The N-terminal Capping Propensities of the D-helix Modulate the Allosteric Activation of the Escherichia coli cAMP Receptor Protein*

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**Background:** Residue 138 situates in the hinge region connecting the DNA- and cAMP-binding domains of CRP. The goal of the current study is to elucidate the role of residue 138 in modulating the allosteric activation event.

**Results:** A correlation was established among N-capping propensities and cooperativity of cAMP binding and affinity for lac-DNA. Our results provide a quantitative characterization of the N-capping properties of residue 138 in the allosteric activation event.

**Conclusion:** Our results provide a quantitative characterization of the N-capping properties of residue 138 in the allosteric activation event.

**Significance:** The results provide the thermodynamic basis to the structural model of allostery.

Transduction of biological signals at the molecular level involves the activation and/or inhibition of allosteric proteins. In the transcription factor cAMP receptor protein (CRP) from Escherichia coli, the allosteric activation, or apo-holo transition, involves rigid body motions of domains and structural rearrangements within the hinge region connecting the cAMP- and DNA-binding domains. During this apo-holo transition, residue 138 is converted as part of the elongated D-helix to the position of the N-terminal capping residue of a shorter D-helix. The goal of the current study is to elucidate the role of residue 138 in modulating the allosteric activation event. By systematically mutating residue 138, we found that mutants with higher N-terminal capping propensities lead to increased cooperativity of cAMP binding and a concomitant increase in affinity for lac-DNA. Furthermore, mutants with higher N-terminal capping propensity correlate with properties characteristic of holo-CRP, particularly, increase in protein structural dynamics. Overall, our results provide a quantitative characterization of the role of residue 138 in the isomerization equilibrium between the apo and holo forms of CRP, and in turn the thermodynamic underpin to the molecular model of allostery revealed by the high resolution structural studies.

One of the fundamental questions yet to be resolved about allostery is the identity of the physical properties of the protein that are responsible for modulating the phenomenon of allostery. Escherichia coli cAMP receptor protein (CRP) is chosen as a model system to address this important yet unanswered question. Cooperativity between the two high affinity cAMP-binding sites and DNA binding by the cAMP-activated CRP are the consequences of homotropic and heterotropic allosteric effects, respectively (1).

Recently, we uncovered two fundamental features of allostery in CRP. First, the nature of cooperativity can be modulated from negative to positive via mutations at sites not directly involved in ligand binding or in subunit contacts. Second, negative cooperativity and positive cooperativity arise from the modulation of the dynamics of the protein. This conclusion is based on our observation of a linear relationship between the energetics of cooperativity in cAMP binding and protein dynamics (2). A similar conclusion can be derived from the DNA binding results.

One of the critical dynamic motions in CRP during the apo-holo transition is the reorientation of the DNA- and cAMP-binding domains (3, 4). Kalodimos and co-workers (3) recently resolved the structure of apo-CRP by NMR, whereas Steitz and co-workers (4) resolved the structure by x-ray crystallography. Both groups observed a rotation and translation of the DNA-binding domain relative to the cAMP-binding domain (Fig. 1), although the x-ray data revealed an additional structural change that involved a change in the length of the D-helix during the apo-holo transition (3, 4). A consequence of this domain rotation is the exposure of the DNA recognition F-helices to position them for binding DNA (Fig. 1) and another concomitant change involving a coil-helix conversion including residues Val126 through Phe136. Residues 126–136 constitute part of the C-helix in the holo form, whereas in apo-CRP, the same residues assume a coil configuration (Fig. 1). Moreover, in the apo-CRP structure, the length of the D-helix is extended to include residue Asp-138, leading to an interdomain antiparallel helix-helix interaction between the two D-helices of adjacent subunits (4). Thus, these two recent structural studies provide greater details to the earlier model (5–8), in particular, showing residue Asp-138 as a part of a longer helix in the apo state and as an N-terminal capping residue of a shorter helix in the holo state.

