ and $\langle S_j \rangle$ denote the average folding state of residues i and j , respectively, over the ensemble. A positive value of the RSC indicates that a stabilization of residue i or j results in a stabilization of residue j or i (i.e., they display positive cooperativity), whereas a negative value indicates a stabilization of residue i or j leads to a destabilization of residue j or i (i.e., they display negative cooperativity).

RESULTS

SAXS Solution Structural Study.

We wish to determine if the microstates of apo CRP were redistributed in solution and which might be the dominant form, if there is such a form. Hence, we employed SAXS as such an approach. Prior to such experiments, we needed to establish the resolving power of SAXS to distinguish those isoforms; hence, SAXS scattering curves were simulated on the basis of the coordinates of CRP structures from X-ray using CRY SOL software.²⁶

Simulated SAXS Scattering Curves.—The three microstate structures basically reflected the different orientations assumed by the DBDs with respect to the NBDs. On the basis of the coordinates of the three crystalline structures, scattering curves were calculated. The simulated curves were expressed as a logarithmic Guinier plot or as a Kratky plot as shown in Figure 3A-D. The simulated curves exhibited distinct features. The scattering plots calculated for the microstate 1 (AB) and microstate 3 (EF) dimers resembled each other, but

that for the microstate 2 (CD) dimer was apparently different. Both logarithmic Guinier and Kratky plots showed that the derived plots were different in the high-angle region of $q = 0.1$ – 0.2 , but the Kratky plot better amplified the difference in this region and provided a clearer picture of the nature of this difference (Figure 3B,D). Hence, this exercise showed that SAXS experiments, in principle, should distinguish differences between apo CRP structures derived from the published X-ray data.

Experimental SAXS Data.—The average of multiple SAXS scans of apo CRP showed a small amount of aggregation, the amount of which was dependent on concentration. Hence, we conducted the rest of our experiments in a concentration range of 4–5 mg/mL, at which the smallest amount of aggregation was detected at low values of q and the best resolution in the q region of 0.1–0.2. The TEK100 buffers at pH 7.5 and 20 °C were chosen to represent the exact experimental conditions for crystallization. A comparison of the experimental scattering curve of wild-type CRP to the scattering plots derived from X-ray crystal structure showed that there was an almost perfect match with the calculated scattering plot of microstates 1 and 3 (AB and EF) dimer from the apo CRP crystal with χ^2 values of 1.8 and 1.9, respectively, as shown in Figure 4A–D. Thus, these experimental results indicate that apo CRP in solution is either a mixture of microstates 1 and 3 or only one is present. The DBDs are known to be very flexible and assume a range of hinge angles between the NBD and DBD in both the holo and apo crystal structures. Therefore, it was expected that the hinge angle in the apo CRP crystal structures may be affected by the environment and may not reflect the value adopted when CRP is in solution. To model this, we performed a rigid body analysis, using CRY SOL, in which we preserved the interdomain interactions, while the NBD and DBD could move independently, being only restrained by a five-residue linker (residues 131–135). The resulting model (Figure 5A,B) showed stronger asymmetry and a larger hinge angle between the DBDs than was present in any of the crystal structure dimers but was closest to microstate 1, which fit the SAXS data better than any other structure. We further allowed for small motions of protein domains from crystal structures to allow them to adopt orientations consistent with the solution scattering. The results of the simulated and experimental Kratky plots and bead models are shown in Figure 6. The fit of the experimental data to the simulated plot is obviously better.

Structural Comparison of the Apo CRP Microstate.

We wish to elucidate the structural differences among the three microstates reported in PDB entry 3HIF. Hence, we compared the differences of the CRP domains with each other, namely, the N-terminal cyclic nucleotide binding domain (NBD) and the C-terminal DNA binding domain (DBD) of their corresponding domain in each chain. We adopted the same annotations of A–F for protein chains in microstates 1–3 as in PDB entry 3HIF and compared the differences among the $C\alpha$ of atoms NBD and DBD separately via root-mean-square deviation (RMSD) analysis. The results of this RMSD analysis are summarized in Table 1. The RMSDs for NBD and DBD in chain A in comparison with chain B in microstate 1 were 1.27 and 1.67 Å, respectively, indicating differences between these domains in chains A and B. Furthermore, the variations among NBDs and DBDs among all microstates were 1.04–1.51 and 0.98–1.80 Å, respectively. Apparently, there were differences between equivalent domains not only among different microstates but also within

the same microstate. The larger differences were observed in DBDs, indicating the asymmetry of the two chains in apo CRP.

Structural Comparison of Microstates of Apo CRP and Holo CRP.

