Effect of Mutations at the Monomer-Monomer Interface of cAMP Receptor Protein on Specific DNA Binding

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Ying Shi, Shenglun Wang, Susan Krueger‡, and Frederick P. Schwarz§

From the Center for Advanced Research in Biotechnology, National Institute of Standards and Technology, Rockville, Maryland 20850

To determine the thermodynamic role of binding of an operon to cAMP receptor protein (CRP) in the activation of transcription, isothermal titration calorimetry measurements were performed on the binding of three 40-base pair DNA sequences to the cyclic nucleoside complexes of CRP and its mutants at 296 K. The three 40-base pair sequences consisted of a consensus DNA (conDNA) duplex derived from the CRP-binding site sequences of the operons activated by CRP and two DNA sequences based on the CRP-binding site sequences of the lac operon (lacDNA) and of the gal operon (galDNA). The mutants of CRP consisted of a T127L mutant, a S128A mutant, and a mutant containing both mutations (CRP*) which not only alter the transcriptional activity of the CRP complexes but also are involved in the monomer-monomer interfacial interactions of the CRP dimer. The binding reactions of the DNA duplexes to the fully cNMP-ligated CRP-mutant complexes were endothermic with binding constants as high as 6.6 ± 1.1 × 10^6 M^{-1} (conDNA-CRP(cAMP)_2). ConDNA binding to the unligated T127L and CRP* mutants was observed as well as conDNA and lacDNA binding to CRP with cAMP bound to only one monomer. The reduction of the binding constants with increase in KCl concentration indicated the formation of two ion pairs for the cAMP-ligated CRP and S128A complexes and four ion pairs for the cAMP-ligated T127L and CRP* complexes. Reduction of the DNA binding constants upon substitution of D_2O for H_2O in the buffer, the large heat capacity changes, and the enthalpy-entropy compensation exhibited by the binding reactions indicate the importance of dehydration in the binding reaction. Small angle neutron scattering measurements on the lacDNA-CRP(cAMP)_2 complex in D_2O/H_2O mixtures show that the DNA is bent around the cAMP-ligated protein in solution.

The transcription of over 20 different operons encoded for enzymes involved in carbohydrate metabolism in Escherichia coli is activated by the binding of 3',5'-cyclic adenosine monophosphate (cAMP) receptor protein (CRP)\(^1\) to the operon. CRP is a 45,000 g mol\(^{-1}\) dimer, consisting of a 3',5'-cyclic adenosine monophosphate (cAMP)-binding site in the amino-terminal domain and an α-helical DNA-binding region in the carboxyl-terminal domain of each monomer. Upon binding of cAMP to CRP, CRP undergoes a change to a conformation which binds specifically to the operon and results in binding of the operon around the RNA polymerase. The nature of this conformational change is not clear but may be induced by a stronger monomer-monomer interaction in CRP since this interaction is enhanced in the presence of cAMP (1). X-ray crystallographic studies of the CRP(cAMP)_2 complex show that the sequence at the monomer-monomer interface is similar to that of the leucine zipper motif and that a serine (Ser\(^125\)) from the other monomer crosses over the interface and forms a hydrogen bond with the cAMP (2). More recent x-ray crystallographic studies of the CRP(cAMP)_2 complexed with DNA (3, 4) show that the DNA is symmetrically bound across the α-helices of the two monomers of CRP. Mutations at the monomer-monomer interface of CRP alter the activity of CRP in the cell as is evident by a doubly mutated (T127L/S128A) CRP mutant (CRP*) which activates transcription in the absence of cAMP, a CRP (T127L) mutant which also activates transcription in the presence of an analog of cAMP, 3',5'-cyclic guanosine monophosphate (cGMP), and a CRP (S128A) mutant which does not activate transcription in vivo (5).

Since it has been shown from isothermal titration calorimetry (ITC) measurements that changes in the in vivo transcriptional activity arising from these interfacial mutations are not dependent on the binding affinity of the cyclic nucleotide monophosphate (cNMP) to the CRP-mutant (6), the transcriptional activity of cNMP-ligated CRP-mutants may depend on the influence of these interfacial mutations on the binding of the operon to CRP. It is, thus, important to determine the thermodynamics of DNA binding to CRP and its T127L, S128A, and CRP* mutants. Heretofore, indirect measurement techniques based on filter binding assays and shifts in gel electrophoresis bands have been employed to determine the operon binding affinities for CRP with often contradictory results. Equilibrium constants for binding of lac operon fragments to cAMP-ligated CRP range from 2.3 ± 0.1 × 10^9 M^{-1} for a 216-base pair fragment (7) to 8.4 × 10^10 M^{-1} for a 203-base pair fragment (8) at ambient temperatures. Since it has been shown that the CRP-binding site on the lac operon requires a minimum of 28 base pairs for full binding affinity (9), the binding of shorter lac operon fragments consisting of 40–41 base pairs was determined and, again, the binding constants ranged from 6 × 10^6 M^{-1} in gel electrophoresis measurements at 310 K (9) to 3.2 ± 0.1 × 10^8 M^{-1} from filter binding assays (10). In addition, the DNA binding constants determined from these indirect techniques exhibit a large decrease with increase in salt concentrations (11) in contrary to in vivo transcriptional assays which show high yields even at high salt concentrations (12).

