RNA Polymerase-cNMP-ligated cAMP Receptor Protein (CRP) Mutant Interactions in the Enhancement of Transcription by CRP Mutants*

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The enhancement of the transcription of three synthetic promoters by cNMP-ligated cAMP receptor protein (CRP)/mutant complexes was determined from the transcription yields of a short AAUU transcript in an abortive initiation in vitro transcription assay. The cNMP-ligated CRP and mutants were cAMF, cGMP, and cIMP ligated with CRP, T127L CRP, S128A CRP, and T127L/S128A CRP. The transcriptional activation of a 152-base pair lacUV5 promoter (synlac promoter) with a CRP consensus binding site sequence (syncon promoter) was enhanced by a higher average affinity of 13.2 ± 0.5 with the cAMP-ligated complexes of CRP/mutants and cGMP ligated T127L, although their promoter binding site affinities varied by a factor of 5. However, in the presence of bound RNA polymerase, the binding affinities only ranged from 0.8 ± 0.2 × 10^7 M^−1 for cAMP-ligated CRP* to 1.8 ± 0.3 × 10^7 M^−1 for cAMP-ligated CRP, indicating that the CRP/mutant interacts with the bound RNA polymerase, which would account for the near constancy of the enhancement factors. The corresponding enhancement factors for the synlac promoter and a promoter with a different CRP binding site sequence (syngal promoter) were also nearly the same, 7.2 ± 0.7 and 6 ± 1, respectively. The binding reaction of the syncon promoter to the RNA polymerase is exothermic, with a binding constant (K_b) = 2.1 ± 0.2 × 10^7 M^−1.

The transcription of over 25 operons, which code for enzymes involved in carbohydrate metabolism, is enhanced by the binding of cAMP receptor protein (CRP) to the promoter region of the operon at a site adjacent to the RNA polymerase binding site. Low glucose levels in the cell increase the level of cAMP, resulting in substantial cAMP binding to the amino-terminal domains of the CRP dimer (45,000 g mol^−1), which induces a conformational change in the CRP so that the two carboxy-terminal domains bind specifically to a site in the promoter (1), centered either 70.5, 61.5, or 41.5 base pairs upstream from the promoter transcription start point (P1). Mutations along the helical monomer–monomer interface of CRP significantly alter the in vivo level of transcriptional enhancement by CRP. Convergence of the helical interface to a more perfect leucine zipper by mutating Thr^{127} to Leu (T127L) results in in vivo activation of transcription in the presence of cGMP, an analog of cAMP (2). Mutation of the Ser^{128} residue, which interacts with cAMP in the other subunit, to Ala (S128A) reduces the enhancement of in vivo transcription by CRP (2, 3). A mutant of CRP with both mutations (CRP*) enhances in vivo transcription in the absence of cAMP (2). In addition, the mutation of Thr^{127} to Cys, Ile, or Ser also resulted in the enhancement of in vivo transcription in the presence of CGMP, and it was concluded that the Thr^{127} to Cys, Ile, and Ser mutations in CRP produced structural changes in CRP similar to those induced by cAMP binding to CRP (3). Since the binding affinities of cAMP to CRP and the CRP mutants are nearly the same (3, 4), changes in the enhancement of transcription by CRP must involve processes subsequent to the binding of cAMP to CRP. A recent isothermal titration calorimetric (ITC) study of the binding of three 40-bp DNA duplexes with each one containing the CRP binding site sequence of a promoter to cNMP-ligated CRP, T127L, S128A, and CRP* revealed large differences in the CRP binding site affinities, which could account for differences in the enhancement of transcription by the cNMP-ligated CRP mutants (5). In addition, fluorescence polarization studies show that CRP with bound DNA (6) also interacts with RNA polymerase. Photocross-linking studies indicate that CRP is in close enough proximity to interact with RNA polymerase on the lac promoter (7). Transcription results from surface mutations on CRP show that the most likely RNA polymerase contact points on the CRP are on a loop centered at His^{159} in the RNA polymerase proximal subunit of CRP when the CRP binding site is at −61.5 bp from P1 and also include contacts with a second loop centered at Lys^{160} on the distal subunit when the CRP binding site is at −40 bp from P1 (8). More specifically, Ryu et al. (9), using nitrocellulose filter binding assays, observed a 40% increase in the amount of protein-bound lac promoter in a solution of RNA polymerase and cAMP-ligated CRP relative to the amount of protein-bound lac promoter in the absence of RNA polymerase. They inferred that the RNA polymerase bound to the lac promoter increases the CRP binding affinity to the promoter-RNA polymerase complex by a factor of 1.4. More recently, Leu et al. (10) have shown that the cAMP-ligated Thr^{127} to Cys, Gly, Ile, and Ser mutants form ternary complexes with RNA polymerase and the lac promoter,
although the T127G, T127I, and T127S CRP mutants exhibited low binding affinity to the lac promoter. The results show that the mutation at Thr\(^{127}\) affects the CRP binding affinity to the promoter but has only a minor effect or no effect on the formation of CRP promoter-RNA polymerase complexes (10).