In view of the structural differences of the domains in apo CRP, we wish to compare the structural changes in each domain upon binding of cAMP. The $C\alpha$ values of each NBD and DBD of the three microstates of apo CRP were compared with that of holo CRP (PDB entry 1G6N), which was also determined by the Steitz lab at the same pH (7.5). The results of RMSD analysis are summarized in Table 1. Each microstate underwent a different pattern of structural changes upon binding of cAMP. For microstate 1, binding of cAMP induced the same amount of change in both domains in chains A and B with an RMSD value of 0.81 ± 0.02 Å. However, the RMSD changes for microstates 2 and 3 were asymmetric with greater induced changes in NBDs than DBDs in chains D and F than in chains C and E. In summary, the structures of the subunits in apo CRP are asymmetric, and upon binding of cAMP, the asymmetry is amplified.

Structural Comparison of Microstates of Apo CRP and Holo CRP.

In view of the structural differences of the domains in apo CRP, we wish to compare the structural changes in each domain upon binding of cAMP. The $C\alpha$ values of each NBD and DBD of the three microstates of apo CRP were compared with that of holo CRP (PDB entry 1G6N), which was also determined by the Steitz lab at the same pH (7.5). The results of RMSD analysis are summarized in Table 2. Each microstate underwent a different pattern of structural changes upon binding of cAMP. For microstate 1, binding of cAMP induced the same amount of changes in both domains in chains A and B with an RMSD value of 0.81 ± 0.02 Å. However, the RMSD changes for microstates 2 and 3 were asymmetric with greater induced changes in NBDs than DBDs in chains D and F than in chains C and E. In summary, the structures of the subunits in apo CRP are asymmetric, and upon binding of cAMP, the asymmetry is amplified.

Structural Stability of Apo CRP (PDB entry 3HIF).

In addition to the structural RMSD analysis, computer simulations were employed to define the energy landscapes of these domains. In the original publication, the coordinates of residues G132 and N133 were missing.²² However, the electron density was sufficient for positioning the missing residues. In our computational analysis, those coordinates were included. The structural stability of each domain in microstates was simulated by the COREX/BEST algorithm initially with the structure of microstate 1. Figure 7A-D shows the values of $\ln K_{if}$, the stability constant of the residue throughout chains A and B. The energetic landscapes of the microstate 1 NBD were similar in both subunits, as shown by the filled (subunit A) and empty (subunit B) circles in Figure 7a, although the loops encompassing residues (L50–I60) and (E72–Q80) in subunit A exhibit lower and higher values for $\ln K_{if}$, respectively. The more obvious differences in the energetic landscapes were in the DBDs, for example, in the region encompassing residues T140–P160. This region consists of helix D and part of the loop region between helices D and E. This seemed to be a key region involved in positioning DNA binding helix F (S179–Q193). This difference in the energy landscape of the DBDs among the two subunits of CRP was

evidence of the asymmetry of the CRP subunits. Further computational simulation of the other two microstates of apo CRP in 3HIF resulted in panels B and C of Figure 7. It is evident that the asymmetry of the energetic landscape observed in Figure 7A is a common feature of these three apo CRP structures. Although the general patterns were like that of microstate 1, e.g., residues L50–I60 and E72–Q80 were significantly less stable than the rest of the NBDs; nevertheless, the magnitudes of $\ln K_{if}$ were different. These results show that these different structures do not have the same stability and that the differences in the stability of the molecule are originated from different parts of the molecule; e.g., in Figure 7B, residues L105–R115 and L29–L39 of subunit D of microstate 2 were significantly less stable (lower $\ln K_{if}$) than the equivalent region in other microstates.

The stability patterns of the NBDs in chains A, C, and E of all three microstates were similar; however, the patterns were different in chains B, D, and F, as summarized in Table 3. The NBDs and DBDs of chains A, C, and E exhibited $\langle \ln K_{if} \rangle$ values within a narrow range of 9.4 ± 0.6 and 12.6 ± 0.4 ; however, the values for NBDs and DBDs for chains B, D, and F showed significantly different stability patterns ranging from 7.4 to 10.1 and from 2.7 to 18.3, respectively. Hence, this is a more concrete evidence that the homodimeric CRP is asymmetric in their energy landscapes.

Structural Stability of Holo CRP (PDB entry 1G6N).

Our current understanding in the mechanism of allosteric activation of CRP is advanced by the availability of the apo and holo structures. However, an in-depth understanding of the mechanism of allostery requires mapping the pathway of energy flow, i.e., the trajectory of signal transmission from one cAMP binding site in one NBD to another and to the DNA binding site in DBD. Figure 7D shows the results of the simulations of holo CRP (PDB entry 1G6N). The obvious change is the relative magnitude of the stability of chains A and B. The $\langle \ln K_{if} \rangle$ values, as summarized in Table 3, showed that binding of cAMP destabilized the CRP molecule, particularly the DBDs, although its binding site is in the NBD, as indicated by $\langle \ln K_{if} \rangle$ values of 7.52 and 6.86, respectively. This order of stability was the opposite of that for apo CRP, although the fine features depicting the regions of higher or lower stability in NBD-A were similar to that of apo CRP.