To investigate the effect of monomeric interfacial mutations...
on specific DNA binding, ITC was employed to determine the binding constant ($K_d$) and the changes in enthalpy ($\Delta H$) and entropy ($\Delta S$) for the binding of DNA to cAMP, cGMP, and 3',5'-cyclic inosine monophosphate (cIMP)-ligated CRP, T127L$^\alpha$, S128A, and CRP$^\alpha$.

$$\text{DNA} + \text{CRP(cNMP)2} \leftrightarrow \text{DNA-CRP(cNMP)2} \quad (\text{Eq. 1})$$

in 50 mM potassium phosphate buffer containing 0.2 mM EDTA, 0.2 mM dithiothreitol, and 0.15 M KCl (KPB buffer). The DNA duplexes consisted of 40 base pairs with the following sequences,

\[
\begin{align*}
5'-' & GCAAGCGAATTAAGTGACATGACATTTAAGGCACCC-3' \\
3'-' & TTGGGTTATTTCAGTATAAGTCATGACTGTAATAAATCCGTTGCCC-5'
\end{align*}
\]

ConDNA

and

\[
\begin{align*}
5'-' & GCAAGCGAATTATAAGTGTCCTCAGCTATCTAGCCACC-3' \\
3'-' & TTGCTGTTATTTCAGTATAAGTCATGACTGTAATAAATCCGTTGCCC-5'
\end{align*}
\]

GalDNA

conDNA is a consensus sequence based on the sequences of the 20 operons while lacDNA is the CRP-binding site of the lac operon. The conDNA and lacDNA sequences are from Eibright et al. (10) and are identical from the 1 to 5' terminus and from 23 to the 3' terminus, using the numbering system from Eibright et al. (10). For comparison, galDNA was constructed so that bases from 1 to 21 are the same as the CRP-binding site of the gal operon (29). Although the DNA binding was determined at cyclic nucleoside concentrations where both sites of the CRP-mutants were occupied, the binding of conDNA and lacDNA to CRP was also investigated at subsaturate concentrations of cAMP where significant concentrations of singly occupied CRP, CRP(cAMP), as calculated from previous measurements (13), were present to determine if the DNA binds to CRP(cAMP). In addition, the binding reactions of lacDNA and conDNA with CRP(cAMP)$_2$ were investigated as a function of salt concentration and with D$_2$O substituted for water in the KPB buffer since dehydrosis has been shown to be involved in the binding reaction (7). A preliminary analysis of the binding of the lacDNA bound to the CRP(cAMP)$_2$ complex in water and D$_2$O was performed by small angle neutron scattering (SANS) since the neutron scattering from the DNA can be characterized separately from the protein in H$_2$O/D$_2$O mixtures of the buffer. The results of this study will contribute to a better understanding of the thermodynamics of DNA binding to proteins since binding of the DNA, the helical structure of the DNA-binding site, and the presence of a leucine zipper between the protein subunits are characteristic properties of other DNA-protein complexes.

**EXPERIMENTAL PROCEDURES**

**Materials**—The DNA sequences were synthesized by Oligos, Inc. and purified by high performance liquid chromatography. The DNA was analyzed by capillary electrophoresis and an analytical ion exchange column and found to be at a purity level ($\geq 90\%$). Gel electrophoresis of the single strands revealed essentially one intense band at 12,000 g mol$^{-1}$, the molecular mass of the strand. A procedure similar to that described by Eibright et al. (10) was followed to prepare the duplexes from the single strand DNA sequences. Briefly, milligram quantities of the lyophilized powdered DNA strands were dissolved in 4 ml of 10 mM Tris-HCl buffer containing 1 mM MgCl$_2$ and 0.5 mM NaCl at pH 7.5. Equal amounts of the DNA strand and its complementary strand were mixed together, heated up slowly to 368 K, held at 368 K for about 10 min, and then cooled very slowly at a rate of 10 K h$^{-1}$ to ambient temperature. Gel electrophoresis measurements on aliquots of the DNA solution showed the presence of only one band at the molecular mass of the duplex. The DNA duplex solutions were then concentrated up to 0.3–0.6 mg by centrifugation, dialyzed in the KPB buffer used for dialysis of the protein, and stored in a freezer at 253 K. Prior to the ITC measurements, the DNA duplex concentrations were determined from optical density measurements at 260 nm using the extinction coefficient of 1.32 $\times$ 10$^4$ M$^{-1}$ cm$^{-1}$ per base pair (8). The production from E. coli of CRP and mutants and their purification have been described previously (5) and their activities were checked by an in vitro transcription assay as described by Zhang et al. (14). The concentration of the CRP-mutants was determined from UV measurements at 280 nm using an extinction coefficient of 3.5 $\times$ 10$^{4}$ M$^{-1}$ cm$^{-1}$ (15). The potassium phosphate salts, KCl, Tris, MgCl$_2$, and sodium salts of cAMP, cGMP, and cIMP were reagent grade from Sigma. The dithiothreitol was Ultra-pure brand from Life Technologies, Inc. and the NaEDTA was from Serva Co. The D$_2$O was from Cambridge Isotope Laboratories.