To elucidate the roles of the CRP binding site affinity to the promoter and of the interaction of CRP with bound RNA polymerase in the enhancement of transcription, the \textit{in vitro} activation of transcription of a 152-bp length of the \textit{lacUV5} promoter with a consensus CRP binding site sequence (\textit{syncon} promoter) by cNMP-ligated CRP mutants was determined quantitatively and compared with the cNMP-ligated CRP/mutant binding site affinity to the promoter with and without bound RNA polymerase. The \textit{syncon} promoter, shown in Fig. 1, is the same as the native \textit{lac} promoter with the following exceptions: (i) the TA and GC pairs at positions -8 and -9, respectively, are mutated to AT and AT (11), which removes a second \textit{in vitro} transcription start point located about 20 bases upstream from P1 in the \textit{lac} promoter and enhances the RNA yield (12), and (ii) the 22-bp CRP binding site sequence centered at -61.5 bp from P1 is based on the CRP binding site sequences of the 26 operons. The sequence of the native \textit{lac} promoter with just the mutations described in the first case above is the same as that of the \textit{lacUV5} promoter. The enhancement of transcription was determined from an abortive \textit{in vitro} transcription assay (13), which is designed to eliminate the processes of RNA chain elongation and termination by including only the ribonucleotides, adenyl(\(^3\)P) and radioactive labeled uridine 5'-triphosphate (U) in the reaction mixture. Transcription terminates after yielding the short RNA transcript AAU, since guanosine 5'-triphosphate would be required to continue the transcription as shown in Fig. 1. The enhancement factors determined for the cAMP-ligated CRP/mutants in the \textit{in vitro} transcription assay were compared with their binding affinities to a shorter 104-bp promoter, shown in Fig. 1, and similar to that found in the \textit{gal} promoter. The CRP binding site sequence is replaced by that found in the \textit{lac} promoter and strand. Conversion of the CRP binding site from the sequence in the \textit{syncon} promoter to the sequence found in the \textit{gal} promoter required 10 base mutations, while conversion to the consensus sequence required only seven mutations in the 44-base primer sequence. The amplified products were, thus, shorter 152-bp promoters with the same sequence except for the mutations at the CRP binding site.

The \textit{syncon} promoter used in the \textit{in vitro} measurements was only 104 bp long, from -82 to +22 in Fig. 1, so that it contained the CRP and RNA polymerase binding site sequences. Each complementary strand of the 104-bp promoter strand was annealed by heating equal amounts of each strand in 10 mM Tris-HCl buffer containing 1 mM MgCl\(_2\), and 0.5 M NaCl at pH 7.4 up to 95 °C followed by slow cooling down to room temperature. A mutated 104-bp \textit{syncon} promoter with mutations at -10 to G and -13 to C in the RNA polymerase binding region was also prepared, purified, and annealed using the same procedure. The concentration of the 104-bp \textit{syncon} promoter was determined from OD measurements at 260 nm using an extinction coefficient of 1.3 × 10\(^{4}\) cm\(^{-1}\) M\(^{-1}\) based on an OD of 1 at a 50 °C solution (17) and a molecular mass of 65,000 g mol\(^{-1}\).

