Position 127 Amino Acid Substitutions Affect the Formation of CRP:cAMP:*lac*P Complexes but Not CRP:cAMP:RNA Polymerase Complexes at *lac*P†

Sew-Fen Leu,‡ Cheryl H. Baker,§ Eun Ju Lee,|| and James G. Harman*,§

National Laboratory Animal Breeding and Research Center, National Science Council, Taipei, Taiwan, ROC, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, and Bioneer Corporation, High Tech Park, 124, Chuckbook-Rhi, Namee-Myun, Cheongwon-Kun, Chungbuk 363-810, Korea

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ABSTRACT: The *lac*P DNA binding and activation characteristics of CRP having amino acid substitutions at position 127 were investigated. Wild-type (WT) and T127C CRP footprinted *lac*P DNA in the presence of DNase I in a cAMP-dependent manner. The T127G, T127I, and T127S forms of CRP failed to footprint *lac*P both in the absence and in the presence of cAMP. Consistent with these data, WT and T127C CRP:cAMP complexes exhibited high affinity for the *lac*P CRP site whereas T127G, T127I, or T127S CRP:cAMP complexes exhibited low affinity for the *lac*P CRP site. CRP:cAMP:RNA polymerase (RNAP) complexes formed at *lac*P in reactions that contained WT, T127C, T127G, T127I, or T127S CRP. These results demonstrate that allosteric changes important for cAMP-mediated CRP activation are differentially affected by amino acid substitution at position 127. Proper cAMP-mediated reorientation of the DNA binding helices required either threonine or cysteine at position 127. However, cAMP-dependent interaction of CRP with RNAP was accomplished regardless of the amino acid at position 127. RNAP:*lac*P complexes that supported high-level *lac* RNA synthesis formed rapidly in reactions that contained WT or T127C CRP whereas RNAP:*lac*P complexes that supported only low-level *lac* RNA synthesis formed at slower rates in reactions that contained T127I or T127S CRP. The T127G CRP:cAMP:RNAP:*lac*P complex failed to activate *lac*P. The results of this study lead us to conclude that threonine 127 plays an important role in transduction of the signal from the CRP cyclic nucleotide binding pocket that promotes proper orientation of the DNA binding helices and only a minor, if any, role in the functional exposure of the CRP RNAP interaction domain.

The cyclic 3′,5′-adenosine monophosphate (cAMP)[1] receptor protein (CRP) complexed with cAMP binds to specific sequences located upstream of a number of bacterial promoters and stimulates the formation of active transcription initiation complexes composed of CRP, cAMP, DNA, and RNA polymerase (RNAP) (*1, 2*). Biochemical analyses have shown that cAMP binding to CRP induces conformation changes in the protein that mediate CRP activation as a transcription factor (reviewed in references *1* and *2*). Current understanding of the CRP activation process includes cAMP binding to mediate conformation changes that realign the CRP subunits (*3*−*6*), exposure or reorientation of the CRP DNA binding helices leading to site-specific CRP binding to DNA (*7*−*9*), and a functional exposure or realignment of a short surface loop important for the interaction of CRP with RNAP (*10*−*16*).

Detailed secondary and tertiary structure information for the CRP:$(cAMP)_2$ complex is available (*4*). Interpretation of experimental data in the context of this structure information has contributed significantly to our understanding of CRP structure and function; however, details of the mechanism by which cAMP mediates allosteric activation of CRP remain obscure. Our laboratory (*19, 20*) has confirmed predictions that amino acid residues at positions 72, 82, 83, 127, and 128 play a role in CRP/cAMP interaction and in cAMP-mediated changes in CRP structure (*4*). Amino acid residues glutamate 72 and arginine 82 play a role in establishing the affinity of CRP for cAMP (*19*). Amino acid residues serine (S) 83, threonine (T) 127 and S128 play little, if any, role in cAMP binding but participate in mediating the allosteric effects of cAMP that activate CRP (*20, 21*). Amino acid substitutions at position 127 confer unique properties on CRP structure and on cAMP-mediated activation of CRP as a transcription factor. Threonine 127 plays an important role in maintaining the protease-resistant CRP hinge conformation in the absence of effector. Substitution of cysteine (C), glycine (G), isoleucine (I), or serine for threonine 127 produced forms of CRP that bind cAMP with affinities similar to that of wild-type (WT) CRP yet have hinge-region conformations, in the absence of cAMP, that are similar to that of the WT CRP:cAMP complex (*20*). Despite these similarities, individual position 127 substituted

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* To whom correspondence should be addressed. Phone: 806-742-1297. Fax: 806-742-1289. E-mail: u4jgh@pop.ttu.edu.

‡ National Laboratory Animal Breeding and Research Center.

§ Texas Tech University.

|| Bioneer Corporation.

[1] Abbreviations: cyclic AMP or cAMP, cyclic 3′,5′-adenosine monophosphate; CRP, cyclic AMP receptor protein; *lac*P, lactose operon promoter; RNAP, RNA polymerase; ANS, 8-anilino-1-naphthalenesulfonic acid.

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forms of CRP exhibited large differences in supporting *lac*P activity in vivo (*20*). We report here in vitro analysis of position 127 substituted forms of CRP. The results of this study define the conditions under which ternary complexes form between position 127 substituted CRP, cAMP, and RNAP at *lac*P and the efficacy of these complexes in in vitro *lac* transcription assays. The results of this study demonstrate that the amino acid residue at position 127 affects reorientation of the CRP DNA binding helices in response to cAMP binding. Amino acid substitution at position 127 has little effect on CRP:cAMP complex-mediated formation of RNAP:*lac*P complexes; however, the transcription efficacy of these complexes is shown to be dependent upon the specific amino acid residue at position 127.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Escherichia coli strains used in this study were MZ1 (*his*, *ilv*, *rsp*L, *gal*Kam, *pg*/D8 (*bio-uvr*B), ΔH1) (*22*) and AG1 (F^-, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rk^-, mk^+), *sup*E44, *rel*A1). pRK248cI^{ts}, which encodes a temperature-sensitive λcI repressor and a tet^r determinant, was the gift of D. Helinski (*23*). Plasmid pKL201 (*24*) was used as template for in vitro transcription reactions.

Enzymes and Other Materials. Restriction enzymes, Bio-Rex 70 resin, hydroxylapatite resin, Bio-Gel A-1.5m resin, and 10−20% precast polyacrylamide gradient gels were obtained from Bio-Rad laboratories. Radiolabeled nucleotides α-^{32}P-labeled UTP and γ-^{32}P-labeled ATP were purchased from DuPont/New England Nuclear, nucleoside and deoxynucleoside triphosphates were purchased from Pharmacia, *Taq* and *Pfu* DNA polymerases were purchased from Promega, and the DNA Megalabel kit was purchased from Panvera Corporation. Cyclic AMP, 8-anilino-1-naphthalenesulfonic acid (ANS), ovalbumin, double-stranded and single-stranded DNA-cellulose and CM-sephadex were purchased from Sigma Chemical Co. Synthetic oligonucleotides were synthesized by the Texas Tech University Biotechnology Institute Core Facility. X-ray film (BMR-2) was purchased from Eastman Kodak. Common salts were reagent grade or better.

