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Mutagenesis of the cyclic AMP receptor protein of *Escherichia coli*: targeting positions 83, 127 and 128 of the cyclic nucleotide binding pocket

Eun Ju Lee+, Joel Glasgow, Sew-Fen Leu, Ali O.Belduz§ and James G.Harman*
Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, USA

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ABSTRACT

The cyclic 3′, 5′ adenosine monophosphate (cAMP) binding pocket of the cAMP receptor protein (CRP) of *Escherichia coli* was mutagenized to substitute cysteine or glycine for serine 83; cysteine, glycine, isoleucine, or serine for threonine 127; and threonine or alanine for serine 128. Cells that expressed the binding pocket residue-substituted forms of CRP were characterized by measurements of β-galactosidase activity. Purified wild-type and mutant CRP preparations were characterized by measurement of cAMP binding activity and by their capacity to support *lac*P activation *in vitro*. CRP structure was assessed by measurement of sensitivity to protease and DTNB-mediated subunit crosslinking. The results of this study show that cAMP interactions with serine 83, threonine 127 and serine 128 contribute to CRP activation and have little effect on cAMP binding. Amino acid substitutions that introduce hydrophobic amino acid side chain constituents at either position 127 or 128 decrease CRP discrimination of cAMP and cGMP. Finally, cAMP-induced CRP structural change(s) that occur in or near the CRP hinge region result from cAMP interaction with threonine 127; substitution of threonine 127 by cysteine, glycine, isoleucine, or serine produced forms of CRP that contained, independently of cAMP binding, structural changes similar to those of the wild-type CRP:cAMP complex.

INTRODUCTION

The cyclic 3′, 5′-adenosine monophosphate (cAMP) receptor protein (CRP) of *Escherichia coli* is a dimeric protein that binds cyclic nucleotides. CRP conformation is altered in the presence of cAMP and the CRP:cAMP complex binds to specific DNA sequences (CRP sites) located in a number of *E.coli* promoter regions (1). Binding of the CRP:cAMP complex to promoter CRP sites leads to the formation of an active transcription initiation complex composed of CRP, cAMP, DNA and RNA polymerase (reviewed in 1 and 2).

Analysis of the CRP:$(cAMP)_2$ crystal structure identified five specific amino acid-ligand contacts that are likely to be important in cAMP binding to CRP in solution and/or in mediating cAMP activation of CRP (3). The charged phosphate of cAMP is aligned with arginine 82 to form a salt bridge, and specific hydrogen bond interactions occur between a) the axial phosphate oxygen atom of the 3′, 5′ cyclic phosphate ring and serine 83, b) the 2′ hydroxyl of ribose and glutamate 72, and c) the N^6 amino group of adenine and threonine 127 of one subunit and serine 128 of the other subunit. We have undertaken a study designed to assess the effects of specific amino acid substitutions at these positions to test the predictions of the CRP:$(cAMP)_2$ crystal structure analysis. An earlier publication from this laboratory reported the effects of amino acid substitutions at positions 72 and 82 on CRP function (4).

We report here the results of our analysis of CRP mutants with amino acid substitutions at positions 83, 127 and 128 of the cyclic nucleotide binding pocket. The effects of these substitutions on CRP function were assessed by measuring the capacity of mutant CRP, expressed in a $_{\Delta}cya$ $_{\Delta}crp$ strain of *E.coli*, to support β-galactosidase synthesis in the absence of cAMP or presence of cAMP or in the presence of cGMP. Purified CRP preparations were characterized by measurement of cAMP binding activity, and their structures were assessed by measurement of protease sensitivity and DTNB-mediated subunit crosslinking. The results of this study confirm the importance of serine 83, threonine 127 and serine 128 in cAMP-mediated activation of CRP.

MATERIALS AND METHODS

Bacterial strains and plasmid DNA

E.coli strains CA8445/pRK248 ($_{\Delta}cya$ 845, $_{\Delta}crp$ 45, *thi*/λcI^{ts}, *tet*^{r}) (5) and MZ1 (λcI^{ts}) (6) were used as hosts for recombinant *crp* plasmids. *E.coli* XL-1 Blue [λ$^-$, *rec* A1, *end* A1, *gyr* A96, *thi*, *hsd* 17, *sup* E44, *rel* A1, (*lac*), {F′, *pro* AB, *lac* I^q, *lac* Z_{Δ}M15, Tn10 (tet^r)}] (Stratagene) was used to propagate M13mp18 (7) and its *crp*-containing derivatives. *E.coli* strains MV1190 [$_{\Delta}$(*lac-pro*), *thi*, *sup* E, $_{\Delta}$(*srl-rec* A0306::Tn10 tet^r), {F′:*tra* D36, *pro* AB, *lac* I^q, *lac* Z_{Δ}M15}] (Bio-Rad

*To whom correspondence should be addressed

Present addresses: +Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024-1737, USA and §Karadeniz Technical University, Department of Biology, 61080-Trabzon, Turkey

Laboratories) and CJ236 [*dut*, *ung*, *thi*, *rel* A, {pCJ105 (cm^r)}] (Bio-Rad Laboratories) were used for mutagenesis of *crp*. Plasmid pRK248 (8) was the gift of D. Helinski, University of California at San Diego, La Jolla, CA. Plasmid pKC30*crp* (5) containing wild-type *crp* cloned downstream of the λP_L promoter was used as the source of the *crp* structural gene. Expression vector pRE2 (9) was the gift of P. Reddy, National Institute of Standards and Technology, Gaithersburg, MD. Plasmid pKL201 (5) was used as the DNA template for *in vitro* transcription reactions.

