The thermodynamics of the binding of cyclic adenosine monophosphate (cAMP) and its non-functional analog, cyclic guanosine monophosphate (cGMP), to cyclic AMP receptor protein (CRP) and its T127L mutant were investigated by isothermal titration calorimetry (ITC) in 0.2 and 0.5 M KCl phosphate buffer (pH 7.0) at 24 and 39 °C. Although, the binding of the first cAMP molecule to CRP is exothermic with an enthalpy change (ΔH\text{C}m) of −6 kJ mol\(^{-1}\), a heat capacity change (ΔC\text{p}) of −0.300 kJ mol\(^{-1}\) K\(^{-1}\), and an entropy increase (ΔS\text{C}) of 72 J mol\(^{-1}\) K\(^{-1}\), the overall binding of cAMP to CRP is endothermic and positively cooperative: binding of the first cAMP molecule increases the affinity for the second one by more than an order of magnitude at 24 °C. The binding of the second cAMP molecule is accompanied by large changes of 48.1 kJ mol\(^{-1}\) in ΔH\text{C}m of −1.4 kJ mol\(^{-1}\) K\(^{-1}\) in ΔC\text{p}, and of 255 J mol\(^{-1}\) K\(^{-1}\) in ΔS\text{C}p at 24 °C and 0.5 M KCl phosphate buffer. In contrast, the overall binding of cGMP to CRP is exothermic and non-cooperative with ΔH\text{C}m = ΔC\text{p} = ΔS\text{C}p values close to the those values for binding of the first cAMP molecule to CRP. The point mutation, T127L, switches off the cooperativity between the cAMP ligated binding sites without affecting the binding constant of cAMP and changes the specificity of the protein so that transcription is now activated only upon cGMP binding. All the binding reactions to CRP and the mutant are mainly entropy driven at 24 °C.

The cyclic AMP receptor protein (CRP)\(^1\) from Escherichia coli is a global regulatory transduction protein. In the presence of cAMP, CRP undergoes a conformational change and binds to specific DNA sequences in the regulatory regions of a number of operons to activate or repress transcription (de Crombrugghe et al., 1984; Garges and Adhya, 1985). The global alteration of the CRP structure upon cAMP binding is evident from small angle x-ray scattering measurements (Kumar et al., 1980), analytical gel chromatography (Heyduk et al., 1992), proteolysis digestion studies (Ebright et al., 1985; Moore, 1993), and differential scanning calorimetry (DSC) where DSC scans of CRP exhibit one unfolding transition for CRP and three unfolding transitions for cAMP ligated CRP (Ghosaini et al., 1988). CRP exists as a dimer (molecular mass = 45 kDa) of two identical subunits with one cyclic adenosine monophosphate (cAMP) binding site per subunit. The x-ray structure of cAMP ligated CRP refined to 2.5 Å showed each subunit to be composed of two domains: the carboxyl-terminal domain which binds to the DNA through a helix-turn-helix motif and a larger amino-terminal domain which binds to cAMP (Weber and Stetzel, 1987). More recently, Schultz et al. (1991) determined to 3 Å resolution the structure of the CRP-cAMP\(_2\)DNA complex which showed that the fully ligated CRP dimer interacts directly with 27 of 30 base pairs of duplex DNA. According to the x-ray structure of the cAMP ligated CRP, hydrogen bonds exist between the ribose and phosphate groups of cAMP and the Glu-72, Ser-83, and Arg-82 amino acid residues of CRP, and between the N-6 amino group of the purine ring of cAMP and T127 of the same subunit and Ser-128 of the other subunit. The hydrogen bonds between the cyclic nucleotide and Trp-127 and Ser-128 are particularly important since they, potentially, determine the ligand specificity and participate in intersubunit communication. Biochemical and genetic studies support this model (Moore, 1993; Moore et al., 1992; Ebright et al., 1985).

Cyclic guanosine monophosphate (cGMP), a structural analog of cAMP, also binds to CRP but does not activate specific DNA binding (Ebright et al., 1985). Eilen and Krakow (1977) showed that both cAMP and cGMP, although to a lesser extent, protect CRP against chemical modification by thiol reagents. More recently, Heyduk et al. (1992) showed that cGMP induced a conformational change in CRP similar to that induced by cAMP. However, DSC scans of cGMP ligated CRP can be resolved into only two unfolding transitions (Ghosaini et al., 1988), implying that the conformation of cGMP saturated CRP was different than that of cAMP saturated CRP.