Proteins. (a) CRP Isolation. CRP was induced by heat inactivation of λcI^{ts} in cultures of MZ1 transformed with pRE2*crp* that contained either the wild-type or the appropriate mutant *crp* allele. CRP was purified by sequential chromatography on Bio-Rex 70 and hydroxylapatite (HTP) as described by Harman et al. (*24*). At this stage of purification WT CRP was judged to be greater than 95% pure on coomassie blue stained SDS−polyacrylamide gels. The position 127 substituted forms of CRP were further purified over CM-Sephadex (*24*). These proteins were greater than 95% pure as judged from coomassie blue stained SDS−polyacrylamide gels. CRP concentrations were determined using an extinction coefficient of 3.5×10^4 M^{-1} cm^{-1} at 279 nm.

(b) RNA Polymerase Isolation. Isolation of RNA polymerase from *E. coli* strain AG1 was accomplished as described by Burgess and Jendrisak (*25*) as modified by Lowe et al. (*26*).

DNase I Protection Assay. DNase I footprinting reactions were carried out in a 100 μL volume as described by Tagami and Aiba (*27*). Single-strand labeled *lac* DNA at 2.5 nM, CRP at 100 nM, and as indicated, cAMP at 100 μM were combined and incubated at 37 °C for 5 min. RNAP was then added to 100 nM and the mixture incubated at 37 °C for 30 min. DNase I was added to a final concentration of 50 μg/mL. The tubes were incubated for 1 min at room temperature and the reactions terminated by addition of 25 μL of a solution that was 1.5 M in sodium acetate, 20 mM in EDTA, and contained 100 μg/mL tRNA. DNA was extracted in phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, air-dried, dissolved in loading buffer (7 M in urea, 0.025% in bromophenol blue, 0.025% in xylene cyanol), and separated by electrophoresis on 10% acrylamide gels 7 M in urea. Dried gels were exposed to BMR-2 X-ray film.

DNA Fragment Preparation, Isolation, and Labeling. Amplification of a *lac*P DNA fragment was accomplished in 50 μL PCR reaction mixtures that were 0.2 mM in each of the deoxynucleoside triphosphates, 2.0 mM in $MgCl_2$, 0.5 μM in primer, and 50 fg/μL in pKL201 and contained both 28 units of *Taq* DNA polymerase and 1.75 units of *Pfu* DNA polymerase. The PCR primers were CTGAGCCCGGCCAAGCTTACTCCCCAT and ACTGGAGGGAATTCGTAATCATGGTCATAGC that amplified 220 base pairs (bp) of the pKL201 *lac* sequence between positions −154 and +66 relative to the transcription start site.

Reactions were run in a Perkin-Elmer Cetus thermal cycler for 25 cycles. Each cycle had a 30 s denaturation step at 94 °C, a 30 s annealing step at 59 °C, and a 15 s elongation step at 72 °C. The DNA was isolated on a Qiagen column and further purified on 5% nondenaturing polyacrylamide gels, visualized by UV-shadowing (*28*), excised from the gel, and eluted in 0.3 M sodium acetate at 37 °C. The DNA was ethanol-precipitated, washed with 70% ethanol, dried, and dissolved in TE buffer. The DNA was end-labeled with γ-^{32}P ATP (10 mCi/mL; specific activity: 3000 Ci/mmol) using polynucleotide kinase at 37 °C for 1 h. The kinase was heat-inactivated at 75 °C for 30 min, and labeled DNA was purified on a Qiagen column to eliminate unincorporated label. The *lac*P DNA was digested with *Hin*d III to yield a 202 bp bottom-strand-labeled *lac*P fragment.

The ^{32}P-labeled DNA fragment pool generated from PCR reactions primed with the *lac* primer set contained two radioactive bands that copurified with the 202 bp *lac*P fragment. These ^{32}P-labeled species were primer-dependent, resistant to DNase I, exonuclease VII, and RNase A (data not shown). Their presence did not interfere with the DNase I footprint assay, and we made no attempt to eliminate these species from the *lac* fragment pool.

DNA Binding Assay. ^{32}P-labeled duplex DNA was prepared as described (*12*) from complementary, single-stranded oligonucleotides. CRP:DNA binding assays were carried out in 500 μL volumes. CRP affinity was determined for a 41 bp DNA template that contained the *lac* CRP binding site (LCAP; GCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCC). Duplex DNA at 27 pM was equilibrated in transcription buffer [30 mM in Tris-HCl (pH 8.0), 2.5 mM in $MgCl_2$, 0.1 mM in EDTA, 0.1 mM in DTT, 100 mM in KCl] that contained 5 μg/mL BSA, CRP at the indicated concentration, and cAMP at 100 μM for 60 min

at 23 °C. The mixtures were filtered through 25 mm 0.45 μm nitrocellulose filters (Micro Filtration Systems). The amount of DNA bound to the filter was determined using conventional liquid scintillation counting techniques and corrected for background DNA binding to the filter. CRP DNA association constant (k_{app}^{DNA}) values for the reaction CRP:(cAMP)$_n$ + DNA ↔ CRP:(cAMP)$_n$:DNA where n = 1 or 2 were determined by fitting DNA binding data to eq 1 (*3*)

$$\Delta P = \frac{P k_{app}^{DNA}[CRP]}{1 + k_{app}^{DNA}[CRP]} \quad (1)$$

where P is the background-corrected amount of DNA bound to the filter at a given concentration of CRP.

Cyclic AMP Binding Assay. Fluorescence titration experiments were conducted at 23 °C on protein samples that had been dialyzed against 50 mM Tris-HCl (pH 7.8), 0.1 M KCl, and 1 mM EDTA. All fluorescence measurements were performed on a SPEX FluoroMax spectrofluorometer using DM3000F software. The binding of cAMP to ANS−CRP was studied by the sequential addition of 2−5 μL volumes of concentrated cAMP to a 1.0 mL solution of CRP (5 μM) that contained 10−20 μM ANS. The excitation and emission wavelengths were set at 350 and 480 nm, respectively. The data were corrected for the fluorescence of free ANS in cAMP titration experiments where CRP was omitted from the buffer solution. Changes in intrinsic tryptophan fluorescence were studied by the sequential addition of 2−3 μL volumes of concentrated cAMP to a 1.0 mL solution of CRP (5 μM). The data were corrected for fluorescence of ovalbumin in cAMP titration experiments (3). The excitation and emission wavelengths for tryptophan fluorescence experiments were 300 and 343 nm, respectively. All fluorescence titration data were corrected for solution volume changes.