Materials

Restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were obtained from New England Biolabs. X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), and IPTG (isopropyl β-D-thiogalactopyranoside) were purchased from United States Biochemical Corporation, Inc. DNA sequencing kits were purchased from New England Biolabs and United States Biochemical Corporation, Inc. Calf intestine alkaline phosphatase was purchased from Promega. The Muta-Gene M13 *in vitro* mutagenesis kits, Bio-Rex 70, hydroxylapatite and Bradford protein assay reagent were purchased from Bio-Rad Laboratories. MacConkey indicator media, bactotryptone, casamino acids and yeast extract were purchased from Difco Laboratories. Cyclic nucleotides and subtilisin BPN′ (type XXVII, 7.9 units/mg) were purchased from Sigma Chemical Company. ^3H (2′-8′ labeled) 3′-5′ cAMP (31.3 Ci/mol), α-^{35}S ATP (500 Ci/mol), α-^{32}P ATP (3000 Ci/mol) and α-^{32}P UTP (3000 Ci/mol) were purchased from New England Nuclear. XAR-5 X-ray film was obtained from Eastman Kodak. Synthetic oligonucleotides were purchased from Midland Certified Reagent Company or synthesized by the Texas Tech University Biotechnology Institute Core Facility. Qiagen DNA isolation kits were purchased from Qiagen, Inc. Common salts and buffer components were reagent grade or better.

Introduction of cyclic nucleotide binding pocket mutations

The *crp* gene was mutagenized as described by Kunkel (10) using single stranded uracil-containing M13*crp* *Nde*I DNA (4) as template and the following mutagenic primers. Position 83; GGAACGT(A/G/T)(C/G)CGCATGGG, (GGC = glycine), (TGC = cysteine). Position 127; GCAAGTC(A/G/T)(G/T)(A/G) TCAGAGAA, (AGC = serine), (ATC = isoleucine), (GGC = glycine), (TGC = cysteine). Position 128; GTCACT(A/G/T) (C/G/T)(A/C)GAGAAAGTG, (ACA = threonine), (GCA = alanine). A single sample found to contain the desired mutation was used as the source of the *crp* gene-containing fragment in each case. This fragment was subcloned into pRE2 to generate *crp* expression vector DNA. Sequence analysis of supercoiled DNA confirmed the constructs and ensured that there were no unexpected changes in the structural gene sequence.

β-Galactosidase assay

Cell growth and sample preparation were conducted as described by Belduz *et al.* (4). β-Galactsidase assays were conducted according to Miller (11).

Proteins

CRP isolation. Wild-type CRP was isolated from cultures of *E.coli* MZ1/pRE2*crp* that were grown on LB medium supplemented with glucose at 1%. CRP was isolated through the hydroxylapatite column step according to the protocol of Harman *et al.* (5) substituting Bio-Rex 70 resin for phosphocellulose. Analysis of the protein preparation was carried out by 15% sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-page) as described by Laemmli (12). Protein was stained in a solution that was 0.125% (w/v) in coomassie blue R-250, 50% in methanol and 10% in acetic acid and the gel was destained in a solution that was 5% in methanol and 7% in acetic acid. Wild-type CRP was judged greater than 95% pure from densitometric analysis of the gel.

Mutant CRP was isolated from cultures of *E.coli* MZ1/pRE2*crp* grown as described by Belduz *et al.* (4). Cell pellets were suspended in PC buffer [potassium phosphate, pH 7.5, at 50 mM, EDTA at 2 mM, β-mercaptoethanol at 1 mM, and 5% (v/v) in glycerol] that contained KCl at 0.1 M, the cells were ruptured in a French pressure cell and dialyzed overnight against 1 liter PC buffer 0.1 M in KCl. Extracts were applied to Bio-Rex 70 columns, washed with 3 sequential 5 ml volumes of PC buffer 0.1 M in KCl followed by a 5 ml wash with PC buffer 0.2 M in KCl. The CRP was eluted in 5 ml of PC buffer 0.4 M in KCl. The protein present in the mutant CRP preparations, visualized after SDS-page as described above, was conservatively judged 80% to 85% CRP by densitometry, with CRP the single major peak. CRP concentrations were determined using the extinction coefficient $3.5 \times 10^4\ M^{-1}\,cm^{-1}$ at A_{280} nm (13).

RNA polymerase isolation. RNA polymerase was purified by the method of Burgess and Jendrisak (14) as modified by Lowe *et al.* (15) and contained the full compliment of subunits ($\alpha_2\beta\beta'\sigma$) as determined by SDS-page. RNA polymerase concentration was determined using the extinction coefficient (1 %) $6.5\ M^{-1}\,cm^{-1}$ at A_{280} nm (14).

Cyclic AMP binding assay

Cyclic AMP binding studies utilized the solid phase partition assay of Santa-Coloma *et al.* (16) as modified by Belduz *et al.* (4).

Protease digestion

Protease digestion reactions were carried out as described by Belduz *et al.* (4). Reactions were terminated by addition of PMSF to 5 mM.

DTNB-mediated crosslinking

CRP was dialyzed against buffer that was 50 mM in Tris−HCl (pH 7.9), 100 mM in NaCl and 1 mM in EDTA. Reaction mixtures (25 μl total volume) contained CRP at 93 μM, Tris−HCl at 50 mM, NaCl at 100 mM, EDTA at 1 mM, dithiobis-2-nitrobenzoic acid (DTNB) at 50 μM and when present, cAMP at 100 μM. The crosslinking reaction was initiated by addition of DTNB and allowed to proceed at 30°C for 30 min. Reactions were terminated by addition of SDS-page sample buffer (12) that lacked a disulfide reducing agent. Monomer and disulfide-crosslinked dimer CRP were separated by electrophoresis on 15% polyacrylamide gels, stained, and quantitated by densitometry as described above.

In vitro transcription reactions

Reactions, carried out in a volume of 10 μl at 37°C, were 40 mM in Tris−HCl (pH 8.0), 8 mM in $MgCl_2$, 2 mM in spermidine, 50 mM in KCl, 0.25 mM in ATP, 0.25 mM in GTP, 0.25 mM in CTP, 0.05 mM in α-^{32}P UTP (20 Ci/mmol), and

contained 500 mg BSA/ml, RNA polymerase at 44 nM and pKL201 DNA at 2.5 nM. The wild-type and mutant CRP concentrations were 250 nM; where indicated, cAMP was added at 100 μM.