In \textit{In Vitro Transcription Assays}—The \textit{in vitro} assay followed the protocol described by Zhang et al. (13). Twenty-five-\(\mu\)l reaction mixtures containing an 80 nm concentration of the CRP/mutant, 0.5 nm operon, 40 nm RNA polymerase, 50 nm [\(\alpha\)-\(\text{\textsuperscript{32}P}\)]UTP (from Amersham Pharmacia Biotech), and 200 \(\mu\)M cNMP were pre-equilibrated for 10 min at 37 °C. The final concentration of cNMP ensured that at least 90% of the CRP/mutant was complexed with the cNMP. The buffer was 40 mM Tris-HCl at pH 8 with 100 mM KCl, 10 mM MgCl\(_2\), 2 mM mercaptoethanol, and 5% glycerol. Reactions were initiated by the addition of ApA to make up a 0.25 mM ApA reaction mixture and were allowed to proceed for 15 min at 37 °C. The transcription was then terminated by the addition of 25 \(\mu\)l of formamide loading buffer (80% formamide, 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), 0.05% bromphenol blue, 0.05% xylene cyanol) to the reaction mixture. The reaction product, \(33\text{P}-\text{PAUU}\), was resolved by electrophoresis on a 20% polyacrylamide, 8 % urea gel. The amount of product was quantitated in units of product volume by using a storage phosphor autoradiography method in conjunction with a Molecular Dynamics 3000 E PhosphorImager equipped with ImageQuant software. The linear range of the imager was ascertained by the linear response between the sample volume from 5 to 25 \(\mu\)l and the measured volume count of the product band.

The enhancement factors were determined from the amount of product in the gel electrophoresis measurements. A background volume count of the gel at the product band position (B) was determined from a sample of the assay mixture with just the RNA polymerase and the ribonucleotides present. Then a volume count was taken of the product

![FIG. 1. Sequence of the \textit{syncon} promoter. Boldface letters represent the CRP binding site sequence. The broken line is the sequence of the 104-bp promoter used in the ITC measurements.](image-url)
band with just the RNA polymerase, ribonucleotides, and the promoter present to determine the amount of RNA transcribed in the absence of the cNMP-ligated CRP/mutants (RNA (0)). This was compared with the product volume count determined in the presence of the cNMP-ligated CRP/mutant complex (RNA (cNMP-ligated CRP/mutant)). The enhancement of transcription by CRP, the transcription factor (ε), was then determined as follows.

ε = (cRNA (cNMP-ligated CRP/mutant))

\[ \text{− } B((\text{RNA (0)}) − B) \] (Eq. 1)

