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Mutagenesis of the cyclic AMP receptor protein of *Escherichia coli*: targeting positions 72 and 82 of the cyclic nucleotide binding pocket

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ABSTRACT

The 3′, 5′ cyclic adenosine monophosphate (cAMP) binding pocket of the cAMP receptor protein (CRP) of *Escherichia coli* was mutagenized to substitute leucine, glutamine, or aspartate for glutamate 72; and lysine, histidine, leucine, isoleucine, or glutamine for arginine 82. Substitutions were made in wild-type CRP and in a CRP*, or cAMP-independent, form of the protein to assess the effects of the amino acid substitutions on CRP structure. Cells containing the binding pocket residue-substituted forms of CRP were characterized through β-galactosidase activity and by measurement of cAMP binding activity. This study confirms a role for both glutamate 72 and arginine 82 in cAMP binding and activation of CRP. Glutamine or leucine substitution of glutamate 72 produced forms of CRP having low affinity for the cAMP and unresponsive to the nucleotide. Aspartate substituted for glutamate 72 produced a low affinity cAMP-responsive form of CRP. CRP has a stringent requirement for the positioning of the position 72 glutamate carboxyl group within the cyclic nucleotide binding pocket. Results of this study also indicate that there are differences in the binding requirements of cAMP and cGMP, a competitive inhibitor of cAMP binding to CRP.

INTRODUCTION

The cyclic 3′:5′-adenosine monophosphate (cAMP) receptor protein (CRP) of *Escherichia coli* is a dimeric protein that binds to both cyclic nucleotides and, in the presence of cAMP, to specific DNA sequences located in a number of *E.coli* promoter regions (1). Binding of CRP:cAMP to a promoter region either stimulates or represses the activity of that promoter depending upon the relative locations of the CRP and RNA polymerase binding sites. The activation of CRP as a transcription control element is initiated by the binding of cAMP. Biochemical evidence clearly indicates that cAMP binding alters CRP conformation and induces DNA sequence specific recognition (reviewed in 1 and 2). Cyclic nucleotides other than cAMP (i.e., cGMP) bind CRP but fail to activate the protein (3–5).

Three conformational states of CRP are known: native CRP and complexes having either one or both of the cAMP binding sites occupied by cAMP (6). The CRP:$(cAMP)_1$ conformer appears to be the relevant active form *in vivo*, considering both the intracellular cAMP concentrations in bacteria and the 1:1 stoichiometry of CRP to cAMP observed in the CRP:cAMP:*gal* DNA and CRP:cAMP:*lac* DNA complexes formed *in vitro* (7–9). Details of the changes in CRP structure that result upon binding one or two molecules of cAMP are incomplete; crystals of the CRP and the CRP:$(cAMP)_1$ conformers have not yet been obtained. Detailed structure analysis information available for the CRP:$(cAMP)_2$ conformer (10) has provided a basis for understanding both cAMP-mediated and mutational activation of CRP (11–14), and proposals have been advanced on the probable effects of cAMP on wild-type CRP.

Analysis of the CRP:$(cAMP)_2$ crystal structure identified five specific amino acid-ligand contacts that are considered important in cAMP binding to CRP and/or in mediating cAMP activation of CRP (13). The authors suggested that the charged phosphate of cAMP interacts with arginine 82 to form a salt bridge, and that 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 in CRP to test the predictions of the CRP:$(cAMP)_2$ crystal structure analysis for CRP in solution. This study includes analysis of substitutions contained in both wild-type CRP and in a cAMP-independent form of CRP (CRP*) that mediates CRP-dependent gene expression in the absence of cAMP (15).

Many *crp** alleles have amino acid substitutions located outside of the cAMP binding pocket (12, 14–17). One such mutant, 91 CRP (reference 15, hereafter referred to as $CRP^{*[A144T]}$), contains threonine in place of alanine 144 located in the DNA binding domain of CRP. This substitution renders CRP conformation similar to that of the active cAMP-modified conformation of the wild type CRP:cAMP complex (15). The inherent transcription control activity of many forms of CRP*

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(including CRP*[A144T]) is stimulated by both cAMP and cGMP. CRP* forms of CRP are thought to be intermediate in both conformation and activity between the active and inactive conformations of wild type CRP; they are capable of undergoing a conformation change upon binding of cyclic nucleotide, and have lost the capacity to discriminate between cAMP and cGMP.