RNA polymerase, CRP and where indicated, cAMP, were incubated with DNA at 37° C for 30 min, or where indicated, for 30 sec. A solution of nucleoside triphosphates and heparin was added to the mixture to initiate a single round of transcription from pre-formed open complexes. The elongation reaction was allowed to proceed for 15 min at 37°C and was terminated with addition of an equal volume of a solution that was 10 M in urea and 0.04 % (w/v) in bromophenol blue and heated to 75°C for 5 min. RNA was resolved on 6.0 % polyacrylamide (19:1, acrylamide:methylene-bisacrylamide) gels (0.05 cm×20 cm×40 cm) that were 7 M in urea. RNA was visualized by autoradiography, analyzed by densitometry and the *lac* and *rep* RNA bands were quantitated from the peak areas.

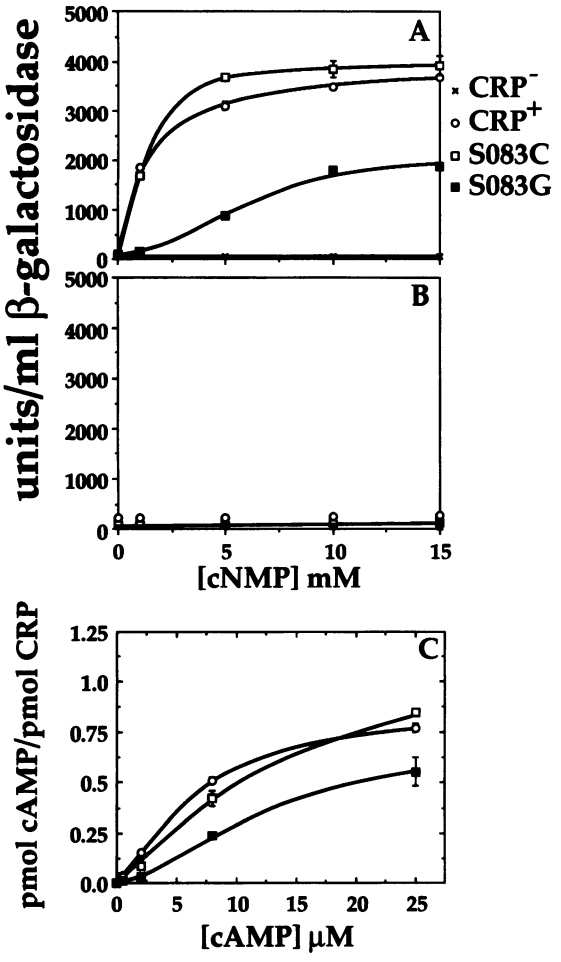

Figure 1. β-Galactosidase activity in cells containing wild-type CRP and position 83 CRP mutants. CRP was expressed from pRE2*crp* plasmids in *E.coli* strain CA8445/pRK248. Each value is the mean of at least two independent experiments with the standard error of the determinations indicated by error bars. The lack of error bars at any data point indicates that the range of error is covered by the symbol. Panel **A.** β-Galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B.** β-Galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C.** Cyclic AMP binding assays were conducted on CRP purified from *E.coli* strain MZ1 that contained the pRE2*crp* plasmid DNA expressing either the wild-type CRP or position 83 CRP mutants. An equivalent amount of MZ1/pRE2 extract did not bind cAMP above background levels. CRP^- designates the control culture of *E.coli* CA8445/pRK248 that contained pRE2 vector DNA alone. CRP^+ designates the control culture of *E.coli* CA8445/pRK248 that contained pRE2 vector DNA with the wild-type *crp* allele cloned downstream of the λP_L promoter. Cultures that expressed mutant CRP are represented by the amino acid residue of wild-type CRP, its position number, followed by the substituted amino acid. Amino acid one letter designations are: C, cysteine (open square); G, glycine (filled square).

RESULTS

Characterization of the CRP mutants described here included measurements of β-galactosidase expression to quantitate the effects of mutations on CRP function *in vivo*. CRP synthesis that originates from pRE2*crp* plasmids requires temperature inactivation of λ cI repressor to activate the λ P_L promoter located upstream of *crp* (9). Control experiments (Figures 1, 2 and 4) showed that high level β-galactosidase synthesis in *E.coli* CA8445/pRK248 was dependent upon the expression of CRP and the addition of cAMP to cultures of cells that contained wild-type CRP. Cyclic GMP did not substitute for cAMP in mediating β-galactosidase synthesis in cells that contained wild-type CRP. Cyclic AMP binding measurements, conducted on purified protein, were used to quantitate the effects of mutations on CRP affinity for cAMP.

The activation of CRP as a transcriptional control element is accompanied by structural changes in or near the hinge region of the protein and realignment of the cysteine 178 residues in the DNA-binding portion of the protein (17, 18). We have utilized both protease sensitivity and DTNB-mediated intersubunit crosslinking measurements to investigate the effects of amino acid substitutions on the microenvironments near the hinge region of CRP and on the DNA binding domain, respectively. Tsugita *et al.* (19) reported that subtilisin cleaves the CRP:cAMP complex at amino acid residue 116 and yields a protease-resistant core fragment of approximately 13,000 Da. Under the reaction conditions used in this study, subtilisin cleaves the CRP:cAMP complex to a core fragment of 16,000 Da. Amino acid sequence analysis of cyanogen bromide fragments of the 16,000 Da core confirmed that the core extends beyond position 116 and terminates at glycine 141 (Glasgow, San Francisco and Harman, unpublished). Subtilisin-mediated cleavage of CRP:cAMP complex at glycine 141 under the conditions utilized in this study clearly monitors the microenvironment of the CRP hinge.

