Effect of cAMP Binding Site Mutations on the Interaction of cAMP Receptor Protein with Cyclic Nucleoside Monophosphate Ligands and DNA*

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Although cAMP binding to wild type cAMP receptor protein (CRP) induces specific DNA binding and activates transcription, cyclic nucleoside monophosphate (cNMP) binding to the CRP mutant Ser128→Ala does not, whereas the double CRP mutant Thr127→Leu/Ser128→Ala activates transcription even in the absence of cNMP. Isothermal titration calorimetry measurements on the cNMP binding reactions to the S128A and T127L/S128A mutants show that the reactions are mainly entropically driven as is cAMP binding to CRP. In contrast to cAMP binding to CRP, the binding reactions are noncooperative and exothermic with binding enthalpies (ΔHrxn) ranging from -23.4 ± 0.9 kJ mol⁻¹ for cAMP binding to S128A at 39 °C to -4.1 ± 0.6 kJ mol⁻¹ for cAMP binding to T127L/S128A at 24 °C and exhibit enthalpy-entropy compensation. To account for the inactivity of the S128A mutant, in vitro and in vivo DNA binding experiments were performed on the cAMP-ligated S128A mutant. The cAMP-ligated S128A mutant binds to the consensus DNA binding site with approximately the same affinity as that of cAMP-ligated CRP but forms a different type of complex, which may account for loss of transcriptional activity by the mutant. Energy minimization computations on the cAMP-ligated S128A mutant show that amino acid conformational differences between S128A and CRP occur at Ser179, Glu181, and Thr182 in the center of the DNA binding site, implying that these conformational changes may account for the difference in DNA binding.

Many fundamental biological processes are profoundly affected by small conformational changes induced by the binding of very specific ligand "signal" molecules as well as by single amino acid mutations in the protein. These conformational changes may be evident in the thermodynamics of the ligand-protein/ligand-mutant interaction. The binding of cAMP to the cyclic AMP receptor protein (CRP), a dimer (M, = 45,000) of two identical subunits with one cAMP binding site in each subunit, induces a conformational change in CRP (1), and this change is evident in the cooperative binding between the two binding sites of CRP and in the endothermic nature of the binding reaction (2). This conformational change is followed by the binding of CRP to specific DNA sequences in the promoter regions of many catabolite-sensitive operons and by the interaction of CRP with RNA polymerase to initiate transcription (3–5). More specifically, cAMP binds to at least six amino acid residues including Thr127 in the subunit and Ser128 from the other subunit in the amino-terminal domain of CRP (6, 7). In the carboxyl-terminal domain, specific amino acids within the helix-turn-helix portion of the E and F α-helices interact with the specific DNA sequences (8), and a surface exposed loop around amino acids 156–164 interacts with the α-subunit of RNA polymerase (5, 9–11). The binding of other cyclic nucleotides (cNMP) differ in their ability to induce conformational changes in CRP (1, 12). Indeed, the binding of cGMP to CRP does not exhibit cooperative behavior between the two binding sites, is exothermic (2), and does not initiate transcription.

Single or double amino acid mutations in CRP, as well as minor substitutions in the structure of cAMP, also result in changes in the activity of CRP from the complete absence of transcriptional activity (1) to activation in the absence of cNMP (13–18). Studies on the CRP mutants known to activate transcription in the absence of ligand (CRP*) have been helpful in understanding the conformational change induced in CRP by the ligand. Another class of CRP mutants, positive control mutants, have been particularly helpful in dissecting the transcriptional activation function of CRP. These proteins are able to bind DNA but are unable to efficiently activate transcription (19–22). Typically, positive control mutations are on the surface exposed areas of CRP and are thought to affect protein-protein interactions between CRP and RNA polymerase. The highly specific dependence of transcriptional activation on amino acid substitution in CRP is particularly evident, as will be shown in this investigation by the conversion of the positive control mutant S128A to a CRP* upon the introduction of a second mutation, Thr127→Leu in the cAMP binding site. Earlier, it was shown that the single Thr127→Leu mutation in CRP results in activation of transcription by the binding of cGMP and not cAMP (23). Interestingly, the positive control nature of the mutant S128A results from a mutation at the cAMP binding site instead of in the RNA polymerase contact region.