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5 The abbreviations used are: CRP, cAMP receptor protein; H/D, hydrogen/deuterium; ITC, isothermal titration calorimetry.
Because residue 138 is part of the secondary structural change during the apo-holo transition, we hypothesize that a change in the N-terminal capping propensity of the Asp-138 side chain should influence the equilibrium distribution of the apo-holo states. As a consequence, the allosteric properties of CRP should be altered. Hence, the functional energetics of cAMP binding and DNA binding are correlated to the structural energetic landscape of CRP and to the N-terminal capping propensities of amino acid substitutions at position 138.

**EXPERIMENTAL PROCEDURES**

**Materials**—cAMP and proteases (subtilisin and chymotrypsin) were purchased from Sigma-Aldrich. Sodium azide was purchased from Kodak. The absorption coefficient of cAMP was 14,650 M⁻¹ cm⁻¹ at 259 nm. Calibration proteins for chromatography were products of Roche Applied Science. All of the experiments were conducted in TEK100 buffer (50 mM Tris, 1 mM EDTA, and 100 mM KCl at pH 7.8 and 25 °C).

**Protein Preparation**—Wild type and all Asp-138 mutants of CRP were purified from isopropyl thiogalactopyranoside-induced E. coli strains HMS174DE3 with a published procedure (9–11). The protein purity was evaluated by SDS-PAGE gel stained by Coomassie Blue. Purified protein was stored at −20 °C freezer with ~0.2 M KCl and ~10% glycerol at pH 7.5. Protein solutions were concentrated by passage through a hydroxyapatite gel column. CRP solution was dialyzed against TEK100 buffer overnight with three buffer changes before being used. The concentration of stock CRP solution was determined spectrophotometrically by using the extinction coefficient of 40,800 M⁻¹ cm⁻¹ at 278 nm for the CRP dimer (12). The ratio of absorbance at 278 nm to that at 260 nm was larger than 1.85, indicating that contaminated DNA was removed. The presence of the desired substitutions was confirmed by mass spectrometry and automated dideoxy sequencing of the DNA templates.

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**cAMP Binding Monitored by ITC**—All ITC titration experiments were carried out on the VP-ITC MicroCalorimeter (MicroCal, Inc., Northampton, MA), which was calibrated with electrically generated heat pulses as recommended by the manufacturer. Concentrations of all CRP mutants were higher than 180 μM in TEK100 buffer. All ITC experiments were conducted in TEK100 buffer, and the method for data analysis was the same as previously described (1). Heats of dilution of cAMP were corrected in the calculation for the heats of reactions.

**DNA Binding as a Function of cAMP Concentration**—The relationship between DNA binding affinity of CRP and cAMP concentration was monitored by our previously published method of fluorescence anisotropy (13, 14).

**Size Exclusion Chromatography**—The experiments, repeated three times, were carried out using a Phenomenex BioSEC S2000 HPLC gel filtration column 2000 × 7.8 mm. Constant temperature was maintained by a water bath set at 25 °C. The protein samples (~30 μM) were warmed up to room temperature before using. The steel-jacketed column was washed with 500 ml of degassed H₂O and then equilibrated with 500 ml of filtered and degassed TEK100 buffer. The column was operated with mechanical injection within a fully automated BioCad SPRINT HPLC system. The elution volumes of protein sample, blue dextran, and sodium azide were obtained, and the partition coefficient (Kᵥ) was calculated with Equation 1 (15),

\[
Kᵥ = \frac{(V_{protein} - V_{blue dextran})(V_{azide} - V_{blue dextran})}{V_{protein}} \quad (Eq. 1)
\]

where \(V_{protein}\), \(V_{blue dextran}\), and \(V_{azide}\) represent the elution volumes of protein sample, blue dextran, and azide, respectively.

Before each experiment, the column was calibrated using proteins with Stokes radii taken from the literature (15). 1/Kᵥ was shown to be proportional to the Stokes radius (Rₛ) of protein as described by Equation 2, which was used for estimating the dimensions of native protein.

\[
1/Kᵥ = 0.154Rₛ - 1.289 \quad (Eq. 2)
\]

**Determination of Vprotein from the Size Exclusion Chromatography Elution Profile**—The range of data chosen for numerical analysis encompassed the entire protein peak for CRP wild type and all mutants. To determine the numerical value of the maximum (or peak), a nonlinear least squares fitting routine using a sixth degree polynomial was employed to simulate the elution profile. The first derivative of the fitted data were employed to determine the numerical values corresponding to slope zero, which was the maximum value of the elution profile. The fitting procedures were performed using SigmaPlot 8.0. Numerical calculations were performed using Mathematica 4.0 (Wolfram Research).