We analyzed ANS cAMP titration data, where two components were evident in the cAMP binding isotherm, according to eq 2 (*3*)

$$\Delta P_{obs} = \frac{\Delta P_1 K_1[cAMP] + \Delta P_2 K_2[cAMP]^2}{1 + K_1[cAMP] + K_2[cAMP]^2} \quad (2)$$

where K_1 and K_2 are Adair constants for the formation of CRP(cAMP)$_1$ and CRP(cAMP)$_2$ according to the relationship (*3*)

$$\text{apo-CRP} + \text{cAMP} \overset{K_1}{\rightleftharpoons} \text{CRP(cAMP)}_1 + \text{cAMP} \overset{K_2}{\rightleftharpoons} \text{CRP(cAMP)}_2$$

Cyclic AMP association constant values k_{app}^{cAMP1} and k_{app}^{cAMP2} were determined from the ANS−CRP titration studies given the relationships $K_1 = 2k_{app}^{cAMP1}$ and $K_2 = k_{app}^{cAMP1} k_{app}^{cAMP2}$. The cAMP association constant k_{app}^{cAMP2} was determined directly from intrinsic tryptophan fluorescence studies by fitting the data to eq 1, substituting the terms [cAMP] for [CRP] and k_{app}^{cAMP2} for k_{app}^{DNA} (*3*). All titration data were fitted to eq 1 or eq 2 using a nonlinear regression program running the Marquart algorithm.

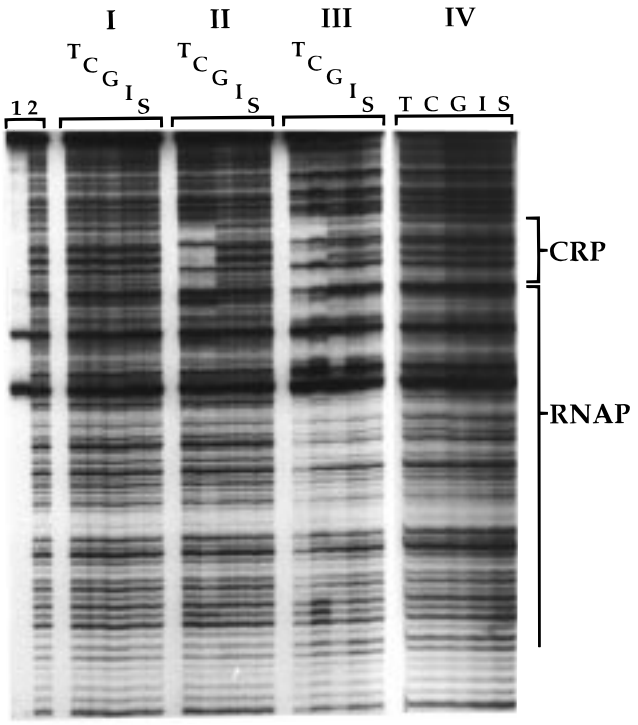

FIGURE 1: DNase I footprinting of *lac*P. Reactions were run in the presence of CRP (series I), CRP and cAMP (series II), CRP, cAMP, and RNAP (series III), or CRP and RNAP (series IV). Final concentrations of CRP, cAMP, and RNAP were 100 nM, 100 μM, and 100 nM, respectively. Control designations are: lane 1, untreated DNA; lane 2, DNA treated with DNase I. Lanes for individual groups were loaded, from left to right sequentially, with samples from reaction mixtures that contained WT CRP (T), T127C CRP (C), T127G CRP (G), T127I CRP (I), or T127S CRP (S). The CRP and RNAP binding sites in *lac*P DNA are indicated.

In Vitro Transcription Assay. Transcription reactions were carried out in a 10 μL volume. Reaction mixtures were 30 mM in Tris-HCl (pH 8.0), 2.5 mM in $MgCl_2$, 0.1 mM in EDTA, 0.1 mM in DTT, and 100 mM in KCl and contained 500 μg/mL BSA. Reaction mixtures that contained DNA at 2.5 nM, CRP at 250 nM, and cAMP at 5 μM were incubated at 37 °C for 30 min. Prewarmed RNAP was added to the reactions at a final concentration of 100 nM to initiate the formation of RNAP:promoter complexes. At selected time intervals after the addition of RNAP, prewarmed nucleoside triphosphates (ATP, GTP, and CTP at 0.25 mM each, UTP at 50 μM, α-^{32}P-UTP at 5 nM) and heparin to 0.1 mg/mL were added to promote a single round of transcription from preformed RNAP:*lac*P open complexes and to prevent the formation of additional RNAP:*lac*P complexes. Transcript elongation was allowed to proceed for 15 min at 37 °C and then terminated by addition of an equal volume of a solution 10 M in urea and 0.04% in bromophenol blue. The solution was then heated to 75 °C for 5 min. RNA was resolved on 6% polyacrylamide gels that were 7 M in urea, visualized by autoradiography, and analyzed by densitometry. The *lac* and *rep* RNA bands were quantitated from the peak areas.

RESULTS

Figure 1 shows the results of DNase I footprint reactions that contained the WT or the position 127 substituted forms of CRP in the absence of cAMP (series I), in the presence of cAMP (series II), in the presence of cAMP and RNAP (series III), or in the presence of RNAP alone (series IV).

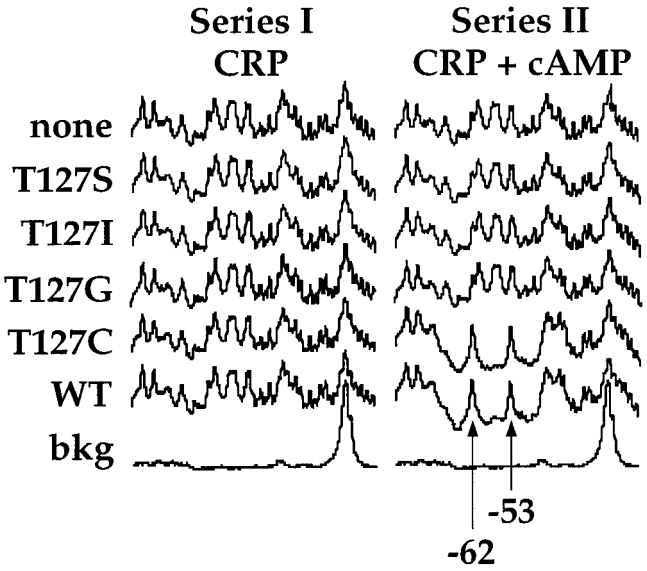

FIGURE 2: Densitometer tracings of the *lac*P CRP binding site. Densitometer tracings are plotted as area under the peak (vertical axis) versus position on the gel (horizontal axis). The designation "bkg" denotes the tracing of the background (Figure 1, lane 1); the designation "none" denotes the tracing of the fragments generated by incubating *lac*P DNA with DNase I in the absence of other proteins (Figure 1, lane 2). Tracings of the reaction products run in the presence of CRP (series I) and CRP and cAMP (series II) are displayed as a vertical array for purposes of comparison.