Differences in the level of activation induced by minor changes in the cNMP structure and by mutations in CRP may be exhibited in differences in the thermodynamics of the binding of cNMP to mutants of CRP because this is the initial step in the activation of the protein. This is typically exemplified by the contrast between the cooperative and endothermicity of the
cAMP to CRP binding reaction, which results in the activation of transcription and the noncooperative nature and exothermicity of the cGMP to CRP binding reaction, which does not activate transcription (2). In the present investigation, the binding constant (Kₜ), the binding enthalpy (ΔHₜ), and the binding entropy (ΔSₜ) are determined from isothermal titration calorimetry (ITC) measurements for cAMP, cGMP, and cMP binding to S128A and the CRP* mutant T127L/S128A. These thermodynamic quantities are compared with the cMP binding properties of CRP and T127L from Gorkovskaya et al. (2). Because, as will be shown, there is little correlation between the cMP binding thermodynamics and the level of activity of the ligated protein, differences in DNA binding, in vivo and in vitro, between CRP and the positive control mutant S128A were also investigated to account for the inactivity of the S128A mutant. In addition, energy minimization computations were performed on the cAMP-ligated forms of CRP, S128A, and T127L/S128A to determine any structural changes in the mutant relative to CRP that might account for the difference in activity. In particular, conformational differences between CRP and the mutants were compared in the DNA binding region, the RNA polymerase contact region, and in the hinge region consisting of residues 130–139, which connect the carboxy-terminal and amino-terminal domains of CRP (6, 7).

**EXPERIMENTAL PROCEDURES**

Materials—A New Brunswick Fermentor Scientific Inc. SF 116 was used for growing the bacterial cultures expressing wild type and mutant CRPs. Wild type and mutant proteins were purified over phosphocellulose and eluted with a linear salt gradient from 0.2 to 1.2 M KCl in 0.05 M potassium phosphate-potassium hydroxide buffer, pH 7.0, containing 0.2 mM sodium EDTA, 0.2 mM diethiothreitol, and 5% glycerol. Peak fractions were dialyzed at 4 °C in the same buffer containing 0.5 mM KCl (0.05 M K₃PO₄ + 0.5 mM KCl buffer) with two changes in the buffer solution. The proteins were determined to be approximately 90% pure by Pharmacia Phast SDS gel electrophoresis analysis. The concentrations of the CRP and the mutants were determined by UV absorption spectroscopy using an extinction coefficient of 3.5 × 10⁴ cm⁻¹ M⁻¹ at 280 nm (24). Differential scanning calorimetry scans of the protein solutions were performed from 25 to 100 °C with a Hart 7707 DSC heat conductive differential scanning calorimeter. Only one thermal transition at 60 °C was apparent for the CRP, in agreement with Gorkovskaya et al. (2), and one transition at the same temperature for the S128A and T127L/S128A double mutant. The ligand solutions were made by dissolving the sodium salt of the cyclic nucleotide in the second dialysate solution so that the ligand solvent was the same as the protein solvent. The concentrations of the ligand solutions were determined using an extinction coefficient of 3.5 × 10⁴ cm⁻¹ M⁻¹ at 280 nm for cAMP, 1.24 × 10⁴ cm⁻¹ M⁻¹ at 250 nm for cGMP (25), and 1.23 × 10⁴ cm⁻¹ M⁻¹ at 250 nm for cMP. The sodium salts of cAMP, cGMP, and cGMP, potassium phosphate, NaOH, EDTA, diethiothreitol, glycerol, Tris, HCl, bromphenol blue, and KCl were reagent grade from Sigma.}