We decided to identify the sources of change and quantify the changes. The difference in structural stability was expressed as $\langle \ln K_{if} \rangle$, which was defined as the difference between the $\langle \ln K_{if} \rangle$ values of holo and apo CRP. Values of $\langle \ln K_{if} \rangle$ were converted to G because $G = -RT \ln K$. A negative value implies that cAMP binding decreased the stability. Figure 8 shows the effect of cAMP on the stability of each of the studied apo CRP microstates. For apo CRP, the binding of cAMP decreased the stability of NBDs and DBDs in chains A, C, and E by 1.3 ± 0.3 and 2.7 ± 0.2 kcal/mol, respectively. However, the values of destabilization of NBDs and DBDs of chains B, D, and F were 0.6 ± 0.5 and 3.5 ± 2.4 kcal/mol, respectively. There were more variations and larger effects on DBDs, particularly for chain F. These differences in pattern may reflect the affinity and/or cooperativity of cAMP binding. If the hypothesis were correct, then this might be the origin of the differences in the binding behavior of cAMP; i.e., a change in the experimental variables or mutations alters the various parameters that define the energetic landscape.

Connectivity Network among Residues, Residue-Specific Connectivity (RSC).

Panels A–C of Figure 7 show the consistency of the pattern of the energy landscape among the three microstates of apo CRP. We wish to elucidate if there were changes in the pattern of communication among these residues in the conversion process from apo to holo CRP upon binding of cAMP. Hence, we constructed a pattern of connectivity in the form of a heat map, residue-specific connectivity (RSC, eq 7), of each residue with respect to all other residues. Panels A–D of Figure 9 are presentations of apo and holo CRP A and B subunits. If two be positive. However, if the folding and unfolding reactions of two residues were out of phase, i.e., one folds while the other unfolds, then RSC assumed a negative value. It is obvious that many residues in NBD were in some degree of synchrony in their folding–unfolding dynamic motions. For example, residues F69–E72, W85–R103, and L116–R123 were in synchronized dynamic motions in a positive manner, as indicated by positive (red) RSC values with the rest of the subunit, including those in DBD. There are also residues with negative synchronization shown in negative (blue) RSC values.

Figure 10 shows the locations of structural elements in synchronized dynamic motions. On the basis of the heat maps, it is evident that both apo and holo CRP retain the same or a very similar network of connectivity, although in some cases larger numbers of residues adjacent to the existing network are involved. Clearly, the network is extensively involved with the binding site of cAMP. The network within DBD is more diffused; i.e., more residues are connected though less synchronized.

DISCUSSION

Allosteric activation of CRP by cAMP is a complex process and has been studied by multiple approaches for many decades. One of the remaining issues is the network of signal transmission. Our recent study showed differential modulation of energy landscapes of CRP as a regulatory mechanism for class II CRP-dependent promoters.³¹ In that study, we also concluded that the effect of a mutation is the sum of the consequences of modulating the synchrony of dynamic motions of residues at a distance leading to differential effects on the three activating regions in different subunits.

The availability of high-resolution structures of apo CRP²² provides a unique opportunity to elucidate the basic structural and energetic characteristics of different modes of allostery embedded in these microstates. In this study, we show that (1) the various apo CRP structures exhibit different energetic landscapes, (2) NBD and DBD structures are altered upon binding of cAMP, although the relative magnitude of changes in the energetic landscape is different, and (3) the relative stability of a specific amino acid sequence is different without a significant change in the energetic landscape pattern of NBDs, although the landscapes of DBDs are susceptible to changes in both pattern and magnitude. The obvious next step is to elucidate the functional consequences in the differential changes in energy landscapes of these microstates.

Changes in structural stability involve exchange of energy. Where does that energy come from to destabilize or stabilize the domains? Ligand binding is most likely the source. Hence, the “total” energy of binding is

$$\Delta G_{\text{tot, binding}} = \Delta G_{\text{obs}} + \Delta G_{\text{conf}} \quad (8)$$

where G_{obs} and G_{conf} are the free energy changes observed in binding and conformational change, respectively. On the basis of Table 3, we calculated the values of G for averaged residue stability because $G = -RT\langle \ln K_{i,f} \rangle_x$, where x is the same domain in either apo or holo CRP. Furthermore, we estimated $G_{x,\text{conf}}$ by

$$\begin{aligned} \Delta \langle \ln K_{i,f} \rangle_x &= \langle \ln K_{i,f} \rangle_{\text{holo},x} - \langle \ln K_{i,f} \rangle_{\text{apo},x} \text{ and } \Delta G_{x,\text{conf}} \\ &= \Delta G_{x,\text{holo}} - \Delta G_{x,\text{apo}} \end{aligned} \quad (9)$$