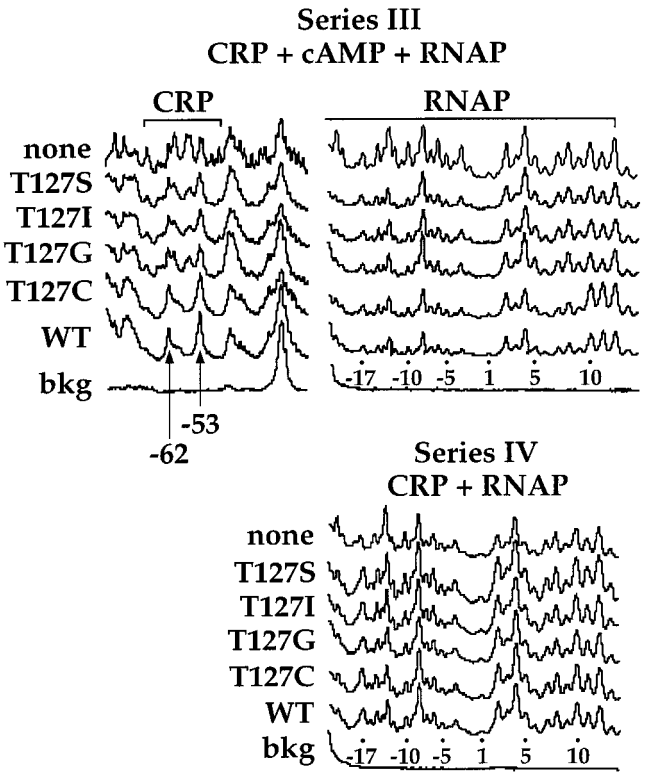

FIGURE 3: Densitometer tracings of the *lac*P CRP and RNAP binding sites. Display of the densitometer tracings is as described in the legend to Figure 2. Tracings of the reaction products run in the presence of CRP and RNAP (Figure 1, series IV) and CRP, cAMP, and RNAP (Figure 1, series III) are displayed as a vertical array for purposes of comparison. The data extend from positions −21 to +13 relative to the transcription start site. Tracings of the series III reaction products were normalized to a band located downstream of position +35. Normalization factors were as follows: WT (1.0), T127C (0.7), T127G (0.92), T127I (0.68), and T127S (0.8). The series IV reaction products displays did not require normalization.

Neither the WT nor the position 127 substituted forms of apo-CRP protected the *lac*P CRP binding site from DNase I digestion (series I and IV). CRP:cAMP complex footprints were observed in those reactions that contained WT CRP or the T127C CRP (series II). These data demonstrate cAMP-mediated WT CRP binding to *lac*P that produced a DNase I footprint extending from position −44 to position −73. The footprint contained hypersensitive phosphodiester bonds between positions −52/53/54 and −61/62/63. Further, these data show that cAMP induced CRP site-specific DNA binding in reactions that contained T127C CRP but not in those that contained the T127G, T127I, or T127S forms of CRP (Figures 1 and 2).

Phosphodiester bonds in both the *lac* CRP binding site and the RNAP binding site were protected from DNase I-mediated hydrolysis in reaction mixtures that contained CRP, cAMP, and RNAP regardless of the CRP included in the reaction (series III, Figures 1 and 3). Three fine-structure details of the *lac*P:CRP:cAMP:RNAP ternary complexes are revealed in these gels. First, the DNase I cleavage pattern of the DNA sequences upstream of the CRP binding site indicates that all of the ternary complexes were associated with upstream DNA (Figure 1, *29*). Second, densitometer tracing of the DNase I gels showed that for all series III reactions the RNAP binding site protection between positions −17 to +1 was equivalent (Figure 3). However, CRP binding site protection was incomplete in reactions that contained the T127G, T127I, or T127S CRP:cAMP complexes (Figure 3). In these reactions the phosphodiester bonds between positions −52/53/54 and −61/62/63 were less sensitive to DNase I digestion, and adjacent bonds were less protected in those reactions that contained the T127G, T127I, or T127S CRP compared to those reactions that contained the WT or T127C CRP. Third, the RNAP footprint extended beyond +1 to position +9 in those reactions that contained the WT or T127C CRP:cAMP complexes (Figure 3). This indicates that the interactions between the T127G, T127I, or T127S CRP and the *lac*P CRP binding site differ from those of the WT and T127C CRP. In addition, the ternary complexes that contained the T127G, T127I, or T127S CRP differed from those that contained either the WT or T127C CRP.

The series III footprint results clearly show that RNAP promoted the formation of ternary complexes at *lac*P in reaction mixtures that contained position 127 substituted forms of CRP and cAMP. If cAMP was omitted from the reactions, these ternary complexes did not form (Figures 1 and 3, series IV).

To extend the DNase I footprint results we have measured the affinity of CRP for the *lac* CRP binding site contained on a 41 base pair duplex DNA (LCAP) (Figure 4, Table 1). The apo form of WT CRP bound LCAP DNA with an apparent association constant (k_{app}^{LCAP}) of 1.3×10^5 M^{-1}. The apo forms of the T127C, T127G, T127I, and T127S CRP bound LCAP DNA with affinities that were 1.5−8.5 times that of WT CRP. The WT and T127C CRP:cAMP complexes bound LCAP DNA with 192−546 times greater affinity than was observed for apo-WT CRP having k_{app}^{LCAP} values of 2.5×10^7 M^{-1} and 7.1×10^7 M^{-1}, respectively—values that agree well with those reported previously (*10*,

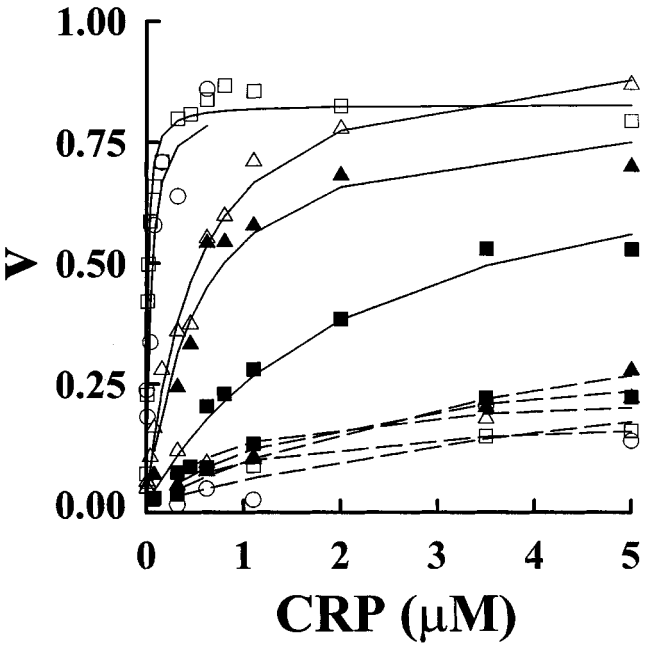

FIGURE 4: CRP:cAMP:LCAP complex formation. Reactions were as described in Materials and Methods. Reactions contained CRP at the indicated concentration, LCAP at 27 pM, and cAMP at 100 μM. Reactions were performed using WT CRP (open circles), T127C CRP (open squares), T127G CRP (closed squares), T127I CRP (open triangles), and T127S CRP (closed triangles). The data were fit to eq 1. The solid lines fit the data from reactions run in the presence of cAMP. The dashed lines fit the data from reactions run in the absence of cAMP. Each data point is the average of duplicate experiments. The values for k_{app}^{LCAP} are presented in Table 1.