**ITC Measurements**—The binding affinity of the cAMP-ligated CRP/mutants to the 104 bp syncon promoter-RNA polymerase complex was determined by ITC using a Microcal, Inc. VP Titration Calorimeter. The VP titration calorimeter consists of a matched pair of sample and reference vessels (1.409 ml) enclosed in an adiabatic enclosure and a rotating stirrer-syringe for titrating ligand solutions into the sample vessel. The ITC measurements were performed at 25.0 °C. The sample vessel contained either the RNA polymerase, the RNA polymerase-104-bp syncon promoter complex, or the promoter alone in the phosphate buffer, while the reference vessel contained just the buffer solution. The phosphate buffer solution was 50 mM K₃PO₄ and contained 1 mM cAMP, 0.2 mM DTT, 0.2 mM EDTA, and 0.15 mM KCl at pH 7.0. (The 1 mM cAMP concentration in the buffer ensured that the CRP/mutant was all complexed with the cAMP.) First, 2–4-μl aliquots of the 0.03–0.1 mM promoter solution were titrated 3–4 min apart into the 1–3 mM RNA polymerase sample solution until the binding was saturated as evident by the lack of a heat exchange signal. Then, 10-μl aliquots of a 0.03-0.06 mM cAMP-ligated CRP/mutant solution were titrated into the promoter-RNA polymerase complex solution. In a separate titration, the cAMP-ligated CRP/mutant solution was titrated into the sample vessel containing just a 1-3 mM promoter solution. For each of the titrations, the additions were continued for 2–3 times past saturation so that a heat of dilution of the titrant could be determined from these additional peak areas. For the promoter into RNA polymerase titrations, these extra additions amounted to about a 7% excess of the promoter to RNA polymerase concentration. Some of the heats of dilution, particularly the large values, were checked by titrating the titrant directly into the buffer solution. The heats of dilution were then subtracted from the heats obtained during the titration prior to analysis of the data.

A nonlinear, least square minimization software program from Microcal, Inc., Origin 5.0 (18), was used to fit the incremental heat of the i-th titration (ΔQi(i)) of the total heat, Q, to the total titrant concentration, X, according to the equations,

\[ Q = nC\Delta H/\sqrt{V1 + X/nC} + 1/nKcC_i - (1 + X/nC_i + 1/nK_cC_i)^{1/2}/2 \] (Eq. 2)

and

\[ \Delta Q(i) = Q(i) + dVi/2V(Q(i) + Qi(i) − Qi(i − 1)) − Qi(i − 1) \] (Eq. 3)

where C, is the total RNA polymerase or promoter concentration in the sample vessel, V is the volume of the sample vessel, and n is the stoichiometry of the binding reaction, to yield values of Kc, ΔH, and n. In this investigation, only the pertinent values for the binding constant are reported, and the other thermodynamic quantities of the binding enthalpy and entropy will be reported in a subsequent paper on the thermodynamics of transcription.

**RESULTS**

**In Vitro Transcription Results**—Typical gel electrophoresis results from transcription assays are shown in Fig. 2. The results are from an abortive initiation in vitro transcription assay performed with the syncon and with the synlac promotors. The higher molecular mass bands are the radioactive labeled pApApU*U* transcripts. The intensities of the bands were quantified in terms of a volume count of radiolabeled product by scanning the bands on the PhosphorImager. By sampling the reaction mixture at 5-min intervals, it was observed that the volume count of the transcript product increased linearly with reaction time up to 20 min, as shown in Fig. 3. Thus, the 15-min reaction time used in the assays was within the linear range of transcription and below any saturation limitation of product formation. The enhancement factors determined from the product volume counts generated by the 36 combinations of cNMP-ligated CRP/mutants and the three promotors are presented in Table I. Each enhancement factor is averaged from at least two separate transcription assays performed with the cNMP-ligated CRP/mutant-promoter complex. Although the volume counts of product varied from run to run, they were always compared with the results of the synlac promoter transcription assays performed at the same time with the same samples of UTP* and analyzed in the same electrophoresis gel. In this way, the product yields were normalized for variations in the determinations due to different levels of UTP* activity and different electrophoresis gel conditions. The uncertainties in the enhancement factors reflected the precision in the results from the different transcription assays. Additional transcription assays were performed with cAMP-ligated CRP and the three promotors at the same time, and the product yields were compared on the same gel. These results yielded enhancement factors in agreement with the enhancement factors in Table I. The enhancement factors cover a range from 1.0 (no enhancement of the activation) to 13.1 ± 1.0 for cAMP-ligated CRP binding to the syncon promoter in Table I. Malan et al. (12) determined from a kinetic analysis of the activation of transcription by CRP an enhancement factor of 22 for the 203-bp lacUV5 promoter, while in Table I the enhancement factor for the synlac promoter, a 152-bp lacUV5 promoter, is 7.6 ± 0.4 at the 80 nM CRP concentration used in this assay.