As shown in columns 6 and 11 of Table 4, all $\langle \ln K_{i,f} \rangle_x$ values led to positive G_x values for microstate 1 (AB) and microstate 3 (EF); i.e., binding of cAMP destabilized all of the domains. The magnitudes of $G_{\text{tot,conf}}$ values for chains A, C, and E in all microstates were similar. The patterns for chains B, D, and F were very different. The $G_{x,\text{holo}}$ values of the NBD and DBD in these chains were identical; however, the corresponding values in the apo states were very different. Consequently, the $G_{\text{tot,conf}}$ values of chains B, D, and F varied significantly, and the source of the difference was the apo state.

At pH 7.8, the functional thermodynamic parameters of cAMP binding of the first and second ligand are enthalpically and entropically driven, respectively, as defined by ITC measurements.^{17,32,33} The microscopic binding constants of cAMP are as follows: $k_1 = (4 \pm 0.5) \times 10^4$, and $k_2 = (6.9 \pm 1) \times 10^4 \text{ M}^{-1}$. The corresponding $G_{1,\text{binding}}$ and $G_{2,\text{binding}}$ are 6.3 and 6.6 kcal/mol, respectively. On the basis of these values of estimated total $G_{x,\text{conf}}$ summarized in Table 4 and the experimental values of $G_{1,\text{binding}}$ and $G_{2,\text{binding}}$, let us seek for consistency between the two sets of energetic data.

Binding of cAMP to Microstate 1.

If the first or second cAMP binds to chain A, the estimated binding constants would be $k_{1,\text{tot}} = 5.4 \times 10^7$ and $k_{2,\text{tot}} = 8.8 \times 10^7 \text{ M}^{-1}$. However, if the first or second cAMP binds to chain B, the corresponding k_1 and k_2 values would be 6.5×10^5 and $1.1 \times 10^6 \text{ M}^{-1}$. Under the scenario of cAMP first binding to chain A, a negative cooperativity phenomenon should be observed. However, the reverse scenario of binding first to chain B and then positive cooperativity binding should be observed, which is consistent with the experimental observation (Table 5).

Binding of cAMP to Microstate 2.

A similar analysis of the data for the binding of cAMP to microstate 2 was conducted. Binding of cAMP to chain C first or second would lead to $k_1 = 3.2 \times 10^7$ and $k_2 = 5.3 \times 10^7 \text{ M}^{-1}$. Conversely, constants for binding to chain D would be $k_1 = 4 \times 10^3$ and $k_2 = 6.7 \times 10^3 \text{ M}^{-1}$. Consequently, negative and positive cooperativity phenomena would be observed by binding to the C and C chain first, respectively.

Binding of cAMP to Microstate 3.

A similar analysis leads to positive cooperativity if cAMP binds first to chain E but negative cooperativity if the first binding were to chain F.

Which microstate is the most probable state present at pH 7.5?

Microstate 2 was not detected by SAXS measurement, which showed that the predominant states are either a mixture of microstates 1 and 3 or one of these two. On the basis of Table 4, the highly consistent values of stability for chains A, C, and E (columns 2–5) versus the larger fluctuation of stability for chains B, D, and F (columns 7 and 9), particularly the values for the corresponding DBDs, it is likely the subunit to which cAMP binds first is chain A or E. If one accepts this rationale, then EF is likely the predominant microstate at pH 7.5. It is the only one among the three that would provide data consistent with experimental data.

In this calculation, a major assumption is that the source of energetic differences between the experiment and that derived from structure is totally from conformational changes. This assumption may not be totally correct because it has been reported that ligand–protein interaction can dissipate some energy in the form of heat.³⁴

In summary, there are three novel observations. (i) The energy changes in subunits upon binding of cAMP are characteristics of individual subunits residing in these microstates. Although most of the changes are in destabilizing the holo state, it could also induce stabilization. (ii) The magnitudes of energy changes induced by cAMP binding are also characteristics of individual domains. The larger fluctuations are mostly due to the different stability of the apo DBD in chains B, D, and F. Thus, these results imply that the stability of the apo DBD may play a dominant role in defining the binding affinity of cAMP. (iii) As a corollary, the energetic landscapes of the microstates for exhibiting positive or negative are differential modulation of the stability of domains with very minor structural differences.