30). The increased affinity of WT and T127C CRP for LCAP DNA was accompanied by increased sequence specificity in DNA binding (Figure 1). In contrast to these results, the T127G, T127I, and T127S CRP:cAMP complexes bound LCAP DNA with affinities that were only 3−15 times that observed for WT apo-CRP. These data, along with the DNase I footprint results (Figures 1 and 2), demonstrate that the substitution of glycine, isoleucine, or serine for threonine 127 interferes with the cAMP-mediated increase in CRP affinity for, if not all CRP-specific DNA sequences, at least the *lac* CRP binding site. Substitution of cysteine for threonine 127, on the other hand, does not interfere with this aspect of cAMP-mediated CRP activation.

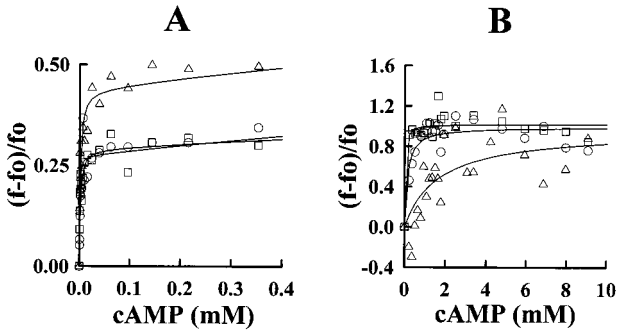

FIGURE 5: Binding of cAMP to the WT and position 127 substituted forms of CRP. Panel A. Fluorescence of the ANS−CRP complexes as a function of cAMP concentration. The data were fit to eq 2. Panel B. Intrinsic CRP tryptophan fluorescence as a function of cAMP concentration. The data were fit to eq 1. Reactions were performed using WT CRP (open circles), T127C CRP (open squares), and T127I CRP (open triangles). Each data point is the average of duplicate experiments. The values for k_{app}^{cAMP1} and k_{app}^{cAMP2} are presented in Table 1.

WT CRP binds two molecules of cAMP in a manner that exhibits negative cooperativity with apparent association constants, k_{app}^{cAMP1} and k_{app}^{cAMP2}, of 2.5×10^4 and 1.0×10^3 M^{-1} (*3*). To address the effect of position 127 amino acid substitutions on CRP cAMP binding affinity we have measured the cAMP binding characteristics of the WT, T127C, and T127I CRP (Figure 5A, Table 1). The values of k_{app}^{cAMP1}, obtained from ANS−CRP fluorescence measurements, were 3.3×10^5, 1.2×10^6, and 3.2×10^5 M^{-1} for WT, T127C, and T127I, respectively. Our value of k_{app}^{cAMP1} for WT CRP is 1 order of magnitude greater than that reported by Heyduk and Lee (*3*). The values of k_{app}^{cAMP2}, obtained from both ANS−CRP and intrinsic tryptophan fluorescence measurements, were 3.4×10^3, 1.8×10^4, and 5.3×10^2 M^{-1} for WT, T127C, and T127I, respectively (Figure 5B, Table 1). Our value for k_{app}^{cAMP2} for WT CRP is in good agreement with that reported by Heyduk and Lee (*3*). The cAMP binding data confirm our earlier conclusion that amino acid substitution at position 127 has little effect on apo-CRP affinity for cAMP; k_{app}^{cAMP1} varied not more than 4-fold (*20*). Amino acid substitution at position 127 did affect CRP affinity for binding the second cAMP molecule. Here, k_{app}^{cAMP2} values varied by 34-fold. We note that this value underestimates the difference in k_{app}^{cAMP2} that exists between

Table 1: Summary of Binding Properties for the WT, T127C, T127G, T127I, and T127S CRP[a]

	k_{app}^{LCAP}					CRP population[c]		
CRP	no cAMP	100 μM cAMP	k_{app}^{cAMP1}	k_{app}^{cAMP2} [a]	$k_{app}^{cAMP1}/k_{app}^{cAMP2}$	apo	1	2
WT	$(1.3 \pm 3.9) \times 10^5$	$(2.5 \pm 1.1) \times 10^7$	3.3×10^5	3.4×10^3	97	3	72	25
C	$(8.8 \pm 2.9) \times 10^5$	$(7.1 \pm 0.8) \times 10^7$	1.2×10^6	1.8×10^4	67	1	35	64
G	$(4.8 \pm 1.6) \times 10^5$	$(4.5 \pm 1.0) \times 10^5$	-	-	-	-	-	-
I	$(1.1 \pm 0.7) \times 10^6$	$(2.0 \pm 0.2) \times 10^6$	3.2×10^5	5.3×10^2	604	3	92	5
S	$(1.9 \pm 0.8) \times 10^5$	$(1.9 \pm 0.5) \times 10^6$	-	-	-	-	-	-

[a] Apparent association constants k_{app}^{LCAP} and k_{app}^{cAMP2} were determined from fits of the data presented in Figures 4 and 5B to eq 1. The apparent association constants k_{app}^{cAMP1} and k_{app}^{cAMP2} were determined from fits of the data presented in Figure 5A to eq 2. For the ANS data, k_{app}^{cAMP1} and k_{app}^{cAMP2} were determined given the following relationships: $K_1 = 2k_{app}^{cAMP1}$ and $K_2 = k_{app}^{cAMP1} k_{app}^{cAMP2}$. Values for K_1 determined from the ANS data were as follows: WT CRP, $(6.6 \pm 1.3) \times 10^5$; T127C CRP, $(2.3 \pm 0.7) \times 10^6$; T127I CRP, $(6.4 \pm 1.1) \times 10^5$. Values for K_2 determined from the ANS data were as follows: WT CRP, $(5.8 \pm 3.9) \times 10^7$; T127C CRP, $(4.1 \pm 2.4) \times 10^8$; T127I CRP, $(1.6 \pm 0.8) \times 10^8$. Errors are expressed as one standard deviation. [b] k_{app}^{cAMP2} is the average of the values determined from the analysis of both the ANS and tryptophan fluorescence data. [c] CRP populations, expressed as a percentage of the total, were determined from the relationships $CRP(cAMP)_n = k_{app}^{cAMP1}[cAMP]/(1 + k_{app}^{cAMP1}[cAMP])$ where $n = 1$ or 2, and $CRP(cAMP)_2$ $k_{app}^{cAMP2}[cAMP]/(1 + k_{app}^{cAMP2}[cAMP])$. CRP populations were determined at 100 μM cAMP.