In Table I, the in vitro enhancement factors with the cAMP-ligated CRP/mutants and cGMP-ligated T127L are almost the same for each promoter and average 12.3 ± 0.5 for the syncon promoter, 7.2 ± 0.7 for the synlac promoter, and 6.3 ± 1.4 for the syngal promoter. Since enhancement factors are also observed with cGMP-ligated CRP (5.79 ± 0.03), cGMP-ligated...

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**Fig. 2.** Gel electrophoresis results of the in vitro activation of transcription for the syncon and the synlac promoter by cNMP-ligated CRP/mutant complexes. The C and CRP/mutants present in the transcription assay mixture are indicated above the product band. A. cAMP; G. cGMP; I. cIMP; C. CRP; S, the S128A mutant. B. the background from the transcription assay mixture without the promoter and the CRP/mutants.
With regard to the activator complex, the cNMP-ligated Thr127 enhancement factors for the syncon promoter are observed even with unligated T127L and CRP* and S128A complexes. Enhancement factors for the syncon promoter are almost the same. This is true for the synlac promoter and to a lesser extent for the syngal promoter. To determine if the promoter binding affinities to the consensus sequence are altered by the presence of bound RNA polymerase, which would account for the constancy of the enhancement factors, the cAMP-ligated CRP/mutant binding affinities to the promoter-RNA polymerase complex were determined from ITC measurements. Typical ITC results are shown in Fig. 4, where 0.06 mM cAMP-ligated CRP was titrated into a 3.0 μM concentration of the 104-bp syncon promoter with bound RNA polymerase. The binding reaction is endothermic with a binding constant of 2.0 × 10^7 M^-1, a value higher than that of 6.6 × 10^6 M^-1, which is observed for binding of 40-bp DNA duplexes containing the 22-bp consensus sequence to cAMP-ligated CRP (5).

Prior to the titration, a slight excess (−7%) of promoter was titrated into the RNA polymerase solution to saturate the binding of promoter to the RNA polymerase as evident by the decrease of heat exchanged between the sample and reference vessels with each addition of the promoter. This is shown in Fig. 5, for a titration of a 0.153 mM 104-bp syncon promoter solution into a 1.5 μM RNA polymerase solution, which exhibits exothermic binding to the RNA polymerase with a binding constant of 2.1 ± 0.5 × 10^5 M^-1 and a binding enthalpy of −150 ± 30 kJ mol^-1. This is in contrast to the endothermic binding of the cAMP-ligated CRP to the promoter-RNA polymerase complex as well as to the promoter alone, described below. This difference may be attributed to large endothermic contributions from the binding of the promoter (1, 9) by the CRP and from conformational changes in the CRP upon promoter binding (19) to the CRP binding enthalpy. The binding constant is lower than the literature value of 6.9 × 10^9 M^-1 determined from a ratio of the kinetic on and off rates of a shorter 70-bp lacUV5 promoter binding to RNA polymerase at 37 °C in a 10 mM Tris buffer containing 10 mM MgCl2 and 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 120 mM KCl (20). The
presence of the glycerol, MgCl₂, and lower ionic strength may affect the binding constant. At the 1.5 mM concentration of RNA polymerase and promoter, about 90% of the promoter is bound to the RNA polymerase, and, thus, the observed binding of the cAMP-ligated CRP/mutant is mainly to the complex. There is undoubtedly some contribution to the binding reaction from binding to a small concentration of the free promoter (10% of the RNA polymerase concentration), which is 2–10 times weaker than the binding affinity with bound RNA polymerase as shown in Table II. The titration results are presented in Table II along with those obtained from titrating the cAMP-ligated CRP/mutants into the 104-bp syncon promoter in the absence of RNA polymerase. As shown in Table II, the binding constants of the cAMP-ligated CRP/mutants to the promoter alone were in agreement with those determined earlier from ITC measurements on the binding of 40-bp consensus DNA to the cAMP-ligated CRP/mutants (5). The binding constants to the promoter, however, increase by factors of 2 (cAMP-ligated CRP) to 10 (cAMP-ligated S128A) with bound RNA polymerase and are due to the interaction of the bound RNA polymerase with the CRP/mutants on the promoter. These results show that the enhancement factors for the syncon promoter are about the same because the concentration of the ternary CRP/mutant-promoter-RNA polymerase is the same with the different cAMP-ligated CRP/mutant activators. To determine if the cAMP-ligated CRP/mutants exhibited the same high binding affinity to the promoter site with unbound RNA polymerase present in solution, separate titrations were performed with cAMP-ligated CRP and cAMP-ligated T127L titrated into a solution of RNA polymerase with the mutated 104-bp syncon promoter that binds weakly to RNA polymerase promoter. This promoter had the same CRP binding site sequence, but mutations at 210 to G and 213 to C in the RNA polymerase binding region of the syncon promoter (Fig. 1) reduced its RNA polymerase binding affinity by a factor of 10–20 so that less than 30% of this mutated promoter is complexed with RNA polymerase. Both cAMP-ligated CRP and cAMP-ligated T127L exhibited lower binding constants, 6 ± 2 × 10⁶ M⁻¹ and 5 ± 1 × 10⁶ M⁻¹, respectively, close to those for binding to the promoter alone. These measurements show that, although the RNA polymerase is present in solution, binding of the CRP/mutant to the syncon promoter is only enhanced with RNA polymerase bound to an adjacent site. Some CRP/mutant binding may occur to the unbound RNA polymerase as observed in fluorescence polarization measurements with fluorescein-labeled CRP, but the binding affinity is 1 order of magnitude weaker (3.3 ± 10⁵ M⁻¹ (21)) than binding to the promoter alone. ITC measurements on the binding of cGMP-ligated T127L, which also exhibits a high enhancement factor (Table I), were unsuccessful because of the low heat exchange of the binding reaction.