The binding of cGMP to the T127L mutant initiates activation of transcription at approximately 35% of the activity of the cAMP ligated CRP and the repression of transcription by the T127L mutant is dependent on cGMP rather than cAMP (Moore, 1993). Although digestion of CRP by subtilisin occurs only in the presence of cAMP, a similar digestion pattern of the T127L mutant is evident in the presence of cGMP. However, the digestion pattern for the cAMP ligated mutant is different (Moore, 1993). Thus, a comparison of the binding properties of CRP and T127L with cAMP and cGMP would help in understanding the mechanism of events which proceed specific DNA binding and the activation of transcription.

Previous investigations of the thermodynamics of the interaction of CRP with cAMP and cGMP showed that the binding exhibited negative cooperativity at low ionic strength of less than 0.4 M (Takahashi et al., 1980) and less than 0.1 M (Heyduk and Lee, 1989). However, Takahashi et al. (1980) observed positive cooperativity at an ionic strength >0.4 M and at low ionic strength in the presence of DNA. Takahashi et al. (1980) also reported that the binding constants for cGMP binding to CRP were very similar to those of cAMP and estimated a...
binding enthalpy of -6.0 kJ mol\(^{-1}\) for cAMP from the dependence of the binding constant on temperature. Fried and Crichton (1984) showed that at low ionic strength, the transfer of cAMP from the specific binding site on DNA to a nonspecific site is accompanied by the net release of one cAMP molecule. Later, Heyduk and Lee (1989) suggested from CRP-specific DNA binding studies in the presence of cAMP that the interaction of CRP with DNA is accompanied by the net release of one cAMP molecule.

In this investigation, the cooperativity and thermodynamics of the binding reactions of cAMP and cGMP to CRP and the T127L mutant are determined in terms of the thermodynamic parameters of the binding constants (K\(_b\)) and changes in the free energy (\(\Delta G^\circ\)), enthalpy (\(\Delta H^\circ\)), entropy (\(\Delta S^\circ\)), and heat capacity (\(\Delta C_P\)), for the following reactions,

\[
\text{CRP} + \text{cAMP} \leftrightarrow \text{CRP} - \text{cAMP} \tag{Eq. 1}
\]

and

\[
K_b = [\text{CRP} \cdot \text{cAMP}] / [\text{CRP}][\text{cAMP}] \tag{Eq. 2}
\]

and

\[
\text{CRP} - \text{cAMP} + \text{cAMP} \leftrightarrow \text{CRP} - 2 \cdot \text{cAMP} \tag{Eq. 3}
\]

where

\[
K_{2b} = [\text{CRP} \cdot \text{cAMP}] / [\text{CRP} \cdot \text{cAMP}] \tag{Eq. 4}
\]

These thermodynamic parameters are determined as a function of ionic strength and temperature from isothermal titration calorimetry (ITC) measurements. It is shown that when considering the complete thermodynamics of the binding reactions, there are, indeed, explicit differences in the interaction of cAMP and cGMP with CRP.

**EXPERIMENTAL PROCEDURES**

Materials—The characterization and mutagenesis of the wild type and mutant of CRP has been described previously by Moore (1993). A New Brunswick Fermentor Scientific, Inc. SF116 was also used in place of the 5-liter shaker flasks for preparations of CRP and the T127L mutant. The protein solutions were dialyzed at 4°C in 50 mM potassium phosphate-potassium hydroxide buffer (pH 7.0) containing 0.2 mM sodium EDTA, 0.2 mM dithiothreitol, and 5% glycerol (phosphate buffer) with either 0.2 or 0.5 M KCl with two changes in the buffer solution. A Pharmacia Phast SDS electrophoresis gel with 0.1 M Tris-HCl buffer performed by comparing the optical densities at 562 nm of samples of the solution to those of bovine serum albumin solutions after adding 4% bromophenol blue, and KCl were reagent grade from Sigma. The concentrations of the CRP and the mutant were determined by UV absorption spectroscopy using an extinction coefficient of 3.5 \(10^4\) cm M\(^{-1}\) at 280 nm (Ghosaini et al., 1988). A DSC scan from 25 to 100°C of the protein solutions performed with a Hart 7707 DSC heat conduction scanning microcalorimeter exhibited only one thermal transition at 65°C for the CRP in agreement with Ghosaini et al. (1988) and Schwarz (1991). Briefly, the titration calorimeter consists of a 1.374-mL cell containing the protein solution and a matched reference cell containing the dicyanide in an adiabatic enclosure. Aliquots of the ligand solution are added via a rotating stirrer-syringe operated with a plunger driven by a stepping motor. The heat absorbed or released accompanying the addition of aliquots of the ligand solution to the protein solution is measured by a thermocouple sensor between the cells. Samples were centrifuged prior to the titration and were carefully scrutinized for precipitant after the addition of titrant, first, a single site data fitting model was used with a site concentration = 2 \(\times\) CRP concentration. The total heat content, \(Q_t\), is related to the total ligand concentration, \(L_1\), via the following equation (Wiseman et al., 1989),