This study reveals, for the first time, that basically the apo and holo states of CRP retain the same connectivity network among the residues. This result implies that the network is engineered into the system and mutations or a change in experimental conditions modulates the magnitude of connectivity to achieve the desirable phenotype. Results from molecular dynamics simulation in addition to force distribution analysis provide insight into the pathways of signaling.¹⁶ Briefly, the cAMP binding site includes V49, G71, E72, R82, S83, and T127 in the same subunit but S128 of the adjacent subunit.²³ Results of the dynamic analysis identified, in one pathway, that the binding of the first cAMP in the signaling pathway involves the L73→(L124-R123-E72) sequence, where L73 resides in the subunit where the cAMP binds and the signal crosses to the adjacent subunit residues (L124-R123-E72). A second pathway involves the R123-R122-E72-I70-F69-D68 sequence of the same subunit while R122 transmits the signal to the adjacent subunit E77-E72-D68 and E77-Q80. Hence, these pathways represent intersubunit communication. It is most interesting to note that these pathways overlap with the highly connected residues shown in Figures 7 and 9, such as residues F69–E72, W85–R103, and L116–R123. Hence, the COREX/BEST algorithm provides data that are complementary to those from molecular dynamics.

CONCLUSION

In combination, the data for the energy landscapes, molecular dynamics, and solution functional and structural data lead to the conclusion that the microstates of apo CRP differ from each other by minor structural perturbations that result in changes in the energy landscapes of the various domains. These differences in energy landscapes presumably affect the energy landscapes of the intermediate and final states of ligand binding. The relative magnitude of changes in domains may define the ultimate outcome of the cooperativity of binding.

ACKNOWLEDGMENTS

The authors acknowledge the Sealy Center for Structural Biology and Molecular Biophysics at the University of Texas Medical Branch at Galveston for providing research resources. The editorial assistance of Dr. Heather Lander of the Sealy Center for Structural Biology and Molecular Biophysics is gratefully acknowledged. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Funding

Supported by National Institutes of Health Grants GM 77551 (J.C.L.) and GM 110591 (Y.W.Y.) and Robert A. Welch Foundation Grant H-0013 (J.C.L.).

REFERENCES

- (1). Monod J, Wyman J, and Changeux JP (1965) On the nature of allosteric transitions: A plausible model. *J. Mol. Biol* 12, 88–118. [PubMed: 14343300]
- (2). Koshland DE, Némethy G, and Filmer D (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* 5, 365–385. [PubMed: 5938952]
- (3). Vogt AD, and Di Cera E (2012) Conformational Selection or Induced Fit? A Critical Appraisal of the Kinetic Mechanism. *Biochemistry* 51, 5894–5902. [PubMed: 22775458]
- (4). Changeux JP, and Edelstein S (2011) Conformational selection or induced fit? 50 years of debate resolved. *F1000 Biol Rep* 3, 19. [PubMed: 21941598]
- (5). Wrabl JO, Gu J, Liu T, Schrank TP, Whitten ST, and Hilser VJ (2011) The role of protein conformational fluctuations in allostery, function, and evolution. *Biophys. Chem.* 159, 129–141. [PubMed: 21684672]
- (6). Swain JF, and Gierasch LM (2006) The changing landscape of protein allostery. *Curr. Opin. Struct. Biol* 16, 102–108. [PubMed: 16423525]
- (7). Hilser VJ, Dowdy D, Oas TG, and Freire E (1998) The structural distribution of cooperative interactions in proteins: analysis of the native state ensemble. *Proc. Natl. Acad. Sci. U. S. A* 95, 9903–9908. [PubMed: 9707573]
- (8). Negre CFA, Morzan UN, Hendrickson HP, Pal R, Lisi GP, Loria JP, Rivalta I, Ho J, and Batista VS (2018) Eigenvector centrality for characterization of protein allosteric pathways. *Proc. Natl. Acad. Sci. U. S. A* 115, E12201–E12208. [PubMed: 30530700]
- (9). Ye X, Lin J, Mayne L, Shorter J, and Englander SW (2019) Hydrogen exchange reveals Hsp104 architecture, structural dynamics, and energetics in physiological solution. *Proc. Natl. Acad. Sci. U. S. A* 116, 7333–7342. [PubMed: 30918129]
- (10). Ackers GK, and Holt JM (2006) Asymmetric Cooperativity in a Symmetric Tetramer: Human Hemoglobin. *J. Biol. Chem* 281, 11441–11443. [PubMed: 16423822]
- (11). Ackers GK, Holt JM, Burgie ES, and Yarian CS (2004) Analyzing intermediate state cooperativity in hemoglobin. *Methods Enzymol.* 379, 3–28. [PubMed: 15051349]