**Mutagenesis and Promoter Activation in Vivo—**Mutagenesis using M13CRP DNA templates has been described previously (23, 26). The mutants were expressed in the plasmid pJLess (23), which carries the λLex temperature-sensitive repressor and was derived from the plasmid pPR (27). Escherichia coli strain CA8445 (ΔcrpAΔcrpC) (28) was used as the host for the expression of recombinant mutant plasmids. Activation of several catalytic-sensitive operons, including those of lactose (lac), galactose (gal), arabinose (ara), and maltose (mal) by CRP and the mutants was phenotypically characterized on MacConkey indicator plates containing 1% of the appropriate sugar, in the absence of cGMP or in the presence of 0.5 mM cAMP or 0.5 mM cGMP. The preparation and application of MacConkey indicator plates has been described by Miller (29). Briefly, the screening of phenotypes by MacConkey indicator plates is performed by monitoring the pink to red coloration appearing on the plates that results from metabolism of the sugar by phenotypically positive colonies. Activation of the T127L mutant was also quantitatively assayed by the production of β-galactosidase as described by Miller (29). Briefly, this assay is based on the amount of o-nitrophenyl-β-D-galactopyranoside hydrolysis into galactopyranoside and o-nitrophenol by β-galactosidase in culture samples and the activity is

\[
\text{o-nitrophenyl-β-D-galactopyranoside} \rightarrow \text{galactopyranoside} + \text{o-nitrophenol}
\]

where A is the optical density of the culture sample, t is the time of reaction, and V is the volume of sample.

In Vivo DNA Binding—A 70-base pair DNA fragment containing the consensus CRP binding site was labeled with [32P]dATP (Pₜ) using the polymerase chain reaction. The template DNA was a modified version of the M13mp18 plasmid that had the consensus CRP binding site in place of the lac CRP binding site (23). Following polymerase chain reaction, the DNA was purified over a Sephadex G-50 spin column. The specific activity of Pₜ was determined to be 1200 cpm fmol⁻¹. Reaction mixtures (500 µl) contained 0.01 M potassium phosphate buffer, pH 7.1, 0.05 M KCl, 2.5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM diethiothreitol, 25 µg/ml bovine serum albumin, 12.5 fmol (25 pm) of [32P]labeled Pₜ, either 11 pm to 11 nM CRP or S128A, and either 0 or 0.1 mM cAMP. The reactions were incubated for 20 min at 24 °C. The CRP or S128A was first mixed with the cAMP, and then the cAMP was added. The reaction mixtures were filtered through nitrocellulose filters (25 mm Millipore type HA, 0.45 µm) that had been pre-soaked in the wash buffer solution (30). The filters were then washed once with 500 µl of the buffer containing 100 µM cAMP, and the radioactivity was measured in a liquid scintillation counter.

In Vivo DNA Binding—A synthetic promoter construct, galP14, which is a derivative of the galP1 promoter and was a kind gift from Dr. Steven Busby and Dr. Andrew Bell, was utilized to assess the in vivo DNA binding activity of CRP and S128A (20, 31). The galP14 promoter contains a 4-base pair deletion between the −10 region and the CRP binding site. CRP sharpens this promoter by blocking access of RNA polymerase (20). In the absence of CRP, the promoter is active (32). The galP14 promoter region was subcloned into a broad host range lac expression vector, pRW2, to monitor promoter activity by measuring β-galactosidase activity (29). Wild type CRP and S128A were expressed from pJLess and co-transformed with the pRW2 plasmid containing galP14 into CA8445 cells, and β-galactosidase activities were assayed. Repression of the promoter can be directly correlated to the ability of the CRP/mutant to bind to the DNA independent of transcriptional activation.

**ITC Measurements—**Data analysis of the exothermic scans that exhibited a continuous monotonic decrease of titration peak area with the addition of titrant consisted of using the identical two-site model (33, 34) described by the following equations.

\[
Q_i = \frac{n_i [CRP] [L] [1 + L_i/n_i [CRP]] + 1/n_i [CRP] - [1 + L_i/n_i [CRP]] + 1/n_i [CRP] [1 + L_i/n_i [CRP]] - 4 [L_i/n_i [CRP]]^{1/2}}{2}
\]

where \( Q_i \) is the total heat, \( L_i \) is the total ligand concentration, \( n_i \) is the stoichiometry, \( K_i \) is an intrinsic binding constant, \( H_i \) is an intrinsic heat of binding, \( [CRP] \) is the total binding site concentration, which is equal to twice the total protein concentration, and \( V_i \) is the cell volume, 1.385 ml. The expression for the heat released per the i th injection, \( Q(i) \), is then (34)