**Protease Sensitivity**—Proteolytic digestion was carried out in a reaction volume of 50 μl containing 15 μM CRP and 8 μg/ml protease at room temperature in TEK100 buffer. The reaction was started by the addition of 2 μl of a stock solution of 200 μg/ml protease and was stopped by adding 1 μl of 100 mM PMSF in 2-propanol to a final concentration of 1 mM. The reaction time was 85 min for subtilisin and 120 min for chymotrypsin. 15-μl aliquots of the reaction mixture were withdrawn and loaded onto a 15% SDS-PAGE slab gel for analysis (16, 17). The
gels were stained by Coomassie Blue. The digestion percentage of each mutant was analyzed by monitoring the intensity of each band using a Kodak camera system (model EDAS 290).

**H/D Exchange Monitored by FTIR Spectroscopy**—FTIR spectra were measured with a Bomem (Quebec, Canada) MB series Fourier transform infrared spectrometer equipped with a DTGS detector and purged constantly with dry air, using the same published procedure (18). For the H/D exchange experiment, the published procedure was employed (18–21). The fraction of the unexchanged amide proton, \( F \), was calculated at various time intervals using Equation 3,

\[
F = \frac{A_{II} - A_{II0}}{A_{II0}}
\]  
(Eq. 3)

where \( A_{II} \) and \( A_{II0} \) are the absorbance maximum of the amide II and II bands, respectively, \( A_{II1} \), is the amide II absorbance maximum of fully deuterated protein, and \( \omega \) is the ratio of \( A_{II0}/A_{II} \), with \( A_{II0} \) and \( A_{II} \) being the respective absorbance maxima for the amide II and amide I bands of protein in H₂O (20, 21).

The exchange kinetic parameters were fitted to Equation 4,

\[
F = A_1e^{-k_1t} + A_2e^{-k_2t} + C
\]  
(Eq. 4)

where \( F \) is amide proton fraction at given time \( t; \) \( k_1 \) and \( k_2 \) are the intermediate and slow exchange rate, respectively; and \( A_1 \), \( A_2 \), and \( C \) are the constants. \( F_0 \) is the remaining amide proton fraction at 1 min. Double exponential expression is necessary for fitting the data.

**RESULTS**

**Functional Energetics**

**cAMP Binding**—We measured the affinity as well as the thermodynamic parameters (enthalpy and entropy change) during cAMP binding to WT CRP and position 138 mutants. The left panel of Fig. 2 shows a typical calorimetric titration of cAMP into a solution of WT-CRP. The right panel of Fig. 2 shows the heat exchange/mole of titrant versus the ratio of the total concentration of ligand to that of protein. The results of all Asp-138 mutant binding reactions are shown in Fig. 3. The solid lines represent the best least squares fits of the data as described (1). The recovered parameters are summarized in Table 1.

These thermodynamic data for WT CRP showed an initial exothermic (\( \Delta H < 0 \)) binding reaction followed by an endothermic (\( \Delta H > 0 \)) phase (1, 14). The magnitude and nature of cooperativity of cAMP binding to the first and second site are expressed as \( \Delta \Delta G, \) i.e., \( \Delta G_2 - \Delta G_1 \), derived from their microscopic binding constants, \( k_2 \) and \( k_1 \) (Table 1). We observed that mutations at residue 138 affect the affinity and various thermodynamic parameters of cAMP binding significantly.