Position 83 mutants

Substitution of cysteine or glycine for serine 83 produced forms of CRP that were insensitive to protease and refractory to DTNB-mediated crosslinking in the absence of cAMP and promoted β-galactosidase synthesis only in the presence of cAMP (Figure 1, panels A and B, Figure 3, Table 1). S83C CRP bound cAMP with approximately the same affinity as wild-type CRP (Figure 1, panel C) and the S83C CRP:cAMP complex was as efficient an activator of *lac*P as the wild-type CRP:cAMP complex (Figure 1, panel A). S83C CRP, while sensitive to protease in the presence of cAMP, was degraded at a slower rate than the wild-type CRP:cAMP complex (Figure 3) and supported lower levels of DTNB-mediated intersubunit crosslinking in the presence of cAMP (Table 1). These data show that while the structural characteristics of S83C CRP and wild-type CRP are qualitatively

similar, there are distinct and as yet undefined differences that have little effect on CRP function.

S83G CRP had somewhat lower affinity for cAMP than did wild-type CRP (Figure 1, panel C) and cells that contained the S83G CRP:cAMP complex supported only half the β-galactosidase activity of cells that contained the wild-type CRP:cAMP complex (Figure 1, panel A). The S83G CRP:cAMP complex was degraded by protease at a slower rate than the wild-type CRP:cAMP complex (Figure 3) and displayed near wild-type levels of DTNB-mediated intersubunit crosslinking in the presence of cAMP (Table 1). Substitution of glycine for serine 83 produced a mutant CRP whose structure changed upon binding cAMP yet failed to complete the structure changes required for efficient, high level CRP-mediated transcription activation.

Position 128 mutants

Substitution of alanine for serine 128 produced a mutant CRP that bound cAMP, had reduced affinity for cAMP, was activated only by cAMP and yielded a mutant CRP:cAMP complex that was a relatively poor activator of *lac*P (Figure 2). The S128A CRP:cAMP complex was considerably less sensitive to protease than the wild-type CRP:cAMP complex (Figure 3) and supported lower levels of DTNB-mediated intersubunit crosslinking (Table 1).

Substitution of threonine for serine 128 produced a mutant CRP with properties virtually identical to those of wild-type CRP. The affinity of S128T CRP for cAMP was identical to that of wild-type CRP and the level of β-galactosidase activity supported by the S128T CRP:cAMP complex was identical to that displayed by cultures that contained the wild-type CRP:cAMP complex (Figure 2, panels A and C). S128T CRP and the S128T CRP:cAMP complex displayed structural properties virtually identical to those of wild-type CRP and the CRP:cAMP complex (Figure 2 and Table 1). S128T CRP differed from wild-type CRP in that it promoted β-galactosidase synthesis in the presence of cGMP (Figure 2, panel B).

Position 127 mutants

Substitutions for threonine 127 had varied effects on β-galactosidase synthesis in cells that expressed the mutant proteins (Figure 4, panels A and B). Cysteine substituted for threonine

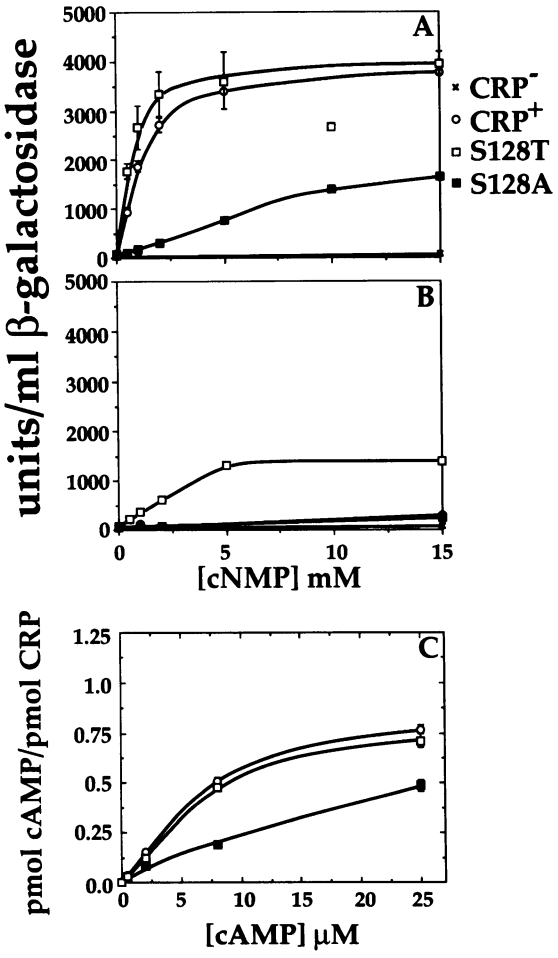

Figure 2. β-Galactosidase activity in cells containing wild-type CRP and position 128 CRP mutants. Panel **A.** β-Galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B.** β-Galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C.** Cyclic AMP binding assays were conducted as outlined in the legend to Figure 2. Amino acid one letter designations are: T, threonine (open square); A, alanine (filled square).

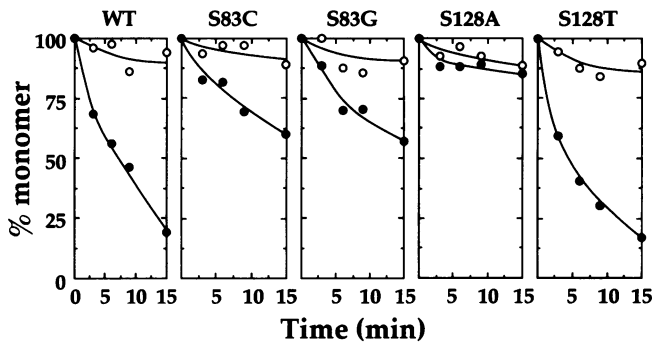

Figure 3. Protease digestion of CRP and position 83 and 128 CRP mutants. Proteins were incubated with subtilisin in the absence (open circles) or presence (closed circles) of cAMP at 100 μM. The reactions were terminated at the indicated time and the samples were processed as described in Materials and Methods. The amount of monomer CRP remaining at each time point was determined by densitometry and expressed as a fraction of the CRP monomer present in control reactions that did not receive protease. Data represent the mean of two independent experiments.