\[
Q_t = n[\text{CRP}] \cdot H_{b1} V (1 + [L_1] / n[\text{CRP}]) + nK_b (1 + [L_1] / [\text{CRP}])^{-1} 
\]

\[
- ([1 + [L_1] / n[\text{CRP}] + 1K_b (1 + L_1)]/2 - 4[L_1] / n[\text{CRP}])^{1/2} 
\]

where \(n\) is the stoichiometry, \(K_b\) is an intrinsic binding constant, \(H_{b1}\) is an intrinsic heat of binding, \([\text{CRP}]\) is the total site concentration, and \(V\) is the cell volume. The expression for the heat released per the ith injection, \(Q(i)\), is then (Yang, 1990),

\[
Q(i) = Q(i) + dV / 2Q(i) (Q(i) - Q(i - 1)) - Q(i - 1) \tag{Eq. 6}
\]

where \(dV\) is the volume of titrant added to the solution.

Each titration calorimetry scan yields values for \(n\), \(H_{b1}\), and since the site concentrations is used, the intrinsic binding constant, \(K_b\). These values were then used in the two identical interactive site fitting model described below where the concentration of sites is replaced by the concentration of protein.

For ligand binding to two sites (Yang, 1990), the fraction of protein bound with one ligand, \(F_1\), where \([L_1]\) is the free ligand concentration is from Equations 1-3,

\[
F_1 = K_{2b}[L_1] + K_{2b}[K_{2b}[L_1]^2] \tag{Eq. 7}
\]

and the fraction of protein bound with two ligands, \(F_2\), is,

\[
F_2 = K_{2b}[K_{2b}[L_1]^2 + K_{2b}[K_{2b}[L_1]^2] \tag{Eq. 8}
\]

so that Equation 5 becomes

\[
Q_t = [\text{CRP}] / [V(F_1 H_{b1} + F_2 H_{b2} + H_{b1} + H_{b2})] \tag{Eq. 9}
\]

Then the heat released per addition of titrant is fitted to Equation 6 using the four parameters \(K_{b1}, K_b, H_{b1}\), and \(H_{b2}\), where \(H_{b1}\) are the enthalpy changes of binding for the two binding reactions (Equations 1-3). If the sites do not interact, the binding constants are related to the intrinsic binding constant, \(K_{b1}\, in\, Equation\, 5\, as\, follows;\n
\[
K_{b1} = 2K_b \tag{Eq. 10}
\]

If the sites interact, the coefficient of cooperativity, \(\alpha\), is,

\[
\alpha = 2K_{b2} / K_b \tag{Eq. 11}
\]

and the energy of interaction, \(\Delta G(\alpha)\), is,

\[
\Delta G(\alpha) = -RT\ln(\alpha) \tag{Eq. 12}
\]

For the titration scans that exhibited a maximum in the peak areas after several additions of ligand solution which may be indicative of a cooperative binding mechanism, the data could only be fitted to Equation 6 using Q, from Equation 9. To facilitate the fitting of the interaction two-site model to the titration data, the initial fitting parameters of \(K_{b1}\) and \(H_{b1}\) were chosen to be the same as for binding of cGMP to CRP.

Values for \(\Delta G^\circ\) and \(\Delta S^\circ\) are determined from the fundamental equation of thermodynamics.
where $R = 8.315 \text{ J mol}^{-1} \text{K}^{-1}$ and $T$ is the absolute temperature. The heat capacity changes, $\Delta C_{p_1}$, were determined from a linear of $\Delta H_{p_1}$ vs $T$. Values of the binding constants at 39.5°C were also determined using the $\Delta H_{p_1}$ and $K_{a}$ values at 24.0°C ($T_0$) and the $\Delta C_{p_1}$ values using the van't Hoff equation.

\[
\ln(K_{a}/T)/K_{a}T_0 = [-\Delta H_{p_1}(T_0) + \Delta C_{p_1}T_0][1/T_0 - 1/T]R
\]