**DNA Binding as a Function of cAMP Concentration**—Side chain substitutions at residue 138 affect the binding affinity of these CRP mutants for lac-DNA, as shown in Table 2 (14). However, all Asp-138 mutants maintain the WT CRP characteristic biphasic feature. It is well established that the relationship between DNA binding affinity of CRP and cAMP concentration is biphasic regardless of DNA sequence (Fig. 4) (16). First, an initial increase in the value of the DNA binding constant is observed as the two high affinity cAMP-binding sites are occupied (1). Then the binding constant decreases as the cAMP concentration reaches saturation of the weak cAMP-binding sites in CRP (1, 13, 16). Hence, mutations at residue 138 affect the affinity for DNA but not the basic mechanism of DNA binding in response to cAMP concentration.

**Protein Structural Dynamics**

**Hydrodynamic Properties: Size Exclusion Chromatography**—As a structural parameter, the Stokes radius reflects the global hydrodynamic conformation of CRP. Gel filtration was used to
determine the Stokes radii of WT CRP and Asp-138 mutants. The elution profiles of CRP wild type and Asp-138 mutants are shown in the inset of Fig. 5A. Small but highly reproducible shifts in the elution profiles are observed, indicating a shift in Stokes radius in CRP induced by side chain replacements at residue 138 (Fig. 5A). We determined the peak value of the elution time of the Asp-138 mutants (Fig. 5). Knowing the flow rate, the elution time can be converted to elution volume, which is related to Stokes radius as expressed by Equation 2. The results are summarized in Table 2. The Asp-138 mutations affect the hydrodynamic property of CRP so much that it is reflected in a change in Stokes radius.

Proteolytic Digestion—The sensitivity of CRP to proteolytic digestion is one of the diagnostic tools to monitor changes in CRP structure (16, 22, 23). WT CRP is only sensitive to protease in the presence of cyclic nucleotide. Both subtilisin and chymotrypsin were employed in this study to detect the protein conformational changes induced by mutations at residue 138. All of the CRP samples behaved identically regardless of the specific protease employed. Similar to WT, the proteolytic digestion pattern of all 138 mutants is identical, namely, the appearance of a single protease-resistant core of very similar molecular weight as detected by SDS-PAGE and biphasic as a function of cAMP concentration as reported by Heyduk and Lee (16). Typical results of the subtilisin digestion are shown in Fig. 6.

Despite these side chain-specific changes in rate of proteolysis, these mutations have not altered the general response of CRP to cAMP binding, namely, a change in intersubunit interaction resulted in an increase in sensitivity to proteolysis. MALDI-TOF mass spectrometric analysis showed that the molecular weights of the protease resistant cores are 15,266 and 15,602 for subtilisin and chymotrypsin digestions, respectively. In turn these values matched the predicted mass of 15,265 and 15,597 for the sequences containing residues Val-2 to Leu-134 and Val-2 to Leu-137, respectively.

Global Dynamics: H/D Exchange Measurement—To explore and compare the protein dynamics of WT CRP and Asp-138 mutants, H/D exchange rates of these mutants were monitored by FTIR. The overall H/D exchange rates were estimated by plotting the fraction of unexchanged amide protons as a function of time (Fig. 7). The fraction of the unexchanged amide protons at the first exchange time point (1 min) for all studied proteins are from 15 to 25%, suggesting that the majority of the amide protons exchanged so rapidly that their exchange was ~80% completed within the time interval of the acquisition of the first time point. Therefore, only the intermediate and slow exchange protons can be practically monitored semiquantitatively over the time range employed in this study. A two-exponential decay model (Equation 4) was used to describe the exchange reaction of the remaining amide protons within the experimental time frame, and the resolved parameters are summarized in Tables 2. Fα, nonexchanged protein fraction at 1 min of exposure to D2O, also reflects the dynamics of the protein. Because of the complexity of the overall H/D exchange reaction and technical limitation, the data shown in Table 2 reveal that the dynamics of Asp-138 mutants can be grossly divided into three classes: D138L is the most dynamic mutant followed by the group consisting of WT and D138K, D138Q, D138G, D138T, and D138S mutants, whereas D138A and D138V constitute the class of the most rigid mutants.