\[
\Delta Q(i) = \Delta Q(i) + \frac{dV}{dV}[Q(i)] + (Q(i) - (i - 1))
\]

where \( dV \) is the volume of titrant added to the solution. Fits of the binding data to an interactive two-site binding model as described previously for the binding of cAMP to CRP (2) increased the standard deviation of the fit as shown below. Each titration calorimetry scan yields values for \( n_i, H_i \) and the intrinsic binding constant, \( K_i \). For simplicity, the intrinsic binding constants are used throughout the paper, even when comparing the results to the CRP and T127L binding results reported earlier (2). To obtain the thermodynamic macroscopic binding constants for each binding step, \( K_i \) is multiplied by the statistical factor 2 for the first step and \( \frac{1}{2} \) for the second step.

The values of \( \Delta G^o_i \) and \( \Delta S_i \) were determined from the fundamental equation of thermodynamics:

\[
\Delta G^o_i = -RT \ln K_i = \Delta H_i - T \Delta S_i
\]
where \( R = 8.315 \text{ J mol}^{-1} \text{ K}^{-1} \), and \( T \) is the absolute temperature. The heat capacity changes, \( \Delta C_p \), were determined from a linear least squares fit of \( \Delta H \) to \( T \). To check the consistency of the thermodynamic quantities, values of the binding constants were calculated at 39 \( ^\circ \text{C} \) using the \( \Delta H_0 \) and \( K_b \) values at 24.0 \( ^\circ \text{C} \) (\( T_0 \)) and the \( \Delta C_p \) values in the van’t Hoff equation,

\[
\ln(K(T)/K(T_0)) = (\Delta H_0(T_0) + \Delta C_p(T_0)(1/T_0 - 1/T) + \Delta C_p(T)\ln(T)/T)/R 
\]  
(Eq. 5)

and compared with the ITC determined values at this temperature.

Energy Minimization Computations—The CRP dimer x-ray crystal structure with bound cAMP is asymmetric where one subunit is in the “closed form” and the other subunit is in the “open form” (6). In the open form, the amino- and carboxyl-terminal domains are more separated from each other than in the closed form. Because only the closed form of CRP binds DNA (35), the energy minimization calculations were performed on the x-ray crystallographic structures of the closed dimer of the CRP-cAMP \( \cdot \) complex from the Protein Data Bank by superimposing a copy of a monomer of the closed form on the monomer that was in the open form. The energy minimizations were performed in a vacuum. The initial structure of the S128A mutant was generated by graphical replacement of the Ser-CH\( \_2 \)OH side chain of the energy-minimized wild type structure with the -CH\( _3 \) side chain of Ala and superimposing all the nitrogen and carbon atoms common to both Ser and Ala. The initial structure of the T127L mutant was similarly generated by graphical replacement of the Thr-CH\( _2 \)OH side chain of the energy-minimized wild type structure with the -CH\( _2 \)-CH(CH\( _3 \))\( _2 \) of Leu. Because the difference between cAMP and cIMP is a single group replacement of the NH\( _2 \) on C-6 by O and the addition of H on N-5, the structure of the CRP-cIMP complex was also energy-minimized using the CRP-cAMP \( \cdot \) complex as the initial structure with the corresponding substitution on the ligand. Structures were then energy-minimized by using the steepest descent method for 1000 iterations after which the conjugate gradient method was used until convergence was achieved (maximum derivative less than 0.04 kJ/mol). Root mean squared displacements of the mutants were computed relative to the wild type after least squares superposition of all heavy atoms common to both proteins. Computational results were obtained using software programs from Bioasyst (MSE of San Diego. Energy minimizations, using the AMBER force field (36), were performed with the Discover \( \text{©} \) program, and structure analyses and comparison were performed with the DeCipher \( \text{©} \) program.

RESULTS

Promoter Activation in Vivo—The ability of S128A and T127L/S128A to activate transcription at the lac, gal, mal, and ara operons was determined by screening the phenotypes on MacConkey indicator plates. The phenotypic results on the MacConkey indicator plates (not shown) indicated that S128A was inactive at all the operons tested. T127L/S128A was active at all the operons tested and even in the absence of cNMP and thus is a CRP*.