**(RESULTS)**

The cGMP binding results are presented first since they were used to analyze the more complex cAMP binding data. A typical calorimetric titration consisting of adding cGMP to CRP at 24°C and 0.5 mM KCl is shown in Fig. 1a and a plot of $\Delta Q(t)$ versus the ratio of $[L]_i/[CRP]_i$ in Fig. 1b. A least squares fit of the data to Equation 6 using $Q_i$ determined from Equation 5 and the site concentration is shown by the solid curve in Fig. 1b. An identical fit was obtained using $Q_i$ determined from Equation 9 and the protein concentration, indicating that the two binding sites do not interact. The thermodynamic parameters for the cGMP-CRP binding reactions are presented in Table I. Each parameter for all the binding reactions in Table I is an average determined from at least two different titration runs with different ligand and protein concentrations. Both cGMP-CRP binding reactions (Equations 1-3) are exothermic and are mainly driven by the increase in entropy. Agreement between the binding enthalpy and entropy at 0.2 and 0.5 mM KCl ionic strength indicates that the binding reaction is independent of ionic strength over this range of KCl concentration. For cGMP, the binding enthalpy decreases with increase in temperature and the heat capacity changes, $\Delta C_{p_2}$, are $-0.300 \pm 0.015 \text{ kJ mol}^{-1} \text{K}^{-1}$.

A typical calorimetric titration of adding cAMP to CRP is

![FIG. 1.](image)
shown in Fig. 2a along with its ΔQ(i) versus [Lj]/[CRP] plot in Fig. 2b. Contrary to the binding of cGMP to CRP, the cAMP-CRP binding reaction is endothermic and appears to be cooperative. The ITC data could only be fitted to the interacting two-site model described by Equation 9 as shown by the ΔQ(i) versus [Lj]/[CRP], in Fig. 2b. The thermodynamic parameters determined from these fits are presented in Table I. The first binding reaction of cAMP to CRP is exothermic and very similar to that for cGMP binding to CRP, and, however, twice the intrinsic binding constant as shown in Table I. As in the case of cGMP binding, the intrinsic binding constant is independent of ionic strength from 0.2 to 0.5 M KCl. In contrast to the binding of cGMP to CRP, the second cAMP binding reaction is strongly endothermic, resulting in the overall endothermic nature of the total two-step binding reaction. Both binding reactions are mainly entropically driven with a much greater ΔS° than ΔH°. The binding of the first cAMP molecule increases the affinity of CRP for the second cAMP molecule by more than an order of magnitude at 0.2 M KCl and 24 °C, yielding a cooperativity parameter, of 11.7 (Table II). Similar results were obtained at pH 8.0 with Tris as a buffer, conditions similar to those of Takahashi et al. (1980). This cooperativity parameter is reduced to 4.8, at 0.5 M KCl ionic strength but does not depend on temperature from 24 to 39 °C. The binding enthalpy for both binding reactions decreases with increase in temperature. The heat capacity change for the first cAMP binding reaction to CRP, ΔCp1, is close to that for cGMP binding to CRP, while the heat capacity change for the second cAMP binding reaction to CRP, ΔCp2, is at least a factor of four more negative (Table II).

ITC data of the cyclic nucleotide binding reactions to the T127L mutant are shown in Fig. 3 along with the corresponding fits, indicated by the solid curves, of Equation 5 for Q, and the site concentration. Identical fits were obtained using Equation 9 for Q, and the protein concentration, indicating that the two binding sites are independent. The thermodynamic parameters for the cAMP and cGMP binding reactions to the mutant are presented in Tables I and II. All the binding reactions to the mutant are exothermic, non-cooperative, and mainly entropically driven. The substitution of Thr-127 by leucine alters the interaction of cAMP with CRP. In spite of little change in Kd and the other thermodynamic parameters of binding of the first cAMP molecule to CRP, this point mutation completely switches off the cooperativity of the cAMP-CRP binding reaction. For both cAMP-mutant binding reactions, the binding enthalpy decreases with increase in temperature yielding a heat capacity change of ΔCp1 = ΔCp2 = -0.47 ± 0.15 kJ mol⁻¹ K⁻¹, only slightly more negative than for binding of the first cAMP molecule to CRP. However, the heat capacity changes accompanying the cGMP binding reactions are almost two times more negative, ΔCp1 = ΔCp2 = -0.76 ± 0.12 kJ mol⁻¹ K⁻¹.

The values for ΔH and Kd at 24 °C in Table I were used along with the ΔCp values to calculate the binding constants at 39 °C using Equation 14. The calculated values were within experimental error of the Kd values at 39 °C in Table I.