The enhancement factors in Table I were determined at a CRP/mutant concentration of 80 nM, and the range of binding affinities in Table II from 0.8 ± 0.2 × 10⁷ M⁻¹ for cAMP-ligated CRP* to 1.8 ± 0.3 × 10⁷ M⁻¹ for cAMP-ligated CRP indicates that the enhancement factor may be more dependent on the CRP* concentration than on the CRP concentration. Thus, additional assays were performed with cAMP-ligated CRP and...
cAMP-ligated CRP\textsuperscript{a} over the concentration range from 10 to 160 nM. The range of concentrations was restricted by the large error in the RNA product yield below 10 nM and the rapid saturation of product formation above 160 nM. The results are presented in Table III and show that the enhancement factor does increase with the cAMP-ligated CRP/mutant concentration over this range of concentrations. Because of the large experimental error, it is difficult to determine if the enhancement factor is more dependent on the cAMP-ligated CRP concentration than on the cAMP-ligated CRP concentration. It is also difficult to determine the relative binding affinities from these assays because of errors in the concentration of reactants as well as in the enhancement factors. However, the results do confirm that the cAMP-ligated CRP and CRP\textsuperscript{a} binding affinities to the promoter-RNA polymerase complex are the same order of magnitude.

**DISCUSSION**

Tagami and Aiba have shown that cAMP-ligated CRP is not required for the processes of RNA elongation and termination (22). This is also indicated for the cAMP-ligated CRP and T127L and S128A mutants by a nearly constant ratio (2.2 \pm 0.4) of the syncon promoter in vitro enhancement factors to the corresponding in vivo enhancement factors determined with plasmids carrying the CRP binding site construct. The in vivo enhancement factors are from Moore (2) and were determined from the amount of β-galactosidase activity in CA8445 cells transformed with plasmids carrying a syncon promoter construct and a plasmid to express either CRP, T127L, or S128A and grown in the presence and absence of CNM P in the culture. Any additional effect of the CRP mutants on the elongation and termination process of the RNA transcript would alter the in vivo enhancement factors, where initiation, elongation, and termination processes are involved, relative to the in vitro enhancement factors where just the initiation of transcription is monitored.