Activation of the lac operon by S128A was quantitatively determined by assaying for the production of \( \beta \)-galactosidase. The results confirm the phenotypes seen on the MacConkey indicator plates. S128A was essentially inactive with only 3-4% of the CRP activity.

ITC Results—A typical titration run is shown in Fig. 1 along with a least squares fit of the independent two-site model to the data. The titration consisted of 5-\( \mu \)l injections of 2.8 mM cAMP solution into a 0.036 mM solution of the S128A mutant at 23.5 \( ^\circ \text{C} \). The titrations were continued until the peak areas were within the noise limit of the baseline. The binding reaction is exothermic, and the average results of fits of the data to Equations 2 and 3 in terms of \( K_b \) and \( \Delta H_b \) are presented in Table I. Attempts to fit the data to an interactive two-site model (34) always increased the standard deviation of the fit, e.g., from 2.8 to 3.1 for the fit in Fig. 1b. Similar results are obtained with the binding reactions of the other cNMPs, which are also presented in Table I. Each set of thermodynamic quantities at a given temperature is the average result from two or more separate titration runs at different protein and ligand concentrations. For this range of \( K_b \) and \( \Delta H_b \) values, these quantities are independent of the stoichiometries that vary about 10% but are close to one as determined by the protein analysis. The \( \Delta H_0 \) and \( K_b \) values at 24.0 \( ^\circ \text{C} \) and \( \Delta C_p \) value from Table I are used in Equation 5 to calculate the binding constants at higher temperatures for the S128A and double mutants. With the exception of the cAMP binding constants for both mutants at 39 \( ^\circ \text{C} \) and the cGMP-doubt mutant at 39 \( ^\circ \text{C} \), the calculated binding constants are within two standard deviations of the experimentally determined binding constants at 30 and 39 \( ^\circ \text{C} \). It is not clear why there are differences between the calculated binding constants and the experimental binding constants at 39 \( ^\circ \text{C} \), but deviations from the van’t Hoff equation have been observed for other protein-ligand binding reactions (37). The principle source of error in the ITC measurements is the noise in the baselines because the heats of reaction were small due to the low concentrations of protein, which were used to minimize possible aggregation.

The results in Table I show that all the cNMP binding reactions are exothermic and do not exhibit cooperativity between the two sites of CRP in contrast to the binding reaction of cAMP to CRP, which is endothermic and exhibits positive cooperativity (2). At 24 \( ^\circ \text{C} \), all the binding reactions are mainly driven by an increase in the entropy of the system. The binding enthalpies decrease with increasing temperature as is evident by the negative heat capacity changes, which are about the same for all the cAMP binding reactions. These range from \(-0.84 \pm 0.04 \text{ kJ mol}^{-1} \text{ K}^{-1}\) to \(-0.87 \pm 0.10 \text{ kJ mol}^{-1} \text{ K}^{-1}\) for the mutants, which are close to \(-0.76 \pm 0.12 \text{ kJ mol}^{-1} \text{ K}^{-1}\) for the cGMP to T127L mutant binding reaction (2). These values are
approximately double the values of ΔCp for the other cGMP and 
dCMP binding reactions, which range from −0.33 ± 0.06 to 
−0.50 ± 0.06 kJ mol⁻¹ K⁻¹.

Differences in the binding site thermodynamic quantities, 
G°b, ΔH°, and ΔS° between the wild type CRP and the mutants 
are presented in Table I. The binding reaction of

\[
\text{CRP + dNMP} \leftrightarrow \text{CRP + cNMP}
\]

(Eq. 6)
is used as the reference reaction along with the intrinsic binding 
site constant of this reaction. Although the overall cAMP 
binding to CRP is endothermic, binding to the first site to form 
the CRP-cAMP complex is exothermic with thermodynamic 
quantities close to those for cGMP binding to CRP (2). In Table 
I, the decrease in ΔG°, for cNMP binding to the double mutant 
appears to result solely from the Ala substitution for Ser128 and 
not the Leu substitution for Thr127 because the ΔΔG°, for 
dNMP binding to the double mutant is the same as the ΔΔG°, 
for cNMP binding to S128A. According to Equation 4, changes 
in ΔG°, are attributed not only to differences in ΔH° but also to 
changes in the entropy. Enthalpy-entropy compensation is 
apparent in these binding differences. This is shown by the 
straight line plot in Fig. 2 where the slope of ΔΔS° versus 
ΔH° is 0.71 ± 0.08.