**ITC Measurements**—All calorimetric titrations were performed according to the methods of Wiseman et al. (16) and Schwarz et al. (17) using a Microcal Omega titration calorimeter. The Omega titration calorimeter consists of a matched pair of sample and reference vessels (1.374 ml) containing the protein solution in the phosphate buffer and the buffer solution, respectively. Four to 10-ml aliquots of the DNA solution at concentrations $\times 10–20$ the protein concentration of 0.01–0.05 mM in the sample vessel were added 3–4 min apart. A separate titration of the DNA solution into the buffer was performed to determine any DNA heat of dilution which was then subtracted from the heats obtained during the titration of the DNA solution into the protein solution. A nonlinear least squares minimization performed by Microcal Origin scientific plotting software was used to fit the incremental heat of the ith titration ($\Delta Q(i)$) of the total heat, $Q_t$, to the total DNA titrant concentration, $nC_t$, according to the following equations (16, 18),

$$Q_t = nC_t \Delta H^i/V(1 + X/nC_t + 1/nK_tC_t) - [(1 + X/nC_t + 1/nK_tC_t)^2 - 4X/nC_t]^{1/2}/2 \quad (\text{Eq. 2})$$

where $n$ is the stoichiometry of the binding reaction, $C_t$ is the total protein concentration in the sample vessel, and $V$ is the vessel volume. Binding entropies, $\Delta S^b_t$, were calculated using the following equation of thermodynamics,

$$\Delta S^b_t = \langle \Delta H^i - \Delta G^i \rangle/T \quad (\text{Eq. 3})$$

where

$$\Delta G^b_t = -R \ln(K_t) \quad (\text{Eq. 5})$$

and $r = 8.31451$ J mol$^{-1}$ K$^{-1}$.

Two methods were employed to determine the dependence of DNA binding on the subsaturate concentration of cAMP in the solution. In one method, aliquots of cAMP in KPB buffer were titrated into CRP solutions containing the DNA duplex and the amount of heat exchanged was monitored as a function of the relative CRP(cAMP) and CRP(cAMP)$_2$ concentrations. Separate ITC runs with just CRP in the protein solution showed very little contribution of the heat of binding of cAMP to CRP under these titration conditions and any observed heat exchange would be from the binding of DNA to CRP(cAMP) and perhaps to CRP(cAMP)$_2$. In the second method, the amount of cAMP added to the CRP solution prior to the DNA titration was well below saturation so that the CRP contained about equal concentrations of CRP(cAMP) and CRP(cAMP)$_2$. In both methods, the concentrations of CRP(cAMP) and CRP(cAMP)$_2$ were determined by solving the following equations, where $K_1$ and $K_2$ are the macroscopic cAMP to CRP binding constants from Gorshkova et al. (13),

$$K_1 = [\text{CRP(cAMP)}]/[\text{CRP}][\text{cAMP}] \quad (\text{Eq. 6})$$

$$K_2 = [\text{CRP(cAMP)}]/[\text{CRP(cAMP)}][\text{cAMP}] \quad (\text{Eq. 7})$$