**DISCUSSION**

A meaningful interpretation of the results of residue 138 mutations depends on whether the mutations only modulate, and not disrupt, the basic mechanism of CRP activity. The features of wild type CRP are characterized by a biphasic pattern in DNA binding as a function of cAMP concentration (16); the presence of two high affinity cAMP-binding sites (1) and higher/lower protease susceptibility at low/high cAMP concentration, respectively (16, 22, 13). Our results for all Asp-138 mutants displayed the same hallmark behavior in DNA binding (Fig. 4), cAMP binding (Table 1), and protease digestion (Fig. 6).

**TABLE 1**

| Protein | K1 × 10^6 s⁻¹ | ΔH1 kcal/mol | ΔS1 cal/K/mol | K2 × 10^6 s⁻¹ | ΔH2 kcal/mol | ΔS2 cal/K/mol |
|---------|---------------|--------------|---------------|---------------|--------------|---------------|
| WT      | 8 ± 2         | -3.9 ± 0.3   | 10            | 6 ± 1         | 6.0 ± 0.3    | 42            |
| D138T   | 9 ± 1         | -3.7 ± 0.1   | 10            | 5 ± 1         | 1.4 ± 0.1    | 26            |
| D138S   | 11 ± 4        | -3.1 ± 0.1   | 13            | 1.1 ± 0.2     | -2.4 ± 0.0   | 10            |
| D138Q   | 6 ± 2         | -9 ± 2       | 7             | 46 ± 6        | 8 ± 2        | 54            |
| D138G   | 10 ± 1        | -3.4 ± 0.1   | 10            | 3.8 ± 0.4     | 3.0 ± 0.1    | 31            |
| D138K   | 36 ± 11       | -0.8 ± 0.2   | 23            | 9 ± 3         | 4.2 ± 0.1    | 37            |
| D138L   | 18 ± 3        | 7.9 ± 0.8    | 50            | 370 ± 70      | 1.5 ± 0.8    | 35            |
| D138A   | 6 ± 1         | -3.4 ± 0.1   | 10            | 1.7 ± 0.1     | -2.8 ± 0.4   | 10            |
| D138V   | 3.1 ± 0.4     | -4.4 ± 0.1   | 6             | 1.7 ± 0.2     | -3.9 ± 0.2   | 7             |
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TABLE 2
Correlations between N-capping frequency and thermodynamic parameters

| Side chain | Residue N-cap frequency | CD | Fluo | Intersubunit interaction | Proteolytic digestion | Stokes radius | H-D exchange out rate $k_1$ | $\Delta G$ | $-T(\Delta S_1 - \Delta S_T)$ | $-\Delta G$ of DNA binding |
|------------|-------------------------|----|------|--------------------------|-----------------------|--------------|-----------------------------|-----|-----------------------------|--------------------------|
| Asp        | 1.58                    | 1.8 ± 0.1 | 2.9 ± 0.1 | 11.7 ± 0.9                | 50                     | 20.4 ± 0.1 | 0.31 ± 0.03               | 0.65 | 9.5                         | 10.7                      |
| Thr        | 1.41                    | 1.8 ± 0.1 | 3.6 ± 0.2 | 13.5 ± 2.0                | 49                     | 21.0 ± 0.2 | 0.32 ± 0.02               | 0.47 | 4.8                         | 10.6                      |
| Ser        | 1.25                    | 2.0 ± 0.1 | 3.8 ± 0.4 | 15.0 ± 1.4                | 30                     | 21.2 ± 0.1 | 0.35 ± 0.08               | -0.54 | -0.9                       | 9.7                       |
| Gln        | 1.05                    | 1.9 ± 0.1 | 4.5 ± 0.4 | 13.0 ± 0.7                | 53                     | 20.5 ± 0.2 | 0.20 ± 0.03               | 2.03 | 14.8                        | 9.8                       |
| Gly        | 0.98                    | 2.2 ± 0.1 | 4.0 ± 0.4 | 11.4 ± 0.8                | 50                     | 20.9 ± 0.1 | 0.43 ± 0.04               | 0.24 | 6.3                         | 9.9                       |
| Lys        | 0.84                    | 1.6 ± 0.1 | 3.6 ± 0.2 | 11.8 ± 0.8                | 55                     | 20.2 ± 0.1 | 0.27 ± 0.03               | 0.4  | 4.2                         | >12                       |
| Leu        | 0.79                    | 1.7 ± 0.1 | 3.2 ± 0.2 | 11.8 ± 0.7                | 68                     | 19.8 ± 0.3 | 0.53 ± 0.01               | 2.6  | -4.5                        | 6.1                       |
| Ala        | 0.67                    | 1.5 ± 0.1 | 4.7 ± 0.4 | 18.6 ± 1.7                | 25                     | 21.6 ± 0.1 | 0.56 ± 0.04               | 0.06 | 0.0                         | 6.7                       |
| Val        | 0.67                    | 1.9 ± 0.1 | 5.6 ± 0.3 | 23.5 ± 1.7                | 23                     | 21.6 ± 0.2 | 0.55 ± 0.03               | 0.47 | -0.3                        | 5.0                       |