In Vitro DNA Binding Results—Results of Pcon binding to 
CRP and S128A are presented in Fig. 3. In both cases, the 
amount of Pcon bound to the protein reaches saturation at 
approximately the same protein concentration but with 
different efficiencies. The DNA binding affinity in the presence of 
cAMP is thus almost the same for both proteins. However, the 
lower amount of DNA bound to S128A at saturation relative to 
the amount of DNA bound to CRP indicates that the mutant 
forms a different type of complex with DNA.

In Vivo DNA Binding Results—The activities of β-galacto-
sidase in CA8445 cells transformed with the lac expression 
vector pRW2 containing the synthetic promoter construct 
galP114 and βl less carrying either CRP or S128A are shown in 
Table III. The results in Table III show that S128A is able to 
repress the galP114 promoter and therefore is able to bind 
DNA in vivo. However, S128A is approximately 50% less active 
than CRP in the presence of cAMP as determined by β-galacto-
sidase activity.

Energy Minimization Results—The energy-minimized struc-
tures of the mutant complexes exhibit changes in the confor-
mation of the amino acid residues relative to those in CRP. 
These differences are expressed in terms of the root mean 
mean displacements between the atoms of each residue in the 
mutant and the wild type. Particular attention was focused on changes at the specific DNA binding site (residues 140, 169–170, 178– 
182, 185, 188, and 201 (38)), the RNA polymerase contact 
region (residues 156–164 (5, 9–11)), and the hinge region 
between the E and F domains (residues 130–139 (7)). In the RNA 
polymerase binding region and the hinge regions, the root 
mean square displacements between the cAMP-ligated com-
plexes of CRP and S128A are all less than 0.05 Å. In the DNA 
binding region, displacements >0.05 Å occur at residues Glu181 
and Thr182 and up to 0.15 Å at Ser179. These residues in the 
DNA binding region of CRP might be important for the initia-
tion of transcription because this is the only region where 
significant displacements occur between the cAMP-ligated 
forms of CRP and completely inactive S128A. The cAMP-
ligated T127L mutant, which is inactive, also exhibits large 
displacements of up to 0.2 Å in this region. Although the energy 
minimized structure of the cAMP complexed double mutant 
must be viewed with caution because this structure differs by 
two mutations from the CRP starting structure, the smallest 
displacement of 0.03 Å occurs on the adjacent amino acid res-
idue, Cys77.
The force field strain energy of the cIMP-CRP energy minimized complex was \( -594 \text{ kJ mol}^{-1} \), almost 20 times less stable than for the energy minimized structures of the cAMP complexes which were all about \( -11\times400 \text{ kJ mol}^{-1} \). This arises out of the strong van der Waals’ repulsion introduced into the complex by the O substituent on C-6 and H on N-5 of the dMP purine ring.

**DISCUSSION**

All the cNMP binding reactions to the T127L(2), S128A, and T127L/S128A mutants are totally different from those of cAMP binding to CRP. They are exothermic, both enthalpically and entropically driven, and noncooperative. Although the mutants exhibit profound differences in their transcriptional activities, differences in their cNMP binding affinities or free energy changes, \( \Delta G_{\text{b}} \), are small, ranging from 1.2 to \(-3.6 \text{ kJ mol}^{-1} \) because large differences in the binding enthalpies are compensated by large differences in the binding entropies. This enthalpy-entropy compensation has been observed in many binding reactions including those involving mutated proteins (39) and may be a consequence of the participation of water in the binding reaction in solution (40). Enthalpy-entropy compensation may also be a consequence of coupling the transi-