So that

$$[\text{CRP(cAMP)}] = K_1[C_c][\text{cAMP}]/P \quad (\text{Eq. 8})$$

and
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RESULTS

Thermodynamics of DNA Binding—Results of a typical ITC measurement consisting of titrating 5.0-μl aliquots of 0.250 mM conDNA into a solution of 0.020 mM CRP(cAMP)₆ complex at 295.6 K along with the binding isotherm are presented in Fig. 1. Prior to this titration, a 10 mM cAMP solution was first titrated into the 0.020 mM CRP solution to completely (99%) ligate the CRP with cAMP. The stoichiometry of the binding reaction as determined by the binding results was always close to one (0.90 ± 0.05). Similar results were obtained with the other cAMP-ligated CRP-mutant complexes and are summarized in Table I for all 12 complexes including binding of conDNA to unligated L127 and unligated CRP*. Binding of the conDNA duplex with unligated CRP and S128A was not observed. The thermodynamic quantities in Table I are average values of at least two ITC scans with different concentrations of ligand and protein and the average stoichiometry was again close to one. As shown in Table I, all the conDNA binding reactions are endothermic with binding enthalpies ranging from 84.1 ± 0.3 kJ mol⁻¹ for binding to CRP(cAMP)₆ to 166 ± 12 kJ mol⁻¹ for binding to L127(cGMP)₂ and binding constants...
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The uncertainties in the tables are standard uncertainties.

| CRP mutant | cNMP | $K$ | $K_b \times 10^{-6}$ | $-\Delta G_b^{0}$ | $\Delta H_b^{0}$ | $\Delta S_b^{0}$ |
|------------|------|-----|---------------------|------------------|----------------|----------------|
| CRP        | cAMP | 295.8 ± 0.2 | 66 ± 11 | 38.6 ± 0.4 | 84 ± 4 | 123 ± 5 |
|            | cGMP | 295.8 ± 0.1 | 3.9 ± 0.3 | 31.7 ± 0.2 | 165 ± 5 | 197 ± 5 |
|            | cIMP | 296.0 ± 0.1 | 2.8 ± 0.4 | 30.9 ± 0.3 | 155 ± 6 | 186 ± 6 |
|           | T127L | 296.7 ± 0.1 | 1.9 ± 0.6 | 30.0 ± 0.7 | 166 ± 12 | 196 ± 12 |
|            | cAMP | 296.0 ± 0.6 | 34 ± 5 | 37.0 ± 0.4 | 129 ± 9 | 167 ± 9 |
|            | cGMP | 297.2 ± 0.1 | 23 ± 8 | 36.2 ± 0.9 | 160 ± 5 | 196 ± 5 |
|            | cIMP | 295.7 ± 0.1 | 16 ± 2 | 35.1 ± 0.4 | 148 ± 5 | 183 ± 5 |
|           | S128A | 296.1 ± 0.6 | 12.3 ± 1.5 | 34.5 ± 0.3 | 160 ± 10 | 195 ± 10 |
|            | cAMP | 294.4 ± 0.1 | 0.81 ± 0.09 | 27.7 ± 0.3 | 122 ± 5 | 150 ± 5 |
|            | cGMP | 294.4 ± 0.1 | 1.2 ± 0.1 | 28.6 ± 0.3 | 121 ± 5 | 150 ± 5 |
|            | cIMP | 296.2 ± 0.1 | 1.6 ± 0.3 | 29.5 ± 0.5 | 147 ± 9 | 177 ± 9 |
|           | CRP* | 296.2 ± 0.1 | 10.5 ± 0.9 | 34.1 ± 0.2 | 120 ± 4 | 154 ± 4 |
|            | cAMP | 297.0 ± 0.7 | 2.5 ± 0.3 | 30.7 ± 0.3 | 133 ± 7 | 164 ± 7 |
|            | cGMP | 296.0 ± 0.8 | 8.8 ± 2.2 | 33.7 ± 0.6 | 121 ± 5 | 155 ± 5 |

...the heat capacity change of $-5.7 ± 1.6$ kJ mol$^{-1}$ K$^{-1}$ is the same as the heat capacity change observed for binding of conDNA to CRP*cAMP$^2$ ($-4.0 ± 0.4$ kJ mol$^{-1}$ K$^{-1}$). All the DNA binding reactions exhibited enthalpy-entropy compensation as shown in Fig. 5 where the slopes of the $T\Delta S_b^{0}$ versus $\Delta H_b^{0}$ plots are $0.95 ± 0.06$ for conDNA, $0.98 ± 0.06$ for lacDNA, and $0.98 ± 0.06$ for galDNA binding to CRP-mutant(cNMP)$^2$. A comparison of all the DNA binding constants is shown in Fig. 6 where it is observed that the naturally occurring sequences exhibit a greater range of values, i.e., specificity than the synthetic conDNA.