Table 1. DTNB-mediated crosslinking of the wild-type and mutant forms of CRP

CRP	% Crosslinked CRP −cAMP	+cAMP
wild-type	7 (0.4)	73 (1.6)
S83C	4 (1.3)	36 (3.4)
S83G	9 (2.4)	66 (3.4)
S128T	10 (1.6)	73 (1.3)
S128A	9 (1.8)	40 (1.4)
T127C	96 (0.8)	98 (0.9)
T127G	4 (1.3)	10 (2.7)
T127I	26 (6.2)	63 (6.6)
T127S	6 (2.5)	26 (8.3)

CRP was incubated with DTNB in the absence or presence of cAMP. Monomer, and disulfide-crosslinked dimer CRP were separated, visualized and quantitated as described in Materials and Methods. Data are the mean of three independent experiments. Numbers in parentheses are experimental standard deviations.

127 produced a form of CRP that activated *lac*P in the presence of either cAMP or cGMP. Isoleucine and serine substitutions at position 127 produced CRP mutants that showed limited capacity to activate *lac*P in the presence of cAMP, and significant levels of *lac*P activity in the presence of cGMP. The T127G mutant did not activate *lac*P in the presence of either cAMP or cGMP.

None of the threonine 127 substitutions had a significant effect on the affinity of CRP for cAMP (Figure 4, panel C); concentrations of cAMP required to bind 0.5 pmol cAMP/pmol CRP varied from 8 μM for wild-type CRP and the T127C CRP to approximately 20 μM for the T127G and T127S forms of the protein.

All substitutions for threonine 127 affected the structure of CRP in and around the hinge region of the protein, displaying protease sensitivity in the absence of cAMP similar to that of the wild-type CRP:cAMP complex (Figure 5). These results demonstrate that threonine 127 plays an important role in maintaining the protease-resistant structure of CRP.

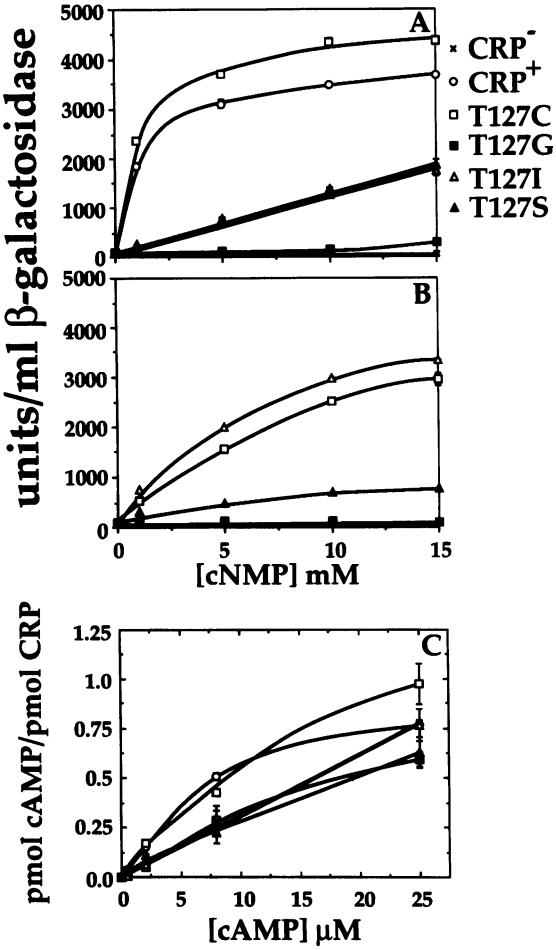

Figure 4. β-Galactosidase activity in cells containing wild-type CRP and position 127 CRP mutants. Panel **A.** β-Galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B.** β-Galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C.** Cyclic AMP binding assays were conducted as outlined in the legend to Figure 2. Amino acid one letter designations are: C, cysteine (open square); G, glycine (closed square); I, isoleucine (open triangle); and S, serine (closed triangle).

Structural change(s) induced in CRP by threonine 127 substitution had varied effects on the positioning of the cysteine 178 residues in the dimer forms of mutant CRP (Table 1). Isoleucine substitution yielded a protein that, in the absence of cAMP, crosslinked at levels three to four times higher than wild-type CRP, and to near wild-type levels of in the presence of cAMP. Substitution of either glycine or serine for threonine 127 yielded CRP mutants refractory to DTNB-mediated crosslinking in the absence and in the presence of cAMP.

Cysteine substitution for threonine 127 produced a unique form of crosslinked CRP (Figure 6) that migrated, under non-reducing conditions, as dimer CRP in the absence or presence of DTNB. T127C CRP incubated with DTNB in the presence or in the absence of cAMP and subsequently digested with subtilisin yielded 32,000 Da core fragments (Figure 6, panel A). Under reducing conditions, the 32,000 Da cores migrated with a mass of 16,000 Da (Figure 6, panel B). These results clearly show that T127C CRP subunits are covalently coupled through a disulfide bond that lies amino-proximal to the hinge, most likely involving cysteine 127.