Large changes in the binding heat capacity, \( \Delta C_p \), occur for the second cAMP binding reaction to CRP (\(-1.47 \pm 0.17 \text{ kJ mol}^{-1}\text{K}^{-1} \)) and for cGMP binding to the T127L mutant (\(-0.76 \pm 0.12 \text{ kJ mol}^{-1}\text{K}^{-1} \))(2), which are both active in the initiation of transcription (23), compared with \( \Delta C_p \) for the first cAMP binding step (\(-0.30 \pm 0.08 \text{ kJ mol}^{-1}\text{K}^{-1} \)) and cGMP binding to CRP (\(-0.47 \pm 0.15 \text{ kJ mol}^{-1}\text{K}^{-1} \))(2). It was proposed that the large differences in \( \Delta C_p \) might reflect the structural changes necessary for activation of transcription (2). However, a large heat capacity change of \(-0.87 \pm 0.10 \text{ kJ mol}^{-1}\text{K}^{-1} \) is also observed for binding of cAMP to S128A, which does not activate transcription. The more negative heat capacity changes may reflect structural changes upon cNMP binding but not necessarily structural changes for activation of transcription. Apparently, there is no direct correlation between the thermodynamic cNMP binding quantities and the initiation of transcription by CRP. Consequently, changes in the activation of transcription must arise from changes in other regions of CRP. The most probable regions are the DNA binding region, the hinge region, or the RNA polymerase contact region of the cNMP-ligated CRP.

**FIG. 2** Differences in \( \Delta S_b \) for binding of the cNMP to the mutants relative to binding of the cNMP to CRP are plotted as a function of the same differences in \( \Delta H_b \) at 24 °C. The straight line determined by a least squares fit of all the data except for cAMP binding to S128A has a slope of \( 0.71 \pm 0.08 \).

**FIG. 3** Dependence of \(^{32}\)P-labeled 70-base pair DNA fragment containing the consensus CRP binding site, \( P_{\text{con}} \), on DNA concentration as determined from nitrocellulose filter assays. The results indicated by \( \Delta \) were obtained in the absence of cAMP.

**TABLE III**

| CRP                | \( \beta \)-Galactosidase activities | No ligand | +cAMP | +cGMP |
|--------------------|-------------------------------------|-----------|-------|-------|
| No CRP             | \( \beta \)-Galactosidase activities | 758 ± 76  | 784 ± 78 | 719 ± 72 |
| Wild type          | \( \beta \)-Galactosidase activities | 769 ± 77  | 126 ± 13 | 703 ± 70 |
| S128A              | \( \beta \)-Galactosidase activities | 701 ± 70  | 433 ± 43 | 605 ± 61 |

\( * \) Estimated uncertainty in the activity, which is based on the amount of hydrolysis of O-nitrophenyl-\( \beta \)-D-galactopyranoside into galactopyranoside and \( \alpha \)-nitrophenol by \( \beta \)-galactosidase in culture samples and is equal to \( 1000(A_{550nm} - 1.75A_{600nm})(tvA_{600nm})^{-1} \) where \( A \) is the optical density of the culture sample, \( t \) is the time of reaction, and \( v \) is the volume of sample.
observed for Ser\textsuperscript{179}, Glu\textsuperscript{181}, and Thr\textsuperscript{182} in the energy minimized differences between the cAMP-ligated CRP and S128A complexes. The interaction of Glu\textsuperscript{181} and Thr\textsuperscript{182} with the lac operon is supported by electrostatic models (38) and chemical studies (42). Ebrigt et al. (8) found that Glu\textsuperscript{181} is directly involved in making sequence specific contact with DNA. More recently, Cheng et al. (43) bound a fluoroscence probe to Cys\textsuperscript{178} and observed that the increase of the fluorescence intensity of the probe correlates with an increase in cAMP concentration, whereas a decrease in the fluorescence intensity of the probe correlates with an increase in cGMP concentration. Thus, the binding of cAMP to the CRP site where the Thr\textsuperscript{127} and Ser\textsuperscript{128} mutations occur directly affected Cys\textsuperscript{178} in the DNA binding region. Although energy minimization results involving two mutations in the starting structure should be examined with care, the minimum displacement between the S128A/T127L mutant and CRP residues occurs at Cys\textsuperscript{178} adjacent to Ser\textsuperscript{179}, and the additional Thr\textsuperscript{127} → Leu mutation in the S128A mutant does restore the activity of the protein.

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