Results from early investigations (23, 24) in the in vitro initiation of transcription by RNA polymerase (RNAP) has lead to the development of the following two-step model for the activation of transcription,

\[
k_1 \text{RNAP} + P \leftrightarrow (\text{RNAPP}) \leftrightarrow (\text{RNAPP})_b
\]

\[
\text{Eq. 4}
\]

where P is the promoter, and the initial RNA polymerase-promoter complex (RNAP-P) is in a “closed” form and then isomerizes to an “open” form, which, in the presence of ribonucleotides, leads to the synthesis of the short AAUU transcript. Although it had been shown earlier that the enhancement of transcription by CRP arises from an increase in the RNA polymerase binding affinity to the promoter, \(k_1/k_{-1}\) (24), more recently the main effect of CRP has been attributed to increasing the isomerization rate constant, \(k_2\) (25), from the closed to the open form. The enhancement factors result from the interaction between the CRP/mutants and the bound RNA polymerase on the promoter, and, thus, the initiation of transcription is enhanced by an increase in the \(k_1/k_{-1}\) constant in Equation 4, i.e. an increase in the RNA polymerase binding affinity through additional bonds to bound CRP on the promoter. These results still do not necessarily rule out the enhancement of the initiation of transcription through an increase in the isomerization rate constant \(k_2\). Leu et al. have shown that the rate of open complex formation is indeed affected by the mutation at Thr\textsuperscript{127}, since the cAMP-ligated T127C CRP mutant forms the open complex more rapidly than the cAMP-ligated T127I and T127S CRP mutants (10). The CRP-RNA polymerase interaction also increases binding of the CRP/mutant to the promoter so that all of the cAMP-ligated CRP/mutants bind to the promoter-RNA polymerase complex with almost the same affinity. Thus, under the concentration conditions of the assay, the concentrations of all the CRP-syncon promoter-RNA polymerase ternary complexes are nearly the same for all the cAMP-ligated CRP/mutants, despite differences in their CRP binding site affinities on the promoter. If the enhancement factors are exclusively dependent on the concentration of the ternary complexes, they would be expected to be nearly the same for the cAMP-ligated CRP/mutants, as is indeed observed in Table I. A similar constancy of enhancement factors is also observed for the synlac and syngal promoters under the assay conditions and may now be attributed to a constancy of the CRP/mutant interaction with these promoter-RNA polymerase complexes.

The interaction between bound CRP and RNA polymerase, which involves a loop consisting of Ala\textsuperscript{156} to Gly\textsuperscript{164} on the surface of CRP (8, 26), would be expected to be the same for the different mutants, since the Thr\textsuperscript{127} \rightarrow Leu and Ser\textsuperscript{128} \rightarrow Ala mutations are at the subunit interface of CRP. However, as shown in Table II, the interaction energy, \(\Delta G_{\text{CRP-RNAP}}^0\), which is the difference in the free energy of CRP binding to the promoter alone (−39.6 to −35.4 kJ mol\textsuperscript{−1}) and to the promoter with bound RNA polymerase (−42 to −39.4 kJ mol\textsuperscript{−1}), ranges from −4.6 kJ mol\textsuperscript{−1} for cAMP-ligated CRP\textsuperscript{a} to −1.8 kJ mol\textsuperscript{−1} for cAMP-ligated CRP. Since the protein-protein interaction between the RNA polymerase and the CRP is expected to be hydrophobic and using an effective hydrophobicity of 150 J

| TABLE II |
| Comparison of the enhancement factors with the cAMP-ligated CRP/mutant binding affinities to the 104-bp syncon promoter with and without bound RNA polymerase and to a 44-bp consensus duplex at 25 °C |