these proteins. While the WT and T127C fluorescence data are accurately fit by eq 1, the T127I tryptophan fluorescence data are not. Cyclic AMP binding to the apo-WT and apo-T127C CRP has no effect on CRP tryptophan fluorescence until the second cAMP binding site begins to fill, at which point tryptophan fluorescence increases proportionally with cAMP concentration (*3*). Intrinsic tryptophan fluorescence of apo-T127I CRP did not display this characteristic in that tryptophan fluorescence decreased by 32% between the cAMP concentrations of 0−200 μM and subsequently increased at higher concentrations of cAMP. We interpret this as evidence that the formation of T127I CRP:$(cAMP)_1$ quenches intrinsic tryptophan fluorescence. This yields a cAMP binding isotherm that, when fit to eq 1, overestimates k_{app}^{cAMP2}.

To evaluate the efficacy of the ternary complexes formed at *lac*P in the presence of CRP, cAMP, and RNAP we quantitated full-length *lac* RNA and *rep* RNA produced in an in vitro *lac* transcription system composed of purified components and containing supercoiled pKL201 as template (*24*). In these reactions the *rep* transcript, originating from a CRP-independent promoter, served to normalize individual reactions in a reaction series and to normalize reaction series. Transcription reactions that contained WT CRP, T127C, T127G, T127I, or T127S CRP were found to yield equivalent amounts of *rep* transcript in the presence or absence of cAMP (data not shown). The WT CRP:cAMP complex reaction series produced maximum *rep*P activity within 30 s after the addition of RNAP; there was little variation in the absolute amount of *rep* transcript produced when the time for open complex formation was varied from 30 s to 60 min (data not shown). Similar results were obtained from reactions that contained the position 127 substituted forms of the protein, indicating that none of the CRP:cAMP complexes had either a specific or patterned time-dependent effect on *rep*P activity.

The rates of formation of the open complexes formed between RNAP and *lac*P DNA, expressed as the *lac*/*rep* ratio, are shown in Figure 6. Low-level *lac*P activity was observed in control reactions that did not contain CRP. Reactions that contained either WT CRP or T127C CRP and cAMP rapidly formed open complexes at *lac*P and produced *lac/rep* ratios 14 times those observed in control reactions. These levels of open complex were maintained for 20 min and then decreased with increasing incubation time. Reactions that contained the T127G CRP and cAMP produced levels of *lac* RNA comparable to those of control reactions. The *lac*/*rep* ratio in reactions that contained either T127I or T127S CRP and cAMP, however, progressively increased production of *lac* RNA over a period of 15 min, maintained for 15 min a *lac*/*rep* ratio only twice that of control reactions, and then decreased in activity with increasing incubation time. These data are completely consistent with our in vivo data presented earlier (*20*) and show that cysteine substitution of threonine 127 yields a form of CRP that, in the presence of cAMP, activates *lac*P. Substitution of glycine, serine, or isoleucine for threonine 127 yields forms of CRP that either fail to activate *lac*P or promote only marginal *lac*P activity in the presence of cAMP.

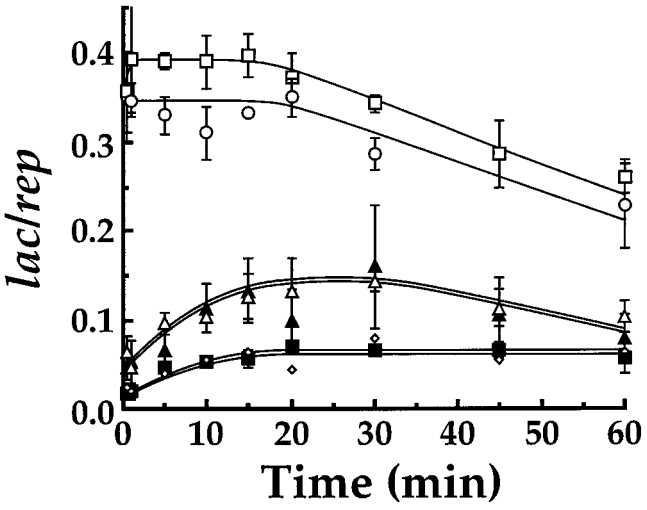

FIGURE 6: Time course display of the *lac/rep* ratio. Reaction mixtures were incubated with RNAP at 37 °C for the indicated times prior to addition of nucleoside triphosphates and heparin. Autoradiographs of the RNA products separated on 6% polyacrylamide gels 7 M in urea were scanned on a laser densitometer, and the *lac* and *rep* RNA bands quantitated. Reactions were performed using WT CRP (open circles), T127C CRP (open squares), T127G CRP (closed squares), T127I CRP (open triangles), and T127S CRP (closed triangles). Each data point is the average of three separate experiments; one standard deviation of the averaged data is shown by the vertical error bar.

DISCUSSION

The data presented in this report confirm and extend previous studies regarding the effect of position 127 amino acid substitutions on CRP (*20*). Apo-CRP that contains a position 127 amino acid substitution binds cAMP with affinity similar to that of WT CRP (Table 1, Figure 5A, *20*, *32*). Gorshkova et al. (*32*) reported that leucine substitution for threonine 127 "switches off" cooperativity in cAMP binding without having an appreciable affect on k_{app}^{cAMP1}. In a system having *dependent* ligand binding sites, such as CRP, cooperativity between those sites may be qualitatively assessed by comparing the ratio of k_{app}^{cAMP1} and k_{app}^{cAMP2} (Table 1). Our study shows that while cysteine or isoleucine substitution for threonine 127 produced less than a 4-fold effect on k_{app}^{cAMP1}, the effects of these amino acid substitutions on k_{app}^{cAMP2} were varied. Using WT CRP as the standard, T127C CRP showed a 5-fold increase in k_{app}^{cAMP2} and T127I CRP showed a 6-fold decrease in k_{app}^{cAMP2}. Clearly, the amino acid at position 127 can either increase negative cooperativity between cAMP binding sites, as in the case of T127I CRP, or decrease negative cooperativity between cAMP binding sites, as in the case of the T127C CRP and the T127L CRP (*32*).