**Thermodynamics of DNA Binding to CRP as a Function of cAMP Concentration**—The results of titrating 0.435 mM cAMP in KPB into a solution containing 0.023 mM CRP and 0.023 mM conDNA in KPB are presented in the form of a binding isotherm in Fig. 7 along with the calculated concentrations of CRP(cAMP)$^2$ and CRP(cAMP)$^3$, the calculated heats absorbed by the CRP(cAMP)$^2$ alone, and the calculated heats absorbed by both CRP(cAMP) and CRP(cAMP)$^3$ using the binding constant and binding enthalpy values from Table I. Similar results were obtained with titrating the cAMP solution into a solution containing lacDNA instead of the conDNA. As shown in Fig. 7, at least 90% of the total amount of heat absorbed with each addition of the cAMP solution can only be attributed to DNA binding to both CRP(cAMP)$^2$ and CRP(cAMP)$^3$. An ICT titration of a 0.2 mM lacDNA solution into a 0.05 mM CRP solution containing 0.064 mM cAMP so that equal concentrations of CRP(cAMP)$^2$ and CRP(cAMP)$^3$ were present yielded a binding constant of $1.9 ± 0.4 \times 10^6$ liter mol$^{-1}$ and a binding enthalpy of $82 ± 4$ kJ mol$^{-1}$. These values are within two standard uncertainties of the corresponding averages of $4.0 ± 6.0 \times 10^5$ and $98 ± 4$ kJ mol$^{-1}$ for binding to the fully cAMP-ligated CRP (Table II). An ICT measurement under similar concentration conditions with conDNA yielded a binding constant of $6.3 ± 3.7 \times 10^5$ and a binding enthalpy of $88 ± 2$ kJ mol$^{-1}$ close to the results in Table I. The ICT results, thus, show that the DNA duplex can also bind to CRP(cAMP) with the same binding affinity and enthalpy as to the CRP(cAMP)$^2$ species. Equal binding affinities of specific DNA binding to both CRP- (cIMP)$^2$ and CRP(cIMP)$^3$ were also observed earlier by Takashashi et al. (23) in equilibrium dialysis measurements on specific DNA binding to CRP as a function of cAMP concentration.

Solvent Effects on the Thermodynamics of DNA Binding—Since DNA binding to CRP involves electrostatic complementarity (24), ICT measurements were performed on the binding of conDNA to CRP mutants as a function of KCl concentration from 0.15 to 0.60 M and are summarized in Table III. A typical ICT scan of titrating 5.0 μl of 0.71 mM conDNA into 0.12 mM CRP(cAMP)$^2$ at 0.5 M KCl in KPB buffer is shown in Fig. 8. An increase in the KCl concentration reduces the binding constant through an increase in the endothermic binding enthalpy and...
only a partial compensating increase in the binding entropy. The decrease in the binding constant could result from competitive anion binding to the DNA-binding site on CRP or electrostatic screening of the ion pair interactions between the DNA bases and the CRP amino acid residues. For B-DNA binding to proteins based solely on ion pair interactions, it has been shown (25) that,

\[ \log K_b \approx 0.88 \times m \log [KCl] \] (Eq. 13)

where \( K_b \) is the binding constant at 1.0 M KCl and \( m \) is the number of CRP-mutant/DNA ion pairs. The results in Table III yield a value of 1.76 ± 0.20 for conDNA binding to CRP(cAMP)\(_2\), close to 2 ion pair interactions between CRP-(cAMP)\(_2\) and conDNA. For conDNA binding to the other cNMP-ligated CRP mutants, \( m \) can be estimated from

\[ m = 2 \times \log(K_b(0.15 M KCl)/K_b(0.5 M KCl)) \]

which yields close to 4 ion pairs for T127L(cAMP)\(_2\), T127L(cGMP)\(_2\), and CRP*(cAMP)\(_2\) complexes and again close to 2 ion pairs for the S128A(cAMP)\(_2\) complex. The number of ion pairs of 2 for conDNA binding to CRP(cAMP)\(_2\) is not in agreement with the number of 8 ion pairs determined from the filter binding assays (11). According to Equation 13, 8 ion pairs would result in a reduction of the conDNA binding constant by a factor of 4.8 × 10\(^3\) from 0.15 to 0.50 M KCl in the KPB buffer, which is clearly not observed by comparing Figs. 1 and 8. In addition, CRP is able to function in osmotically stressed cells grown at high salt concentrations (12) and this would not be the case if the DNA binding constant is reduced by more than 3 orders of magnitude. Thus, the lower number of ion pairs determined from the ITC measurements is more consistent with the observations from the osmotically stressed cells. The doubling of the number of ion pairs from 2 to 4 formed between conDNA and the T127L(cNMP)\(_2\) and CRP*(cAMP)\(_2\) complexes reflects some alteration of the topography of the DNA-binding sites in cNMP-ligated T127L and CRP* complexes.