- (12). Dai J, Lin SH, Kemmis C, Chin AJ, and Lee JC (2004) Interplay between site-specific mutations and cyclic nucleotides in modulating DNA recognition by Escherichia coli cyclic AMP receptor protein. *Biochemistry* 43, 8901–8910. [PubMed: 15248748]
- (13). Harman JG (2001) Allosteric regulation of the cAMP receptor protein. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol* 1547, 1–17.
- (14). Liu B, Hong C, Huang RK, Yu Z, and Steitz TA (2017) Structural basis of bacterial transcription activation. *Science* 358, 947–951. [PubMed: 29146813]
- (15). Khankal R, Chin JW, Ghosh D, and Cirino PC (2009) Transcriptional effects of CRP* expression in Escherichia coli. *J. Biol. Eng* 3, 13. [PubMed: 19703305]
- (16). Louet M, Seifert C, Hensen U, and Grater F (2015) Dynamic allostery of the Catabolite Activator Protein revealed by interatomic forces. *PLoS Comput. Biol* 11, e1004358. [PubMed: 26244893]
- (17). Lin SH, and Lee JC (2002) Communications between the high affinity cyclic nucleotide binding sites in E. coli cyclic AMP receptor protein: effect of single site mutations. *Biochemistry* 41, 11857–11867. [PubMed: 12269830]
- (18). Gekko K, Obu N, Li J, and Lee JC (2004) A linear correlation between the energetics of allosteric communication and protein flexibility in the Escherichia coli cyclic AMP receptor protein revealed by mutation-induced changes in compressibility and amide hydrogen-deuterium exchange. *Biochemistry* 43, 3844–3852. [PubMed: 15049691]
- (19). Yu S, and Lee JC (2004) Role of Residue 138 in the Interdomain Hinge Region in Transmitting Allosteric Signals for DNA Binding in Escherichia coli cAMP Receptor Protein. *Biochemistry* 43, 4662–4669. [PubMed: 15096034]
- (20). Yu S, Maillard RA, Gribenko AV, and Lee JC (2012) The N-terminal Capping Propensities of the D-helix Modulate the Allosteric Activation of the Escherichia coli cAMP Receptor Protein. *J. Biol. Chem* 287, 39402–39411. [PubMed: 23035121]
- (21). Seok SH, Im H, Won HS, Seo MD, Lee YS, Yoon HJ, Cha MJ, Park JY, and Lee BJ (2014) Structures of inactive CRP species reveal the atomic details of the allosteric transition that discriminates cyclic nucleotide second messengers. *Acta Crystallogr., Sect. D: Biol. Crystallogr* 70, 1726–1742. [PubMed: 24914983]
- (22). Sharma H, Yu S, Kong J, Wang J, and Steitz TA (2009) Structure of apo-CAP reveals that large conformational changes are necessary for DNA binding. *Proc. Natl. Acad. Sci. U. S. A* 106, 16604–16609. [PubMed: 19805344]
- (23). Passner JM, Schultz SC, and Steitz TA (2000) Modeling the cAMP induced allosteric transition using the crystal structure of CAP-cAMP at 2.1 Å resolution. *J. Mol. Biol* 304, 847–859. [PubMed: 11124031]
- (24). Popovych N, Sun S, Ebright RH, and Kalodimos CG (2006) Dynamically driven protein allostery. *Nat. Struct. Mol. Biol* 13, 831–838. [PubMed: 16906160]
- (25). Shi Y, Wang S, and Schwarz FP (2000) Intersubunit association induces unique allosteric dependence of the T127L CRP mutant on pH. *Biochemistry* 39, 7300–7308. [PubMed: 10852730]
- (26). Pan H, Lee JC, and Hilser VJ (2000) Binding sites in Escherichia coli dihydrofolate reductase communicate by modulating the conformational ensemble. *Proc. Natl. Acad. Sci. U. S. A* 97, 12020–12025. [PubMed: 11035796]
- (27). Yeh L-CC, Falcon WE, Garces A, Lee JC, and Lee JC (2012) A Host-Guest Relationship in Bone Morphogenetic Protein Receptor-II Defines Specificity in Ligand-Receptor Recognition. *Biochemistry* 51, 6968–6980. [PubMed: 22894880]
- (28). Evangelista W, Yeh L-CC, Gmyrek A, Lee JC, and Lee JC (2015) Long-Range Communication Network in the Type 1B Bone Morphogenetic Protein Receptor. *Biochemistry* 54, 7079–7088. [PubMed: 26562759]
- (29). Hilser VJ, Dowdy D, Oas TG, and Freire E (1998) The structural distribution of cooperative interactions in proteins: Analysis of the native state ensemble. *Proc. Natl. Acad. Sci. U. S. A* 95, 9903–9908. [PubMed: 9707573]
- (30). Svergun DI, Barberato C, and Koch MHJ (1995) CRY SOL - a Program to Evaluate X-ray Solution Scattering of Biological Macromolecules from Atomic Coordinates. *J. Appl. Crystallogr* 28, 768–773.