Complexed with cAMP, the position 127 substituted forms of CRP separate into three distinct categories (*20*). One category, represented by T127C CRP, stimulated *lac* mRNA (in vitro) and β-galactosidase synthesis (in vivo) to WT CRP levels. A second category, represented by the T127I and T127S CRP, stimulated *lac* mRNA and β-galactosidase synthesis to levels well below those observed for WT CRP yet significantly above background. The third category, represented by T127G CRP, failed to stimulate *lac* mRNA or β-galactosidase synthesis significantly above background levels. The results of our current study lead us to two

principle conclusions regarding the function of threonine 127 in cAMP-mediated CRP activation.

Conclusion 1. Threonine 127 Plays a Role in Orienting the DNA Binding Helices. Cyclic AMP binding to CRP results in a conformation change in CRP that renders the hinge region sensitive to proteases (*1*, *2*). This change in CRP conformation appears important for proper orientation of the DNA binding helices. Protease sensitivity of the hinge alone is not solely responsible for the differences between apo-CRP and CRP:cAMP complex DNA binding characteristics. The T127C, T127G, T127I, and T127S forms of CRP are, in the absence of cAMP, all more sensitive to protease than is WT CRP (*20*). Despite this, the position 127 substituted forms of CRP displayed significant differences in DNA binding characteristics (Figures 1, 2, and 4, Table 1). Only the WT and T127C CRP demonstrated increased affinity for LCAP DNA in the presence of cAMP and increased DNA site-specific binding in footprinting the *lac* CRP binding site. The amino acids glycine, isoleucine, or serine at position 127 rendered CRP unable to undergo the complete series of cAMP-mediated conformation changes required to properly orient the DNA binding helices. This conclusion is drawn from the fact that T127G, T127I, and T127S CRP:cAMP complexes failed to bind specifically to the *lac* CRP binding site.

Threonine 127 side chain structure is involved in maintaining the protease-resistant hinge conformation adopted by CRP in the absence of cAMP and in establishing a CRP DNA binding helices orientation that allows sequence-specific DNA interaction (Figures 1, 2, and 4, Table 1, *20*). In response to cAMP, the threonine side chain presumably repositions to promote a hinge conformation that is protease-sensitive and to reorient the DNA binding helices. Replacement of threonine 127 by glycine, serine, or isoleucine does not provide essential side chain structure to allow these residues to functionally substitute for threonine. Cysteine substitution of threonine 127 likely eliminates the potential for CRP hydrogen bond contact with the N^6 amino group of cAMP yet renders CRP fully capable of site-specific DNA binding upon binding cAMP. Complementarity of steric interactions may explain why cysteine can substitute for threonine at position 127. The amino acid side chain volumes of buried residues are 122, 118, 66, 168, and 99 $Å^3$ for threonine, cysteine, glycine, isoleucine, and serine, respectively (*31*). Buried cysteine and threonine side chains occupy similar volumes, within 3.3%, whereas the volumes of glycine, isoleucine, and serine differ from that of threonine by −46%, +38%, and −19%, respectively. An alternative explanation may reside in the fact that the T127C CRP subunits are cross-linked through a disulfide bond that is accessible to solvent only under denaturing conditions (*20*, C. H. Baker and J. G. Harman, unpublished). Definitive proof that the disulfide is important for T127C CRP:cAMP complex activity at *lac* awaits further investigation.

WT CRP:cAMP complex is required for *E. coli* to ferment lactose, mannitol, xylose, maltose, sorbitol, ribose, and melibiose (*2*, *3*, Table 2). Cells that contain the position 127 substituted forms of CRP show significantly different phenotypes. The T127C CRP:cAMP complex supports the fermentation of lactose, melibiose, and only weakly, fermentation of mannitol and xylose (Table 2). Cells that contain the T127G, T127I, or T127S CRP:cAMP complexes display essentially the CRP^- phenotype; T127S was found to support weak fermentation of mannitol. While cysteine substitutes for threonine 127 to yield a CRP variant that effectively mediates *lac*P activation, it is clear that the position 127 amino acid side chain requirements for CRP to function in a global manner are uniquely filled by threonine alone (*20*, Table 2).

Table 2: Fermentation Characteristics of *E. coli* 8445/pRK248 Containing the WT, T127C, T127G, T127I, or T127S CRP[a]

carbon source	WT	T127C	T127G	T127I	T127S
fructose + cAMP	+	+	+	+	+
	+	+	+	+	+
lactose + cAMP	−	−	−	−	−
	+	+	−	−	−
mannitol + cAMP	−	−	−	−	−
	+	±	−	−	±
xylose + cAMP	−	−	−	−	−
	+	±	−	−	−
maltose + cAMP	−	−	−	−	−
	+	−	−	−	−
sorbitol + cAMP	−	−	−	−	−
	+	−	−	−	−
ribose + cAMP	−	−	−	−	−
	+	−	−	−	−
melibiose + cAMP	−	−	−	−	−
	+	+	−	−	−

[a] Cells were plated on MacConkey agar plates containing the indicated fermentable carbon source at a final concentration of 1% and, where indicated, cAMP at a final concentration of 5 mM. The plates were incubated at 37 °C and scored for fermentation at 12−24 h after plating. The fermentation scores were as follows: +, red colonies; ± pink to pink with red centers; −, white colonies.

Conclusion 2. Threonine 127 Plays Little, If Any, Role in CRP Structure Changes Required for the Formation of CRP:cAMP:RNAP Complexes at lacP. Results presented in Figures 1 and 3 show that, in the presence of both cAMP and RNAP, all of the position 127 substituted forms of CRP promoted CRP:cAMP:RNAP:*lac*P complex formation. We know that the CRP:cAMP complex and RNAP interact (*15*−*18*). Zhou et al. (*17*) described a class of CRP mutant (CRP^{pc}) that results from amino acid substitutions in a carboxyl-proximal RNAP interaction loop. These mutants eliminate essential contacts important for CRP interaction with RNAP and CRP-dependent promoter activation without affecting CRP:cAMP complex binding to DNA (*17*). Changes induced in CRP^{pc} structure upon binding cAMP include reorientation of the DNA binding helices and exposure of a nonfunctional RNAP interaction domain. The T127G, T127I, and T127S forms of CRP upon binding cAMP display altered *lac*P DNA binding characteristics compared to WT CRP and expose a functional RNAP interaction domain. RNAP interaction with the T127G, T127I, and T127S CRP:cAMP complexes, which do not footprint DNA (Figure 1, series II), promotes the formation of CRP:cAMP:RNAP complexes at *lac*P (Figure 1, series III). This provides strong evidence that the allosteric signal required to reorient the DNA binding helices is, for the most part, distinct from the signal that functionally exposes the RNAP interaction loop.