Since dehydration has been shown to play an important role in DNA binding to CRP (7), ITC measurements on the binding of DNA to CRP(cAMP)\(_2\) were performed in D\(_2\)O-buffered solutions. Substitution of H\(_2\)O by D\(_2\)O in the buffer results in a reduction in the binding constant for both the conDNA and lacDNA duplexes due to an increase in the endothermicity of the binding reaction as shown in Table IV. The same results were obtained after the solutions were left standing for 24 h prior to the ITC measurement, indicating that the deuterium had completed any exchange with the hydrogen on the DNA duplex and on the protein surface.
nent of the complex was $23 \pm 1$ angstroms in agreement with recent SANS measurements on CRP(cAMP)$_2$ (20). The radius of gyration for the lacDNA duplex in the complex was $32 \pm 1$ angstroms which is close to the value of 29.7 angstroms calculated for the DNA component in the x-ray crystal structure of the DNA-CRP(cAMP)$_2$ complex (3). The distance distribution function, $P(r)$ (20) for the lacDNA component of the lacDNA-CRP(cAMP)$_2$ complex in the $0.085 \text{mM}$ solution is shown in Fig. 9 along with the distance distribution function of the DNA component of the x-ray crystal structure. The shape of the two curves are similar in that they both contain a maximum in distance distribution function at a radius around 20 angstroms, distance distribution function decreases slowly to a shoulder around 60 angstroms, and then distance distribution function decreases rapidly to 0–95 angstroms. This last rapid decrease occurs when “rigid” rods are bent into a “U” shape, thereby eliminating the larger distances in the distribution function. However, the lacDNA in the lacDNA-CRP(cAMP)$_2$ complex appears to exhibit a broader distribution around the maximum than the DNA in the DNA-CRP(cAMP)$_2$ crystal structure. This is probably due to a globular component in the distance distribution function from aggregates of the complex in the 70 and 100% (v/v) D$_2$O-buffered solutions. It can be concluded that the SANS data show that the DNA in the lacDNA-CRP(cAMP)$_2$ complex is bent, independent of the concentration of D$_2$O in the sample.
DISCUSSION

As observed for other protein-DNA associations (26, 27), the specific binding of DNA to cNMP-ligated CRP-mutant complexes is endothermic, thus, entropically driven, and involves dehydration. The binding enthalpy can be attributed to endothermic bending of the DNA ($\Delta H_{bn}^{0}$) and to the formation of ionic pair interactions, van der Waals interactions, and hydrogen bonding interactions between the amino acid residues at the CRP-binding site and the DNA bases (direct interactions) and the ribose-phosphate backbone (indirect interactions) all of which would be exothermic ($\Delta H_{bx}^{0}$).

$$\Delta H_{bn}^{0} = \Delta H_{bx}^{0} + \Delta H_{bs}^{0}$$  \hspace{1cm} (Eq. 14)

Contributions to $\Delta H_{bn}^{0}$ would principally arise from the energy absorbed in the bending of the DNA duplex about 90° around the CRP and would expected to be the same for the different DNA sequences bending around the CRP(cAMP)$_2$ complex. Differences in the direct and indirect interactions based on the x-ray crystallographic structure of the DNA-CRP(cAMP)$_2$ complex by Parkinson et al. (4) are itemized in Table V. Since the number of A, T, G, and C bases is the same for conDNA, lacDNA, and galDNA, it can be assumed that the interaction between the isolated DNA duplexes and water is approximately the same for all three DNA sequences and, thus, differences in the intermolecular binding interactions can be conceptualized in terms of differences between the CRP-binding site sequences of the DNA duplexes.

Attacks have been made to correlate the binding affinities ($-\Delta G_s^{0}$) of the catabolite operons with the 22-base pair sequence of the CRP-binding site in the operon (28, 29). In all the catabolite operons, the 4 TGTGA sequence is conserved and there is a center of pseudo 2-fold symmetry between bases 11 and 12 (28). ConDNA with the highest binding affinity exhibits perfect 2-fold symmetry and this 2-fold symmetry is reduced in the more naturally occurring lacDNA and galDNA duplexes which also exhibit weaker binding affinities. By determining
the frequency a specific nucleotide appeared in each of the 1–22 bases of the CRP-binding site in 26 operons, Berg and von Hippel (29) derived a quantitative model predicting the binding affinity of an operon from the difference between its CRP-
(cAMP)$_2$ species alone in solution (●), and the calculated total heat absorbed by both species employing the binding enthalpy and constant from Table I (●). The greater the deviation of each half of the 22-base sequence from the perfect 2-fold sequence of conDNA, i.e., the lower the symmetry, the weaker the CRP binding affinity. However, their model predicted a weaker binding affinity for galDNA than for lacDNA, contrary to the ITC results. In addition to a correlation between sequence and binding affinity, changes in the relative number of direct and indirect interactions between the sequences, as determined from the known crystal structures of the DNA-
(CRP(cAMP)$_2$ complexes (3, 4), would be also expected to contribute to changes in the binding affinities. As shown in Table V, the number of base changes between conDNA and lacDNA or galDNA are seven and more of the base changes in galDNA are involved in direct interactions with CRP, while more of the base changes in lacDNA are involved in indirect interactions. The lower binding affinity for lacDNA could be accounted for by the change in more of the indirect interactions between the phosphate backbone. This implies that the indirect interactions contribute more than the direct interactions to the binding affinity.