- (31). Evangelista W, Dong A, White MA, Li J, and Lee JC (2019) Differential Modulation of Energy Landscapes of CRP as a Regulatory Mechanism for Class II CRP-dependent Promoters. *J. Biol. Chem* 294, 15544–15556. [PubMed: 31492755]
- (32). Lin SH, and Lee JC (2002) Linkage of multi-equilibria in DNA recognition by the D53H *Escherichia coli* cAMP receptor protein. *Biochemistry* 41, 14935–14943. [PubMed: 12475242]
- (33). Lin S-H, Kovac L, Chin AJ, Chin CQ, and Lee JC (2002) Ability of *E. coli* Cyclic AMP Receptor Protein to Differentiate Cyclic Nucleotides: Effects of Single Site Mutations. *Biochemistry* 41, 2946–2955. [PubMed: 11863432]
- (34). Riedel C, Gabizon R, Wilson CAM, Hamadani K, Tsekouras K, Marqusee S, Pressé S, and Bustamante CJ (2015) The heat released during catalytic turnover enhances the enzymes diffusion. *Nature* 517, 227–230. [PubMed: 25487146]

Figure 1.

Cartoon showing the energy diagram of the three apo CRP microstates, as represented by different shapes and colors. K_i is the apparent binding constant for formation of the cAMP–CRP complex that subsequently binds to different promoter DNA sequences that can bend to different degrees.

Figure 2. Molecular structures of apo CRP (Protein Data Bank entry 3HIF) and holo CRP (Protein Data Bank entry 1G6N). The color code and identity of subunits are as follows: blue, subunit A; red, subunit B. (A) 3HIF AB, (B) 3HIF CD, (C) 3HIF EF, and (D) 1G6N.

Figure 3.

Scattering curves calculated from the molecular structures in Figure 2. (A and B) Kratky plots and (C and D) log Guinier plots for simulated scattering curves derived from crystallographic structures. Panels B and D are focused on $q = 0.1$ – 0.25 to highlight the differences. Solid lines are calculated from the X-ray crystal structures of apo CRP (red, AB dimer; green, CD dimer; blue, EF dimer) under crystallization conditions. The dashed brown line (---) is calculated from holo CRP. R_g values for the simulated scattering curves: X-ray apo AB, 24.3 ± 0.016 Å; X-ray apo CD, 24.49 ± 0.015 Å; X-ray apo EF, 24.2 ± 0.015 Å; holo CRP, 24.1 ± 0.02 Å.

Figure 4.

Comparison of the experimental SAXS data from apo wild-type CRP. (A and B) Kratky plots and (C and D) log Guinier plots. Experimental scattering data from apo wild-type CRP (●). Only alternate data points are shown for the sake of clarity. Fittings of the dimer models: AB dimer (green), $\chi^2 = 1.8$; CD (blue), $\chi^2 = 2.2$; EF (orange), $\chi^2 = 1.7$ (solid line under buffer conditions resembling the crystallographic conditions).

Figure 5.

Bead model of apo CRP. (A) DAMMIF bead model of apo CRP (NSD = 0.47) shown with an X-ray dimer AB fit ($\chi^2 = 1.8$; NSD = 0.95), shown in both front and side views. (B) Same with X-ray dimer CD ($\chi^2 = 1.6$; NSD = 0.95).

Figure 6. Rigid body modeling of the apo CRP homodimer in solution. (A) Comparison of the Kratky plots of simulated and experimental SAXS data from apo wild-type CRP. (B) Bead model of apo CRP.

Figure 7.

Plot of $\ln K$ vs residue number of apo and holo CRP. Symbols and subunit identity: filled circles, subunit A; empty circles, subunit B. Identities of structures: (A) 3HIF AB, (B) 3HIF CD, (C) 3HIF EF, and (D) 1G6N holo CRP.

Figure 8. Change in structural stability from apo to holo. A–F represent subunits in microstates AB, CD, and EF, respectively. Red and green data are for the NBD and DBD, respectively, of subunits A–F.

Figure 9.

(A and B) Connectivity map (RSC) of subunits A and B of microstates 1 and 2, respectively, of apo CRP. The false color scale indicates positive and negative connectivity between residues. Positive connectivity indicates the folding–unfolding reactions of these residues are synchronized at the same time scale whereas negative connectivity indicates asynchronization between these residues. (C and D) Connectivity map (RSC) of microstate 3 and holo subunits A and B of apo and holo CRP. The false color scale indicates positive (red) and negative (blue) connectivity between residues. Positive connectivity indicates the

folding–unfolding reactions of these residues are synchronized at the same time scale whereas negative connectivity indicates asynchronization between these residues.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 10. Connectivity cross points in (left) apo CRP (PDB entry 3HIF) and (right) holo CRP (PDB entry 1G6N). The arrows show the locations of residues among the common connectivity crossing points in the pathways of signal transmission.