The values are from Table I of Aurora and Rose (33).

The values are from Yu and Lee (14).

The only effect of mutation is in the magnitude of modulation, not the basic mode of reaction mechanism of CRP.

Previously we have established a linear correlation between protein dynamics and the magnitude of allosteric effects on cAMP binding (1). That and other observations in the literature (24–27) led to the hypothesis that inter- and intrasubunit interactions are part of the protein dynamics involved in the allosteric mechanism of CRP. Hence, it is logical to identify the mechanism(s) and origin that modulates the dynamics of CRP and to test whether all protein dynamic motions are correlated to the allosteric behavior of CRP.

In this study, there is a change in the dynamic properties of the CRP mutants as monitored by H/D exchange rates. An apparent correlation between the H/D exchange rate and some physical properties of the side chain was observed (Table 2).

Although the high resolution structures of the cAMP-CRP and cAMP-CRP-DNA complexes have been reported quite some time ago (5–8, 28–31), the structure of the apo-CRP has only been published recently (3, 4). These structures clearly demonstrate significant structural changes, unequivocally indicating that the structural isomerization reaction must be included as a finite step in the linked multiequilibria mechanism that describes the CRP function (1, 32). The observed change in the secondary structure involving residues 126–139 implies that perturbation of the equilibrium between the coil to helix and extension/contraction of the D-helix could perturb the equilibrium between the apo and holo isomerization reaction and in turn the allosteric behavior of CRP.

What is the molecular property of the side chains of residue 138 that plays the predominant role in modulating the allosteric behavior of CRP? Residue 138 is placed in the midst of the structural changes involving residues 126–139. In the apo structure, Asp-138 is part of the extended D-helix. However, it becomes the N-terminal capping residue of the contracted D-helix in the holo structure, as shown in Fig. 1. Thus, we hypothesize that residue 138 is a prime candidate for modulating the equilibrium of this interdomain interaction. In this study, we tested this hypothesis by a series of side chain substitutions at residue 138 according to their propensity as the N-terminal capping residue for the D-helix (33–35). A side chain of high N-terminal capping frequency means that it prefers to occupy the N-terminal position of a helix. We hypothesize that substitutions of side chains of high and low N-terminal capping propensity at residue 138 would shift the apo ↔ holo equilibrium toward the holo form and vice versa. Consequently these side chain substitutions would affect the solution biochemical properties of CRP. These shifts in multiple equilibria would ultimately be manifested in a change in allosteric behavior.