Table III

Comparision of thermodynamic quantities of conDNA binding to cNMP-ligated CRP complexes in buffer at different levels of KCl concentration

| CRP mutant | cNMP | KCl concentration | $T$ | $K_m \times 10^{-6}$ | $-\Delta H_m^0$ | $\Delta S_m^0$ | $T \Delta S_m^0$ |
|------------|------|-------------------|-----|---------------------|----------------|----------------|----------------|
| CRP        | cAMP | 0.15              | 295.8 ± 0.2 | 6.6 ± 1.1 | 38.6 ± 0.4 | 84 ± 3 | 123 ± 3  |
|            | cAMP | 0.25              | 295.9 ± 0.1 | 3.5 ± 0.4 | 37.0 ± 0.3 | 77 ± 3 | 114 ± 3  |
|            | cAMP | 0.32              | 296.9 ± 0.1 | 3.3 ± 0.5 | 36.9 ± 0.3 | 73 ± 3 | 110 ± 3  |
|            | cAMP | 0.42              | 296.5 ± 0.1 | 1.8 ± 0.3 | 35.5 ± 0.1 | 62 ± 2 | 98 ± 2   |
|            | cAMP | 0.52              | 296.3 ± 0.1 | 0.9 ± 0.1 | 33.8 ± 0.3 | 63 ± 2 | 94 ± 2   |
|            | cAMP | 0.60              | 296.2 ± 0.1 | 0.8 ± 0.2 | 33.5 ± 0.3 | 73 ± 3 | 107 ± 3  |
| T127L      | cAMP | 0.15              | 296.0 ± 0.6 | 3.4 ± 0.5 | 37.0 ± 0.4 | 129 ± 9 | 167 ± 9  |
|            | cAMP | 0.50              | 297.5 ± 0.1 | 0.07 ± 0.01 | 27.6 ± 0.4 | 83 ± 4 | 111 ± 4  |
|            | cGMP | 0.15              | 297.2 ± 0.1 | 2.3 ± 0.8 | 36.2 ± 0.9 | 160 ± 5 | 196 ± 5  |
|            | cGMP | 0.50              | 295.8 ± 0.1 | 0.031 ± 0.008 | 25.4 ± 0.6 | 87 ± 12 | 112 ± 12 |
| S128A      | cAMP | 0.15              | 296.1 ± 0.6 | 1.2 ± 0.2 | 34.5 ± 0.3 | 160 ± 10 | 195 ± 10 |
|            | cAMP | 0.50              | 297.2 ± 0.1 | 0.11 ± 0.03 | 28.7 ± 0.8 | 75 ± 4  | 104 ± 4  |
|            | CRP* | 0.15              | 296.2 ± 0.1 | 1.05 ± 0.09 | 34.1 ± 0.2 | 120 ± 4 | 154 ± 4  |
|            | cAMP | 0.50              | 295.9 ± 0.1 | 0.021 ± 0.004 | 24.5 ± 0.5 | 177 ± 46 | 202 ± 46 |

FIG. 7 a, the total heat absorbed upon the addition of 5-µl aliquots of 0.435 mM cAMP to a solution containing 0.023 mM CRP and 0.023 mM conDNA (●), the calculated total heat absorbed by the CRP-
(cAMP)$_2$ species alone in solution (●), and the calculated total heat absorbed by both species employing the binding enthalpy and constant from Table I (●). b, the calculated concentrations of CRP(cAMP) (●) and CRP(cAMP)$_2$ (●) generated by the addition of 5-µl aliquots of 0.435 mM cAMP solution in the ITC scan described in a.
from differences in the topography of the DNA-binding site of CRP. Changes in topography between the two T127L mutants and CRP are evident in the KCl results which show that two additional ion pairs are formed between the conDNA and the cAMP-ligated T127L and CRP complexes. Recent SANS measurements (20) on unligated CRP and cAMP-ligated CRP showed that CRP changes from an “open” form, where the DNA binding $\alpha$-helices of the carboxyl-terminal are swung away from the cAMP binding amino-terminal domain, to a “closed” form, where the $\alpha$-helices are swung in toward the amino-terminal domain, and this is the conformation observed in the x-ray crystallographic structure of DNA CRP(cAMP)$^2$ complexes (3, 4). Since cAMP enhances the monomer-monomer interaction in CRP (1), the closed form must be favored by a stronger interaction between the monomers in CRP. The amino acid sequence along the monomer-monomer helical interface exhibits an almost perfect leucine zipper motif consisting of a heptad repeat of leucine residues along one face of the helix with the exception of a Met$^{120}$ and Thr$^{127}$ (6). In the T127L mutant, Thr$^{127}$ is replaced by Leu, thereby creating a more perfect leucine zipper which would lead to stronger association between the monomeric units as is observed in other leucine zipper proteins (30). Hence, the stronger association in the T127L and CRP* mutants would alter the topography of the DNA-binding site more toward the closed form. The tendency toward the closed form in CRP containing the T127L CRP mutation is further substantiated by SANS measurements (20) which show, unlike CRP, little structural difference between the unligated and cAMP-ligated forms of CRP*.