**DISCUSSION**

The process by which the binding of cAMP to CRP switches on specific DNA binding and subsequent activation or repression of transcription is not completely understood. Previous investigations (Eilen and Krakow, 1977; Takahashi et al., 1980; Heyduk et al., 1992), which focused on determining differences between the binding properties of cAMP and its non-functional analog cGMP to elucidate the mechanism for specific DNA binding by CRP, were inconclusive since the binding properties of both cyclic nucleotides were essentially very similar. However, Ebright et al. (1985) showed that there are differences between cAMP ligated CRP and cGMP ligated CRP exhibited by differences in the action of proteases, in the binding specifically to DNA or poly(dA-dT) sequences, and on the level of the activation of transcription. Ebright et al. (1985) proposed that a change in CRP conformation occurs upon cAMP binding which initiates specific DNA binding. The results presented in this investigation show that there is indeed a profound difference between the thermodynamics of cAMP and cGMP binding to CRP.

The binding of the non-activator cGMP to CRP is exothermic, non-cooperative, and driven by an increase in the entropy of the

![Image](image_url)

**Table II** Thermodynamic cooperative binding parameters and heat capacity changes for cAMP binding to CRP and T127L mutant

| KCl concentration | T (°C) | ΔH° (kJ mol⁻¹) | ΔS° (kJ mol⁻¹ K⁻¹) |
|-------------------|-------|----------------|--------------------|
| CRP               |       | ΔH° - ΔH°       | ΔCp1               | ΔCp2               |
| 0.2               | 24.3  | -6.1 ± 0.5      | 38.0 ± 1.5         |                   |
| 0.5               | 24.0  | -3.9 ± 0.4      | 53.7 ± 2.2         |                   |
| 0.5               | 39.3  | -3.6 ± 0.8      | 35.5 ± 3.5         | -0.30 ± 0.08      | -1.47 ± 0.17 |

| T127L mutant      |       | ΔH° - ΔH°       | ΔCp1               | ΔCp2               |
|-------------------|-------|----------------|--------------------|
| 0.5               | 24.0  | 0              | 0                  | 0                  |
| 0.5               | 39.5  | 1.0 ± 0.2      | 0                  | -0.47 ± 0.15      | -0.47 ± 0.15 |
protein-ligand-solvent system. Although, the thermodynamic binding parameters of the first molecule of cAMP to CRP are approximately the same as for cGMP binding to CRP, the overall binding of cAMP is endothermic and is best described by an interactive two-site binding model with positive cooperativity between the two binding sites. This results from the typical thermodynamic parameters of the second binding reaction of cAMP to CRP, \( \Delta H_{2}, \Delta S_{2}, \) and \( \Delta C_{p,2} \), reflect a conformational change in the fully cAMP-ligated CRP which would cause positive cooperativity between the two sites. A cooperativity which is dependent on ionic strength as reflected in the change in the entropy. The amount of energy absorbed is less at the lower ionic strength of 0.2M KCl. Differences in the thermodynamics between cAMP and cGMP binding to CRP show that the amino group in position 6 of the purine ring of cAMP is important, not only for specific recognition of this ligand, but for inducing the cooperativity between the ligand binding sites. The N-6 amino group of cAMP forms a hydrogen bond to the hydroxy group of Thr-127 of CRP. The point mutation at Thr-127 on CRP also changes the specificity of the protein: the mutant activates transcription only upon binding of cGMP, but to a lesser extent than the ligated cAMP-CRP complex. Although the binding reaction of cGMP to the mutant does not exhibit any cooperativity, there is some evidence for a conformational change in the presence of cGMP. The heat capacity changes accompanying both cGMP binding reactions (\(-0.76 \pm 0.12 \text{ kJ mol}^{-1} \text{ K}^{-1}\)) are almost twice as great as for both cAMP binding reactions to the mutant (\(-0.47 \pm 0.15 \text{ kJ mol}^{-1} \text{ K}^{-1}\)). In the presence of cGMP, the mutant is digested by subtilisin and produces the same product pattern as observed for cAMP-ligated CRP (Moore, 1993), implying that cGMP stabilizes the mutant in a conformation similar to that of fully cAMP-ligated CRP. This conformation may be necessary for specific DNA binding and subsequent activation of transcription.

Finally, all the binding reactions of the cyclic nucleotides to CRP and the mutant are mainly entropically driven at 24 °C. The cyclic nucleotides are anions at neutral pH and as charged species in water, they increase the ordered structure of water through the formation of hydration shells. The observed increase in the entropy would result from a loss of the ordered water structure of the cyclic nucleotide anion upon binding to CRP or its mutant. This is expected to be the same for both cyclic nucleotides and both proteins since differences in their structures are small.

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