What is the evidence to support the hypothesis that the N-terminal capping propensity is the dominant property of residue 138 that plays a significant role in modulating the functional and structural behaviors of these mutants? (a) The identity of a ($n + 3$) residue in the D-helix, where $n$ = Asp-138, is expected to influence the results if the side chain of the ($+3$) residue interacts favorably or unfavorably with Asp-138. Indeed, G141Q and L148R CRP mutants both exhibit higher cooperativity in cAMP binding and higher affinity for DNA than WT CRP (1, 2, 12). (b) The NMR data of the DNA-binding domain of G141S CRP mutant show clearly that apo-G141S CRP assumes a structure in equilibrium between the apo and holoWT CRP (31). This result elegantly shows that residue that
FIGURE 5. Numerical analysis of the elution profiles of CRP wild type and Asp-138 mutants. A, time range used for the fitting routine corresponding to 490 – 600 s. The range used contains the observed absolute maximum (inset) of the complete elution profile. The identities of proteins in the order from top to bottom is D138V, D138A, D138S, D138T, D138G, WT, D138Q, D138K, and D138L. B, fitted curves (in red) of the elution profiles of D138A, WT, and D138L (from top to bottom) were performed by nonlinear least square analysis using a sixth degree polynomial. C, residuals from the fittings in B. The highest deviations correspond to less than 5% of the maximum absorbance for each mutant. D, first derivative plots of the absorbance versus time using the parameters obtained from the fitting. Each curve corresponding to each mutant crosses the y axis (zero line) at different times except for D138A and D138V. Analysis of the second derivative verified that those values correspond to an absolute maximum.
occupies the \((n + 3)\) position relative to Asp-138 plays a role in influencing the shift of equilibrium between the apo and holo states. (c) In the apo-state, residue Asp-138 resides as part of the elongated D-helix. If the propensity of residue Asp-138 as an \(\alpha\)-helix former plays a significant enough role in driving the apo-holo isomerization, then one might expect to observe a correlation between the \(\alpha\)-helix propensity of residue Asp-138 and the various functional energetic terms. However, there is no correlation (data not shown). In conclusion, the helix propensity of Asp-138 does not play a significant enough role in driving the apo-holo isomerization reaction.

There is a revealing correlation between the N-terminal capping propensity at residue 138 and DNA binding affinity, as shown in Fig. 8 and Table 2. The highest affinity for DNA is associated with the side chains with the highest propensity for N-terminal capping as predicted by our hypothesis. Conversely, the affinity for DNA decreases with decreasing N-terminal capping propensity. This observed correlation between DNA binding affinity and N-terminal capping propensity implies that the mutations at residue 138 mainly affect the equilibrium distribution of the apo ↔ holo structural isomerization reaction. A higher N-terminal capping propensity would shift the isomerization reaction in favor of the holo form with a shorter D-helix where residue 138 is the N-terminal residue (Fig. 1). Thus, the observed higher DNA binding affinity of some CRP mutants could arise, in part, because less binding energy would be expended in the structural isomerization step.

Data for D138K is not included in Fig. 8 because its binding affinity is out of range compared with the other mutants shown in Table 2. The lack of correlation for this particular substitution suggests that other side chain properties such as charge can also be involved in DNA binding (i.e., electrostatic interactions between the DNA and the side chain). Alternatively, the data for the Lys mutation may indicate that the substitutions studied here do not merely shift the apo ↔ holo equilibrium that involves the two species of apo and holo-CRP. Within the apo, the holo or both forms, each of which might be populated by multiple conformational microstates. The Asp → Lys mutation might further shift these equilibria to accentuate a different predominant set of behavior of CRP.

Mutations at residue 138 affect the magnitude of cooperativity of cAMP binding, as shown in Fig. 8. In general, the higher the N-terminal capping propensity of the substitution, the higher is the magnitude of positive cooperativity. Not only the magnitude of positive cooperativity is decreased for substitutions of lower N-terminal capping propensity; the nature of cooperativity can actually shift to negative. It is most interesting to note that the binding affinity of DNA tracks the trend of cooperativity of cAMP binding. This tight correlation indicates the intimate thermodynamic linkage between homotropic and
heterotropic allosteric effects as reflected by cAMP and DNA binding, respectively.

The thermodynamic feature of cAMP binding for most of the mutants is consistent with the data for WT CRP: an exothermic binding event is associated with the first site, and in contrast, the occupancy of the second site is endothermic and entropically driven. The top panel of Fig. 9 shows the relation between ΔH of cAMP binding and N-terminal capping propensity. A closer examination shows that the ΔH of binding of cAMP to the first site remains favorable regardless of the nature of side chain substitution, with the exception of the D138L mutant, and remains rather constant in magnitude (Table 2). However, the magnitude of ΔH of cAMP to the second site changes quite significantly, increasingly unfavorable with side chains of high N-terminal capping propensity.