In vitro transcription analysis of wild-type and mutant CRP function

The differences in β-galactosidase expression observed for mutant forms of CRP (Figures 1, 2, and 4) suggests differential CRP

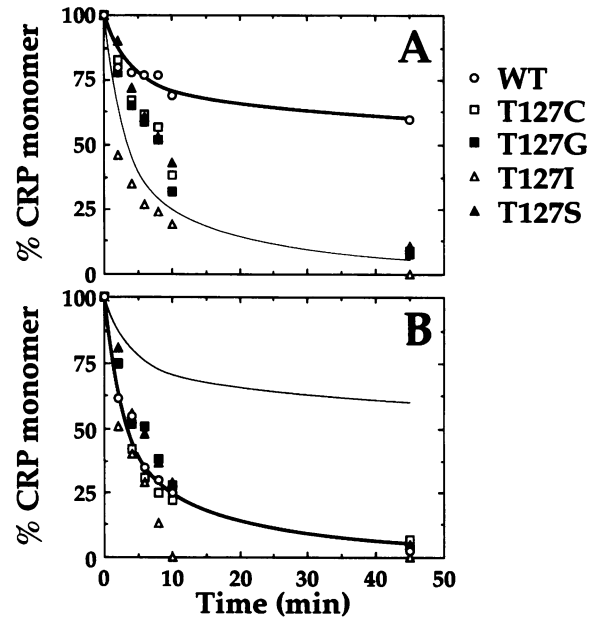

Figure 5. Protease digestion of CRP and position 127 CRP mutants. Wild-type CRP, and position 127 CRP mutants were incubated with subtilisin in the absence (Panel A) or presence (Panel B) of cAMP at 100 μM. Protease digestion was terminated at the indicated time and samples were processed as described in Materials and Methods. The amount of monomer CRP remaining at each time point was determined by densitometry and expressed as a fraction of the CRP monomer present in the control reactions. Data are the mean of three experiments. Lines through the data for position 127 CRP mutants have been omitted for clarity. Panel **A**; the bold curve was fitted to the data obtained with the wild-type CRP; the narrow curve was fitted to the data obtained with the wild-type CRP:cAMP complex. Panel **B**; the bold curve was fitted to the data obtained with the wild-type CRP:cAMP complex; the narrow curve was fitted to the data obtained with the wild-type CRP. Curves were fitted by inspection.

efficacy in activating *lac*P. With the possible exception of the S83G CRP, this interpretation was confirmed through *in vitro* transcription experiments (Figure 7).

The wild-type, the S83C and S83G and the S128T CRP:cAMP complexes supported the formation of equivalent amounts of active *lac*P:RNA polymerase open complex over a 30 min incubation period (Figure 7, panel A). The S128A CRP:cAMP complex promoted 75% the level of active open complexes observed for the wild-type CRP:cAMP. Restricting the time allowed for open complex formation to 30 sec the S83C, the S83G and the S128T CRP produced, respectively, open complexes at 79%, 85% and 62% of the level observed in reactions that contained wild-type CRP:cAMP (Figure 7, panel B). S128A CRP:cAMP produced only 15% of the open complexes observed for wild-type CRP:cAMP. These results show that the S128A CRP:cAMP complex promotes formation of the active *lac*P:RNA polymerase open complex at a slower rate than does wild-type CRP:cAMP. This property could well account for the reduced levels of β-galactosidase observed in cells that contained the S128A form of CRP (Figure 2).

T127C CRP:cAMP supported the formation of the active *lac*P:RNA polymerase open complexes to the level observed with the wild-type CRP:cAMP complex (Figure 7, panel C). In contrast, reduced amounts of active *lac*P:RNA polymerase open complex were formed in the presence of the T127I, the T127S, or the T127G CRP:cAMP complexes at 48%, 23%, and 8% respectively. These results clearly parallel the β-galactosidase data presented in Figure 4, panel A and validate straightforward interpretation of the *in vivo* data.

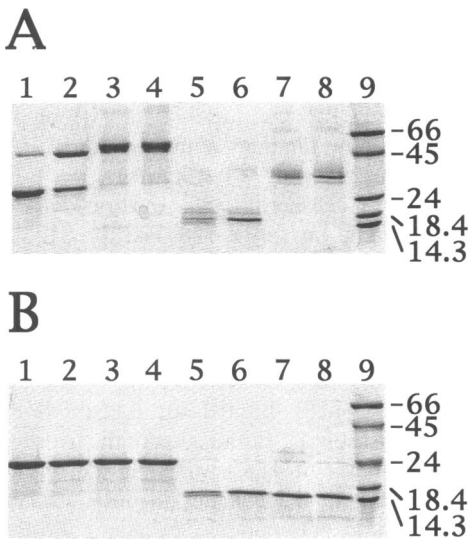

Figure 6. Analysis of T127C CRP. Panel **A**; wild-type CRP (lanes 1, 2) and T127C CRP (lanes 3, 4) were incubated DTNB in the absence (odd numbered lanes) or presence (even numbered lanes) of cAMP at 100 μM. One-half of the samples (lanes 1–4) were denatured in SDS-page sample buffer that lacked a reducing agent. One-half of the samples (lanes 5–8) were incubated with subtilisin for 30 min, treated with PMSF and denatured in SDS-page sample buffer that lacked a reducing agent. Samples were analyzed by electrophoresis through SDS-page. The gel was calibrated with molecular mass markers of the indicated mass in kDa (lane 9). Panel **B**; identical to the conditions described above with the exception that the protein denaturant was SDS-page sample buffer that contained the disulfide reducing agent β-mercaptoethanol.

DISCUSSION

Cyclic AMP binding to CRP

CRP-mediated promoter activation is a multi-component, multi-stage process that involves, as a first step, the binding of cAMP to CRP. Mutant forms of CRP with conservative amino acid substitutions at positions 72 or 82 showed at least a ten-fold decrease in affinity for cAMP; non-conservative amino acid substitutions either reduced cAMP-binding affinity further or eliminated it altogether (4). We show here that neither conservative nor non-conservative amino acid substitutions at positions 83, 127 or 128 have more than a three-fold effect on the affinity of CRP for cAMP. These results, consistent with those of Gronenborn *et al.* (20), clearly show that contacts between cAMP and CRP involving serine 83, threonine 127 or serine 128 are not important determinants of cAMP-binding affinity.