According to a structural model for DNA binding proposed by Garges and Adhya (31), the closed form is more favorably disposed toward DNA binding and, thus, conDNA binds to the unligated T127L and CRP*. The difference in $\Delta G_0$ of $-30$ kJ mol$^{-1}$ for conDNA binding to T127L and CRP* is about 80% of the average $\Delta G_0$ of $-37.8$ kJ mol$^{-1}$ for conDNA bonding to the CRP(cAMP)$^2$ and L127(cAMP)$^2$ complexes. This “tightness” of the monomer-monomer interaction in T127L and CRP* can account for contributing at least 80% to the DNA binding affinity. Interestingly, the S128A mutation in S128A and CRP eliminates the hydrogen bond to cAMP formed in the crossover of the Ser$^{128}$ residue from the other monomer, thereby reducing the monomer-monomer interaction. This reduction results in a decrease of the DNA binding affinity in S128A and CRP* by about 4 kJ mol$^{-1}$. Finally, DNA can also bind to the singly cAMP occupied CRP and, thus only one cAMP is enough to strengthen the monomer-monomer association in CRP, perhaps through formation of the Ser$^{128}$-cAMP hydrogen bond.

The binding entropy can be attributed to entropy changes arising from hydrophobic interactions (dehydration) ($\Delta S_{\text{water}}^0$), and the conformational change in the DNA ($\Delta S_{\text{DNA}}^0$), as well as smaller conformational changes in the DNA-binding site. Following the treatment of Spolar and Record (22), at a temperature ($T$) where the binding entropy is zero then,
the entropy loss due to the loss of rotational and translational degrees of freedom upon binding is the same for protein-ligand interactions, \( \Delta S_{\text{brt}} \), and the entropy change due to the number of ion pairs formed \( (z = 2) \) and the salt concentration \([KCl] = 0.15 \text{ mol liter}^{-1}\), \( \Delta S_{\text{be}} \), is \(-0.88 zR \ln [KCl] = 27 \text{ J mol}^{-1} \text{ K}^{-1}\). The change in the solvent accessible surface area of the nonpolar residues is \(-626 \text{ (angstroms)}\) and, thus, \( \Delta S_{\text{bb}} \approx -852 \text{ (angstroms)} \). Therefore, \( \Delta G_0 = -852 \text{ (angstroms)} \) \( \text{J mol}^{-1} \text{ K}^{-1}\).

Endothermicity of the binding reaction. The increase in binding enthalpy of \(43 \pm 2 \text{ kJ mol}^{-1}\) for conDNA and \(34 \pm 9 \text{ kJ mol}^{-1}\) for lacDNA arises from the enhanced stabilization of the DNA and CRP in D_{2}O relative to the DNA-CRP(cAMP)_{2} complex in D_{2}O. If \(-8.8 \text{ J mol}^{-1} \text{ (angstrom)}^{-1}\) accompanies the transfer of nonpolar amino acid groups from H_{2}O to D_{2}O (32, 33), then the enhanced stabilization of the nonpolar binding surface area \(626 \text{ (angstroms)}^2\) on the protein alone in D_{2}O contributes about 5.5 kJ mol\(^{-1}\) to the difference between the binding enthalpies in D_{2}O and H_{2}O. Apparently, as shown in the entropy calculations, the larger buried polar surface area \(2008 \text{ (angstroms)}^2\) on the protein of CRP must also contribute to the enhanced interaction between the protein and solvent in D_{2}O. Also, if the effect of D_{2}O on the solvent-reactant interaction involved solely solvent reorganization, the binding constant would be unaffected (34) because of enthalpy-entropy compensation. The release of water upon complex formation is also evident in the enthalpy-entropy compensation exhibited by the binding reaction at ambient temperatures and by the temperature dependence of the DNA-binding reaction where the large negative heat capacity changes minimize changes in \( \Delta G_0 \) as the temperature is increased from 288 K to 303 K.

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