Table 1.Summary of RMSD^a Analysis of Apo CRP

		3HIF											
		chain A		chain B		chain C		chain D		chain E		chain F	
		NBD	DBD	NBD	DBD	NBD	DBD	NBD	DBD	NBD	DBD	NBD	DBD
microstate 1	NBD-A			1.27		1.29		1.04		1.20		1.43	
	DBD-A				1.67		1.45		1.11		1.77		0.98
	NBD-B					1.06		1.07		0.98		1.40	
microstate 2	DBD-B						1.38		1.29		1.39		1.80
	NBD-C							1.20		1.05		1.51	
	DBD-C								1.24		1.74		1.61
	NBD-D									1.11		1.29	
	DBD-D										1.30		1.40

^aUnits of angstroms.

Table 2.

RMSD (angstroms) Analyses of Apo (3HIF) and Holo (1G6N) CRP

			1G6N			
			subunit A		subunit B	
			NBD	DBD	NBD	DBD
3HIF	microstate 1	NBD-A	0.81		0.81	
		DBD-A		0.84		0.81
		NBD-B	0.84		0.80	
		DBD-B		0.78		0.79
	microstate 2	NBD-C	0.69		0.92	
		DBD-C		0.22		0.57
		NBD-D	0.43		0.91	
		DBD-D		0.28		0.54
	microstate 3	NBD-E	0.56		0.90	
		DBD-E		0.47		0.91
		NBD-F	0.55		1.07	
		DBD-F		0.47		0.79

Table 3.Summary of $\langle \ln K_{if} \rangle$ Values of Domains in Apo and Holo CRP

	NBD-X ^a	DBD-X	NBD-Y	DBD-Y	subunit A	subunit B
apo CRP						
3HIF-AB	9.90	12.50	8.50	6.80	10.80	7.96
3HIF-CD	9.80	12.10	7.40	2.70	10.64	5.78
3HIF-EF	8.60	13.20	10.10	18.30	10.22	13.02
holo CRP						
1G6N	7.31	7.93	8.18	4.39	7.52	6.86

^aX = chain A, C, or E of apo CRP; Y = chain B, D, or F of apo CRP. Subunits A and B are from holo CRP.

Table 4.

Estimation of $G_{X,conf}$ Values for Conversion from Apo to Holo States in Domains in wt CRP at pH 7.5 and 25 °C

stability in G (kcal/mol)									
subunit A					subunit B				
NBD		DBD		$G_{tot,conf}^a$	NBD		DBD		$G_{tot,conf}$
$G_{x,apo}$	$G_{x,holo}$	$G_{y,apo}$	$G_{y,holo}$		$G_{x,apo}$	$G_{x,holo}$	$G_{y,apo}$	$G_{y,holo}$	
AB	-5.8	-4.3	-7.4	4.2	-5.0	-4.8	-4.0	-2.6	1.6
CD	-5.8	-4.3	-7.1	3.9	-4.4	-4.8	-1.6	-2.6	-1.4
EF	-5.1	-4.3	-7.8	3.9	-6.0	-4.8	-10.8	-2.6	9.4

^aTotal $G_{X,conf}$ of both the NBD and the DBD, assuming no intermediate energetic states other than the observed apo and holo forms because there is no structure of a singly liganded CRP under these experimental conditions.

Table 5.Estimation of Microscopic Binding Constants (k_i) of cAMP Binding.

chain	$G_{1,tot}$ (kcal/mol)	$k_{1,tot}$ (M^{-1})	$G_{2,tot}$ (kcal/mol)	$k_{2,tot}$ (M^{-1})
A	$-6.3 - 4.2 = -10.5$	5.4×10^7	$-6.6 - 4.2 = -10.8$	8.8×10^7
B	$-6.3 - 1.6 = -7.9$	6.5×10^5	$-6.6 - 1.6 = -8.2$	1.1×10^6
C	$-6.3 - 3.9 = -10.2$	3.2×10^7	$-6.6 - 3.9 = -10.5$	5.3×10^7
D	$-6.3 + 1.4 = -4.9$	4×10^3	$-6.6 + 1.4 = -5.2$	6.7×10^3
E	$-6.3 - 3.9 = -10.2$	3.2×10^7	$-6.6 - 3.9 = -10.5$	5.4×10^7
F	$-6.3 - 9.4 = -15.7$	3.6×10^{11}	$-6.6 - 9.4 = -16$	6.0×10^{11}