We report here the results of our analysis of CRP mutants with amino acid substitutions at positions 72 and 82 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, in the presence of cAMP or in the presence of cGMP. Partially purified CRP preparations were utilized to measure mutant CRP affinity for cAMP at physiologically relevant salt and cAMP concentrations. Parallel cAMP binding pocket mutants contained in the CRP*[A144T] background provided a measure of whether specific amino acid substitutions resulted in only local perturbations or in global changes in CRP*[A144T] structure. The results of this study confirm the importance of both glutamate 72 and arginine 82 in cAMP binding and 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}) (15) and D1210HP [*hsd* S20 (rB; mB), *sup* E44, *ara* 14, *gal* K2, *lac* Y1, *pro* A2, *rps* L20, *xyl* -5, *mtl* -1, *rec* A13, (r_B^-; m_B^-), *mcr* A$^+$, *mcr* B$^-$, λcI^{ts}, *xis*$^-$, *kil*$^-$, *int*$^+$] (Stratagene) were used as hosts for recombinant *crp* plasmids. *E.coli* strains TG-1 [$_{\Delta}$ (*lac -pro*) *thi*, *sup* E, *hsd* D5, {F′:*tra* D36, *pro* AB, *lac* I^q, *lac* Z$_{\Delta}$M15}] (18) and 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$_{\Delta}$M15, Tn10 (tet^r)}] (Stratagene) were used to propagate M13mp18 (19) 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$_{\Delta}$M15}] (Bio-Rad Laboratories) and CJ236 [*dut*, *ung*, *thi*, *rel* A{pCJ105 (cm^r)}] (Bio-Rad Laboratories) were used for mutagenesis of *crp*. Plasmid pRK248 (20) was the gift of D.Helinski, University of California at San Diego, La Jolla, CA. pKC30*crp* (15) containing wild type *crp* cloned downstream of the λP$_L$ promoter was used as the source of the *crp* structural gene. Expression vector plasmid pRE2 (21) was the gift of Prasad Reddy, National Institute of Standards and Technology, Gaithersberg, MD.

Materials

Restriction enzymes, T4 DNA ligase, the Klenow fragment of DNA polymerase I and DNA sequencing kits were obtained from New England Biolabs. X-gal (5-bromo-4-chloro-3-indolyl β-D-glactopyranoside), IPTG (isopropyl β-D-thioglactopyranoside) and DNA sequencing kits were purchased from 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. Phosphocellulose (P-11) was obtained from Whatman Biosystems, Ltd. Centricon-30 microconcentrators were purchased from Amicon. MacConkey indicator media, bactotryptone, casamino acids and yeast extract were purchased from Difco Laboratories. 3′-5′ cAMP, 3′-5′ cGMP and subtilisin BPN′ (type XXVII, 7.9 units/mg) were purchased from Sigma Chemical Company. ^{3}H (2′-8′ labeled) 3′-5′ cAMP (31.3 Ci/mol), α-^{35} S-labeled ATP (500 Ci/mol) and α-^{32} P-labeled ATP (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. Qiagen DNA isolation kits were purchased from Qiagen, Inc. Common salts and buffer components were reagent grade or better.

Construction of M13*crp*

The 1321 bp *Alu* I DNA fragment of pKC30*crp* (15) was cloned into *Sma* I-digested, alkaline phosphatase-treated M13mp18 DNA. The ligation mixture was used to transform competent *E.coli* TG-1 cells. Single stranded DNA was isolated and sequenced to confirm that the DNA from one of the resultant plaques, designated M13*crp*, contained the *crp* gene.

Site directed mutagenesis of the *E.coli crp* gene

Introduction of a Nde I site. Mutagenesis of *crp* was accomplished using the method of Kunkel (22) as outlined in the protocol provided by Bio-Rad Laboratories. Uracil-containing M13*crp* DNA was annealed to a mutagenic primer ($_{P}$GATAA-CCGC<u>AT</u>ATGGTGCTTGGC$_{OH}$) and replicated *in vitro* to introduce a *Nde* I restriction endonuclease site that utilized the ATG start codon in the *crp* structural gene. Mutagenized DNA was used to transform competent *E.coli* MV1190 cells. The *Nde* I/*Sac* I fragment containing the *crp* structural gene of one mutant (M13*crp Nde*I) was subcloned into *Nde* I/*Sac* I-digested, alkaline phosphatase-treated pRE2. The ligation mixture was used to transform competent *E.coli* D1210HP. Supercoiled plasmid DNA isolated from from one ampicillin resistant isolate, designated pRE2*crp*, was sequenced using *crp* sequencing primers (15) to confirm the sequence of the construct. DNA sequencing reactions were run as outlined in the protocols provided by New England Biolabs and by United States Biochemical Corporation, Inc.