The binding constants were determined from ITC measurements on the binding of the cAMP-ligated CRP/mutant to the 104-bp syncon promoter with bound RNA polymerase \(K_p (104 \text{ bp} + \text{RNAP})\), on the binding of the cAMP-ligated CRP/mutant to the syncon promoter alone \(K_p (104 \text{ bp})\), and on the binding of the cAMP-ligated CRP/mutant to a 40-bp consensus DNA duplex \(K_p (40 \text{ bp})\). The \(K_p (40 \text{ bp})\) values are from Ref. 5.

| cAMP-ligated CRP/mutant | \(\epsilon\) | \(K_p (104 \text{ bp} + \text{RNAP}) \Delta G_{\text{CRP}}^0 (104 \text{ bp} + \text{RNAP}) | \(K_p (104 \text{ bp}) \Delta G_{\text{CRP}}^0 (104 \text{ bp}) | K_p (40 \text{ bp}) \Delta G_{\text{CRP}}^0 (40 \text{ bp}) |
|------------------------|-----------------|------------------|------------------|------------------|
| cAMP-ligated CRP | 13 ± 1 | 1.8 ± 0.03 | 41.4 ± 0.6 | 0.86 ± 0.22 | 39.6 ± 0.6 | 0.66 ± 0.11 |
| cAMP-ligated T127L | 12.1 ± 0.1 | 1.6 ± 0.4 | 41.1 ± 0.6 | 0.42 ± 0.07 | 37.8 ± 0.4 | 0.34 ± 0.05 |
| cAMP-ligated S128A | 12.4 ± 0.4 | 2.2 ± 1.2 | 42 ± 1 | 0.17 ± 0.03 | 35.4 ± 0.4 | 0.12 ± 0.02 |
| cAMP-ligated CRP\textsuperscript{a} | 11.7 ± 0.5 | 0.80 ± 0.20 | 39.4 ± 0.6 | 0.16 ± 0.02 | 35.4 ± 0.3 | 0.11 ± 0.01 |

| TABLE III |
| The dependence of the enhancement factor on the concentration of cAMP-ligated CRP and CRP\textsuperscript{a} |

| Concentration | cAMP-ligated CRP | cAMP-ligated CRP\textsuperscript{a} |
|---------------|------------------|------------------|
| nm            |                 |                  |
| 10            | 6.9 ± 0.7       | 6.9 ± 0.7        |
| 20            | 11.8 ± 1.2      | 8.2 ± 0.8        |
| 40            | 12.5 ± 1.3      | 10.5 ± 1.1       |
| 80            | 13 ± 1          | 11.7 ± 0.5       |
| 160           | 14 ± 1          | 13.5 ± 1.0       |
the activation of transcription by increasing polymerase and the promoter-bound CRP. This would enhance moter by introducing additional interactions between the RNA polymerase and cAMP-ligated CRP*, exhibits a weaker binding interaction with the bound RNA polymerase, whereas cAMP-ligated CRP* exhibits a stronger binding interaction with the RNA polymerase. This is also evident with the synlac and syntal promoters, which bind with correspondingly weaker binding affinities, at least 1 order of magnitude weaker, to the cAMP-ligated CRP mutants (5) but exhibit enhancement factors that are lower than those of the cAMP-ligated CRP, which exhibits a stronger binding affinity to the promoter than cAMP-ligated CRP*. This results in nearly the same binding affinity within experimental error, 0.80 ± 0.20 to 2.2 ± 1.2 × 10^10 M^-1, of the cAMP-ligated CRP/mutants to the bound RNA polymerase on the promoter. This may compensate for a weak binding affinity to the promoter with a strong binding affinity to the bound RNA polymerase. However, other measurement efforts are under way to determine these binding affinities and confirm this compensatory effect between the promoter and RNA polymerase binding interactions of cAMP-ligated CRP, T127L, S128A, and CRP* with the synlac and syntal promoters. In addition, just how specific mutations in CRP affect its interaction with bound RNA polymerase awaits further investigation.

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