Three cAMP-dependent conformations of CRP exist: apo-CRP, the CRP:$(cAMP)_1$ complex, and the CRP:$(cAMP)_2$ complex (*3*). Experimental evidence suggests that apo-CRP and the CRP:$(cAMP)_1$ complex are the biologically relevant forms of the protein in wild-type *E. coli* (*17*, *18*). We have utilized the cAMP association constants presented in Table

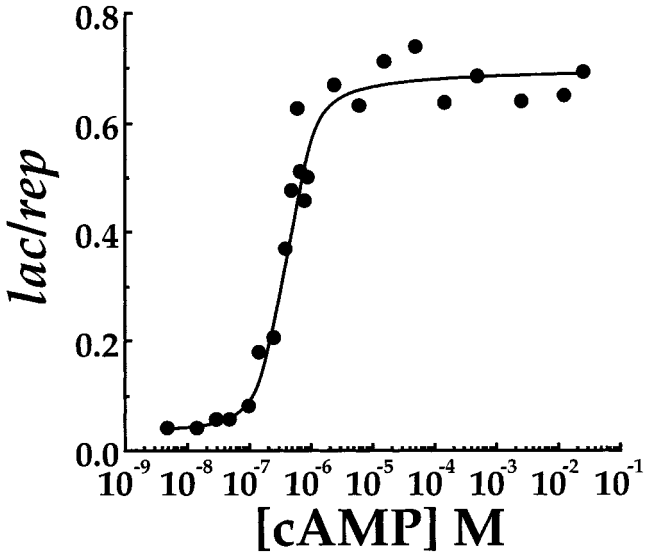

FIGURE 7: Effect of cAMP on the *lac/rep* ratio. Reaction mixtures were incubated with WT CRP, RNAP, and the indicated concentration of cAMP at 37 °C for 15 min prior to addition of nucleoside triphosphates and heparin. Autoradiographs of the RNA products separated on 6% polyacrylamide gels 7 M in urea were scanned on a laser densitometer and the *lac* and *rep* RNA bands quantitated.

1 to determine the relative populations of apo-CRP, CRP:$(cAMP)_1$, and CRP:$(cAMP)_2$ for the WT, T127C, and T127I CRP. In both the DNase I footprinting and DNA binding reactions conducted at a concentration of 100 μM cAMP, the predominant solution species was CRP:$(cAMP)_1$ for the WT and T127I CRP and CRP:$(cAMP)_2$ for the T127C CRP (Table 1). We note that the WT and T127C CRP:cAMP complexes yielded DNase I footprints that were indistinguishable (Figure 1). We conclude from this that either the CRP:$(cAMP)_1$ and CRP:$(cAMP)_2$ complexes behave similarly when bound to DNA, or only the CRP:$(cAMP)_1$ complex is bound to DNA under these reaction conditions. The latter is likely the correct interpretation considering that Heyduk et al. (*5*) determined that CRP:$(cAMP)_1$ has 100-fold greater affinity for the *lac* CRP binding site than does CRP:$(cAMP)_2$. Under the conditions utilized in the transcription reactions, cAMP at 5 μM, the predominant CRP species for all three forms of the protein was CRP:$(cAMP)_1$. To approach the question of whether the CRP:$(cAMP)_2$ complex affects the results of the in vitro transcription system we conducted a cAMP titration in reaction mixtures that contained WT CRP (Figure 7). The data show that cAMP concentrations between 100 nM and 2 μM dramatically affected the *lac/rep* ratio. Cyclic AMP concentrations as high as 25 mM, where 99% of the WT CRP solution population is CRP:$(cAMP)_2$, did not further affect the *lac/rep* ratio. We conclude that it is unlikely that the differences observed between the T127 substituted forms of CRP in the *lac* transcription reactions have their origin in differences in the stoichiometry of cAMP in the CRP:cAMP complex bound to the *lac* promoter.

The synthesis of *lac* mRNA requires protein:protein and protein:DNA interactions that are initiated by the cAMP-dependent interaction of CRP and RNAP followed by association of CRP:cAMP:RNAP and *lac*P to form a closed CRP:cAMP:RNAP:*lac*P complex (*11*,*14*). The series III DNase I footprint results (Figures 1 and 3) show that RNAP footprints were equally intense in the region −17 to +1 for reactions that contained the WT or any of the four mutant CRP:cAMP complexes. Despite this, the yield of full-length transcript was lower in the T127G, T127I, or T127S CRP-containing in vitro transcription reactions. On the basis of these observations we propose that the T127G, T127I, and T127S CRP produce a population of CRP:cAMP:RNAP:*lac*P complexes, only a fraction of which are competent to produce *lac* mRNA (Figure 6). This strongly suggests that the T127G, T127I, and T127S CRP fail to fully activate *lac*P due to a limitation in essential interactions that occur after the formation of the closed complex. It is known that once an *active* open complex is formed, RNAP has no further CRP:cAMP requirement to transcribe downstream *lac* DNA (*27*). This, coupled with the observed differences in the time required to reach maximum *lac/rep* ratios between reactions that contained the WT and T127C CRP and those that contained the T127G, T127I, or T127S CRP, suggests that the corresponding CRP:cAMP:RNAP:*lac*P closed complexes are affected in isomerization to open complexes. There is evidence to suggest that the CRP:RNAP interaction changes in the presence of promoter DNA and that closed complex isomerization to an *active* open complex has a component related to DNA structure (*16*, *33*). Considering that the T127G, T127I, and T127S CRP:cAMP complexes fail to protect completely the CRP binding site in the presence of RNAP (Figures 1 and 3, series III), it is likely that components important to the isomerization reaction are absent in ternary complexes that include T127G CRP and only partially present in ternary complexes that include T127I or T127S CRP.

Amino acid substitution at CRP position 127 yields mutant forms of the protein that, as a group, share important similarities in cAMP binding affinity, in hinge conformation, and in their ability to form ternary complexes with RNAP at *lac*P (*20*, this work). Individual representatives of the group display unique properties with respect to DNA binding and capacity to activate *lac*P. The experimental results presented here extend our earlier report of position 127 substituted CRP (*20*) and reveal two previously unrecognized aspects of the CRP activation process: first, CRP:cAMP DNA binding characteristics are dependent on the amino acid residue at position 127, and second, the functional exposure of the CRP RNAP interaction loop is not dependent on cAMP interaction with threonine 127. Relaxed effector specificity, similar to that displayed by many of the CRP* class of CRP mutant (*8*, *24*), is characteristic of the T127C CRP, the T127I CRP, and to a lesser degree the T127S CRP (*20*). The data presented in Figures 1 and 6 clearly show that for the position 127 substituted forms of CRP both the formation CRP:cAMP:RNAP complexes at *lac*P and the CRP-mediated *lac*P activity are strictly dependent upon cAMP. Position 127 substituted forms of CRP are not CRP*. All clearly mediate the formation of CRP:cAMP:RNAP complexes at *lac*P and, with the exception of T127C CRP, all are defective in mediating positive control of *lac*P. Position 127 substituted forms of CRP represent a unique subset of the CRP^{pc} class of *crp* mutants.

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