Fig. 9B shows the correlation between ΔS of cAMP binding and N-terminal capping propensity. Similar to the observed correlation in Fig. 4A, the ΔS associated with the binding of the first cAMP is rather constant (with the exception of Lys and Leu), whereas the magnitude of ΔS for binding of the second cAMP increases in positive value as the N-terminal capping propensity increases. All of the values of ΔS are positive; hence, the cAMP binding reactions are favored by increase in entropy of the system. ΔS is basically the driving force for the binding of the second cAMP molecule because the ΔH associated with that reaction is positive, as shown in Fig. 9 (top panel).

The thermodynamic data of cAMP binding exhibits a phenomenon of enthalpy-entropy compensation, as shown in Fig. 9 (bottom panel). The molecular events associated with such a pattern of compensation of thermodynamic parameters are usually associated with conformation changes. As the structures of apo and holo-CRP show, there is indeed a significant conformation change involving a rotation of the DNA-binding domains to position the DNA recognition helix to be exposed and interact with the DNA molecule, as shown in Fig. 1.

Our other thermodynamic and structural results provide further insight that is consistent with the thermodynamic data of cAMP binding. A good correlation can be shown between the change in solvent-accessible surface area induced by side chain substitutions and the energetics of subunit assembly. The differences in solvent-accessible area might be revealed by the m value, i.e., slope of ΔGd versus concentration of GdnHCl in the transition region of a chemical denaturation study (Table 2). This assumption is probably valid based on the literature showing that the greater the m value, the greater is the change in the solvent-accessible surface area upon unfolding of the cooperative unfolding unit (36–39). In the context of this study, the change in the solvent-accessible surface area upon unfolding is assumed to include interfacial interaction between subunits and domains.

In CRP mutants where dimer interface of the holo-form is weaker (i.e., in mutants with higher N-terminal capping propensity at position 138), subunit interface may be disrupted before the cAMP-binding domain begins to unfold, resulting in broader transitions and lower apparent unfolding cooperativity. In mutants with low N-terminal capping propensity at position 138, dimer interface is more stable (because the extended D-helix in the apo-form is part of the dimer interface, according to the structural study of Sharma et al. (4)), and subunit dissociation occurs at higher denaturant concentrations. In these cases, subunit dissociation and cAMP domain unfolding occur essentially simultaneously, resulting in higher apparent coop-
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erativity of unfolding resulting in higher apparent $m$ values. Consequently, a roughly linear correlation with a positive slope between $m$ value and the energetics of subunit interaction is observed.

The hydrodynamic properties of the 138 mutants as revealed by their Stokes radii are totally consistent with a model in which the mutations at residue 138 shifts the distribution of the ensemble of CRP between the apo and holo states of CRP. As shown in Table 2, the general trend, with the exception of Leu and Lys substitutions, is a correlation between side chains of lower propensity with larger Stokes radius, although the magnitude of change is small. The data imply that the hydrodynamic shape of CRP becomes more asymmetric when the equilibrium shifts toward the apo form, and the general trend of low propensity substitutions favors the apo form.

New insights on allosteric behavior of CRP—Replacement of side chain at residue 138 does perturb the global structural properties of CRP. The perturbations are subtle and are not obvious at the level of atomic structures; however, the perturbations are amplified in the thermodynamics of cAMP and DNA binding, e.g., the reaction might switch from exothermic to endothermic (Table 2). Nevertheless, the basic mechanism of CRP activity is not altered, although the specific magnitudes of the various linked multiequilibria can be changed significantly. Thus, the molecular mechanism of an allosteric system like CRP can only be revealed by a judicious combination of structure and thermodynamic dissection of the system. This study reveals the intricacy of the fine balance of a network of interactions, which can be modulated by a perturbation defined by the nature of the side chain, namely, the propensity of the side chain to form the N-terminal helix capping residue in this case. Hence, this study demonstrates the significant global effects on the functional energetics of CRP through a local perturbation of a secondary structural element.

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