Cyclic nucleotide discrimination

CRP is a general cyclic nucleotide binding protein; however, only the binding of cAMP mediates the conformational change(s)

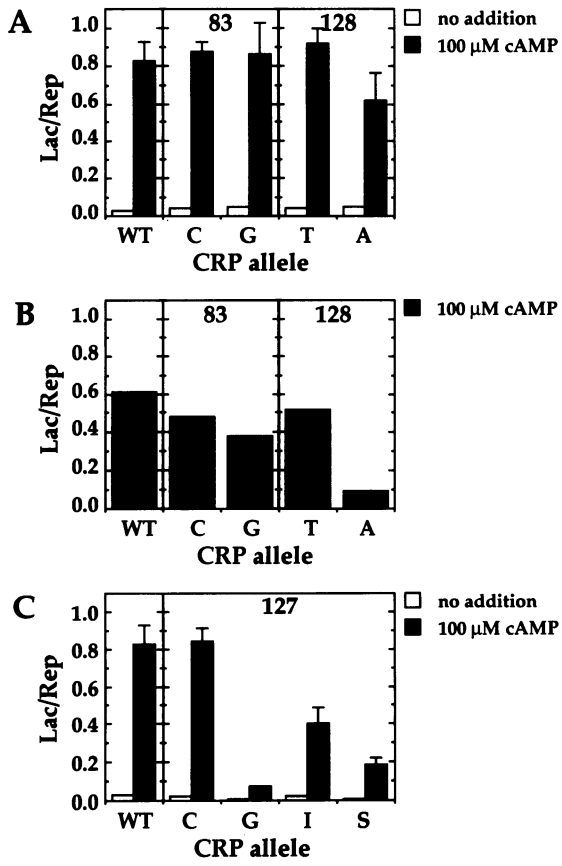

Figure 7. *In vitro* transcription analysis of wild-type and mutant forms of CRP. The histograms present the amount of a cAMP-dependent 136 base *lac* RNA normalized to a cAMP-independent 106 base *rep* RNA derived from pKL201 (5). Panel **A**; 30 min was allowed for open complex formation in reactions that contained the wild-type, the S083C, S083G, S128T, or S128A forms of CRP in the absence (open bars) or in the presence (closed bars) of cAMP. Panel **B**; 30 sec was allowed for open complex formation in reactions that contained the wild-type, the S083C, S083G, S128T, or S128A forms of CRP and cAMP. Panel **C**; 30 min was allowed for open complex formation in reactions that contained the wild-type, the T127C, T127G, T127I, or T127S forms of CRP in the absence (open bars) or in the presence (closed bars) of cAMP.

required for CRP participation in promoter activation (21). Substitution of threonine 127 with cysteine, isoleucine (and, much less effectively, serine), and substitution of serine 128 with threonine produced forms of CRP that were activated by cGMP (Figures 2 and 4), whereas amino acid substitutions at positions 72, 82 and 83 produced CRP mutants that were activated only by cAMP (4, Figure 1).

Eight hydrophobic amino acid residues contribute to the c AMP binding pocket of CRP (3). The side chains of four of these pack against the adenine ring in CRP:$(cAMP)_2$ crystals. Our results indicate that the cyclic nucleotide discrimination of CRP is overcome by introducing a hydrophobic constituent to the amino acid residues at positions 127 and 128 as long as the side chain at position 128 retains polar character. Cysteine and isoleucine at position 127 and threonine at position 128 introduce side chains that could increase hydrophobic packing interactions with the purine base. Serine at position 127 introduces a polar side chain that is not likely to participate in such interactions and produces a CRP mutant that is unresponsive to cGMP. Interactions between threonine 127 and/or serine 128 and the C^6-N^7 face of the cyclic nucleotide play a role in cyclic nucleotide discrimination in wild-type CRP (Figure 8).

Allosteric activation of CRP

Interactions between cAMP and CRP amino acid residues located in the cyclic nucleotide binding pocket promote a change in CRP conformation that converts CRP to a protein fully capable of participating, along with DNA and RNA polymerase, in the process of promoter activation. Secondary CRP structure changes induced by interaction of the CRP:cAMP complex with either DNA or RNA polymerase are reasonable conjectures and have experimental support (22). First, comparison of CRP:$(cAMP)_2$ and CRP:$(CAMP)_2$:DNA crystals suggests DNA-mediated changes in CRP dimer structure. The CRP subunits in CRP:$(cAMP)_2$ crystals are asymmetric, whereas CRP:$(cAMP)_2$:DNA crystals contain symmetrically related CRP subunits and show an asymmetric bend in DNA (3, 23). Whether this change in CRP dimer structure represents a physiologically relevant event or is simply a crystal structure packing artifact is not known (23). Second, comparison of Figure 5, panel A and Figure 6, panels A and B (lanes 5), indicates that TNB modification of cysteine 178 renders the CRP hinge more sensitive to protease than that of unmodified CRP; results consistent with the observation that carboxy-terminal truncated CRP adopts a conformation that has properties similar to those of the CRP:cAMP complex (24). Third, the possibility that RNA polymerase-mediated changes in CRP conformation can occur are suggested by the finding that the CRP:cAMP complex fails to bind *lac*P DNA that contains the L8 mutation in the CRP binding site unless RNA polymerase is added to the reaction mixture (25). These putative secondary CRP conformational adjustments induced by CRP interaction with DNA or RNA polymerase are referred to as second allostery (21). Our interpretation of the results reported here are presented from this perspective.

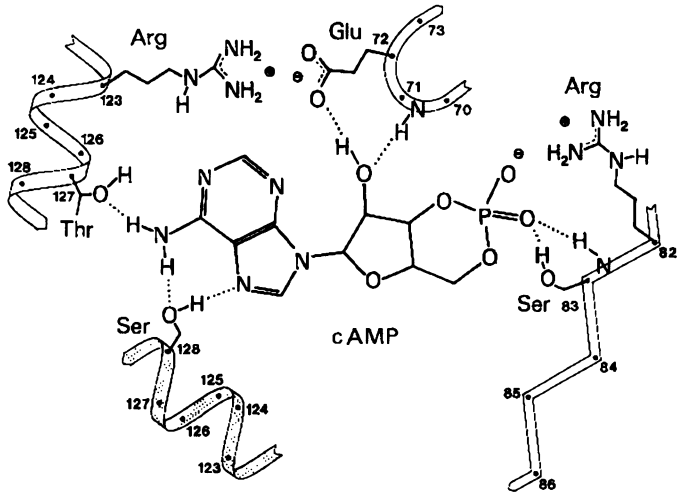

Figure 8. Schematic representation of the cAMP binding sites in the CRP:$(cAMP)_2$ crystals. Amino acid residues that form hydrogen bonds (dotted lines) or ionic interactions (charged groups) with cAMP are illustrated. This figure is copied from reference 3 and is used with permission.

Amino acid substitutions for serine 83 or serine 128 had no detectable effect on CRP structure (Figure 3 and Table 1). The hinge regions of the S83C and S83G CRP:cAMP complexes were more resistant to protease than was the hinge region of the wild-type CRP:cAMP complex, indicdating that these substitutions interfered with some but not all of the changes cAMP causes in CRP structure. Reactivity of the cysteine 178 residues of the cAMP complexes of wild-type CRP, S83C CRP and S83G CRP were qualitatively similar but differed quantitatively (Table 1). Only the wild-type and the S83C complexes fully activated *lac*P *in vivo*. The hydroxyl group of serine was replaced with the less polar sulfhydryl in the S83C complex, and by a (non-polar) proton in the S83G complex. These data suggest that normal allosteric maturation of CRP is disrupted by either increased torsional flexibility of β-strand 7, which is expected to result from glycine substitution, or loss of a weak hydrogen bond between cAMP and position 83 of the CRP. The stage at which this interference occurs (e.g., in the CRP structure in the cAMP binding pocket, and/or in the DNA binding domain and/or in the RNA polymerase interaction site) is not known.

The efficacy of transcription activation was unchanged substitution threonine for serine 128, and the S128T CRP was both structurally and,in the presence of cAMP, functionally indistinguishable from wild-type CRP (Figures 2, 3, and Table 1). Alanine substitution of serine 128 yielded a mutant that was resistant to protease in the presence of cAMP, only moderately sensitive to DTNB-mediated crosslinking of the cysteine 178 residues and functionally limited for transcription activation (Figures 2, 3, and Table 1). Elimination of the hydrogen-bonding hydroxyl group of serine 128 resulted in the failure of either important cAMP-mediated changes in CRP structure, or the response of the complex to putative secondary allosteric effects.

Details of the changes that occur in CRP structure upon the binding of cAMP are incomplete; crystals of the CRP conformer have not yet been obtained; however, structure analysis shows that threonine 127, located in the C α-helix of CRP, is positioned to form a hydrogen bond with the N^6 amino group of cAMP and with the hydroxyl group of serine 128 located in the C α-helix of the adjacent subunit (3). Cyclic AMP interactions with threonine 127 and serine 128 are predicted to play a role in mediating CRP subunit–subunit realignment (3).

Threonine 127 is expected to display a degree of α-carbon torsional freedom (26) and to assume, in the absence of cAMP,

a unique rotational position directed by interaction of side chain methyl and hydroxyl groups with the side chains of surrounding amino acid residues. The data presented in Figure 5 show that removal of either the threonine methyl group by substitution with serine, or the threonine hydroxyl group by substitution with isoleucine, or both (by substitution with glycine or cysteine) produced CRP mutants that were protease sensitive in the absence of cAMP. Wild-type CRP, in contrast, displays protease resistance in the absence of cAMP. Clearly, interactions involving both the methyl group and the hydroxyl group of threonine 127 play important roles in establishing the CRP hinge region conformation in the absence of effector.

Amino acid substitutions that eliminated the threonine 127 methyl (e.g., serine), the threonine 127 hydroxyl (e.g., isoleucine) or both (e.g., glycine) produced CRP mutants that were either limited in or incapable of *lac*P activation in the presence of cAMP (Figures 4 and 7). T127C CRP contains disulfide-crosslinked subunits and was, on the other hand, fully functional in activating *lac*P (Figures 4 and 7). The amino acid residue at position 127 appears to play a role in transmitting the allosteric signal from the cAMP binding pocket by providing a pivot point that allows subunit−subunit realignment. The T127C data strongly suggest that, if the hydrogen bonding of threonine 127to the N^6 amino group of cAMP in CRP:$(cAMP)_2$ crystals is important in CRP function, its role is to promote threonine 127 repositioning in a way that promotes appropriate subunit−subunit interaction in the CRP:cAMP complex.

The effects of substitutions for threonine 127 on CRP structure and function are similar to those observed with class D cAMP analogs. CRP:class D cAMP analog complexes exhibit conformations similar to the CRP:cAMP complex yet fail to bind *lac*P DNA or to activate *cat*P *in vitro* (21). Ebright *et al.* (21) proposed a cAMP-dependent event in the activation of CRP that is distinct from cAMP binding and the formation of the biochemically defined conformation change. Our data suggest an alternate conclusion: a critical degree of rotational freedom and unique spatial positioning of threonine 127 functional groups are important in allowing the step-wise conformational changes that lead to CRP activation. The binding of class D cAMP analogs positions an N^6 aliphatic or hydrophobic ring in close proximity to threonine 127, and we suspect, prohibits participation of threonine 127 in the complete sequence of events required for activation.

Based on the results presented here, we conclude that the interactions that position the threonine 127 side chain are important in stabilizing CRP structure. disruption of these interactions are triggered by cAMP or class D cAMP analog binding to CRP, as well as by amino acid substitution of threonine 127. Precise repositioning of the threonine 127 side chain is required for CRP activation. Our results are consistent with threonine 127 interaction with the N^6 amino group of cAMP (3). Both the correct repositioning of threonine 127 and the presence of polar amino acid residues at positions 83 and 128 appear necessary to promote complete signal transfer through the hinge and yield fully active wild-type CRP:cAMP complex (27−29).

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