Introduction of cyclic nucleotide binding pocket mutations. The *crp* gene was mutagenized as described above using uracil-containing M13*crp Nde*I DNA as template and the following mutagenic primers. *Position 72*; ATTGGC<u>GAC</u>CTGGGCC (G-AC = aspartate), ATTGGC<u>CAA</u>CTGGGCC (CAA = glutamine), ATTGGC<u>TTA</u>CTGGGCC (TTA = leucine). *Position 82*; CCAGGAA<u>(A/C)(A/T)(A/T)</u>AGGCATG (AAA = lysine), (CAT = histidine), (CTT = leucine), (CAA = glutamine), (ATT = isoleucine). The efficiency of mutagenesis varied from 30% to 1%. One sample found to contain the desired mutation(s) was used as the source of an 1186 bp *Nde* I/*Sac* I *crp* gene-containing fragment. This fragment was subcloned into the *Nde* I/*Sac* I-digested, alkaline phosphatase-treated pRE2 to generate *crp* expression vector DNA. Supercoiled DNA sequence analysis confirmed the constructs and ensured that there were no unexpected changes in the structural gene sequence. Confirmed cyclic nucleotide binding pocket mutants in the wild-type *crp* background were utilized as templates to generate cyclic nucleotide binding pocket mutants in the *crp**[A144T] background. The mutagenic oligonucleotide CCGCATT*ACA*CAGACTC, which converts the alanine 144 codon to a threonine codon, was used as the primer for second strand synthesis in these reactions.

β-galactosidase assay

Overnight cultures of *E.coli* CA8445/pRK248/pRE2*crp* grown in LB medium (23) that contained 0.5% fructose, 50 μg/ml ampicillin and 12.5 μg/ml tetracycline were used to inoculate 2 ml of the same medium contained in 16×100 mm tubes to OD_{600} = 0.05. These cultures were vigorously shaken at 37°C. At an OD_{600} = 0.5, 0.5 ml aliquots of culture were transferred to 16×100 mm tubes that contained 0.5 ml pre-warmed (37°C) LB medium that contained 10 mM IPTG with or without 2 mM cAMP or 2 mM cGMP. Cultures were grown at 37°C for 1 hr with shaking. One ml of ice cold LB containing 200 μg/ml chloramphenicol was added to each tube and the tubes were placed on ice. β-galactosidase assays were conducted according to Miller (23).

CRP isolation

Preparation of extracts for cAMP binding measurements. E.coli D1210HP/pRE2*crp* grown at 30°C in 2× superbroth/5×M9 salts mixture (24) supplemented with 10 μg/ml thiamine-HCl, 20 μg/ml proline, 50 μg/ml ampicillin, 25 μg/ml streptomycin, and 0.5% (w/v) fructose were used to inoculate one-liter cultures of the same medium to an OD_{600} = 0.1. Cultures were grown at 30°C to an OD_{600} = 1.2. The culture temperature was shifted to 42°C and maintained at this temperature for 2 hrs and the cells were harvested by centrifugation. Cell pellets were suspended in 50 mM Tris−HCl pH 7.4, 10% sucrose and stored frozen overnight. 1/20 volume of lysozyme (10 mg/ml stock) was added to thawed cells and the cell suspension was incubated on ice for 35 min and subsequently centrifuged at 13,000×g for 30 min. The supernatant fraction was centrifuged at 100,000×g for 1.5 hrs. The clarified supernatant fraction was brought to 60% saturation with the addition of solid ammonium sulfate. The solution was centrifuged at 6,000×g for 30 min. The pellet was dissolved in 5 ml of 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 and dialyzed against 1 l PC + 0.1 M KCl overnight. The extract was applied to a BioRex 70 column, washed with 3 sequential 5 ml volumes of PC + 0.1 M KCl followed by a 5 ml wash with PC + 0.2 M KCl. CRP was eluted in 5 ml of PC + 0.4 M KCl. 1 μg of protein from each sample was electrophoresed through a 12.5% SDS−PAGE gel. Protein was visualized by staining with coomassie blue and the intensities of the CRP bands were determined by densitometry. The integrated area of each CRP band was compared to a standard curve prepared using pure CRP to obtain an estimate of CRP concentration in each sample.

CRP purification. CRP was isolated from six one-liter cultures of strain CA8445/pRK248 that contained the appropriate recombinant pRE2*crp* plasmid. Inocula were grown at 30°C in LB medium that contained 12.5 μg/ml tetracycline, 50 μg/ml ampicillin and 0.5% (w/v) glucose. Fresh medium was inoculated to an OD_{600} = 0.1 and incubated at 30°C with vigorous shaking. At OD_{600} = 1.2 the temperature was increased to 42°C to inactivate the λ repressor and growth was continued at 42°C for 2 hrs. Cells were harvested by centrifugation, suspended in 3 volumes of PC buffer + 0.3 M KCl that contained 130 μg/ml lysozyme, and disrupted in a blender. The extracts were clarified by centrifugation at 12,000×g for 30 min, and subsequently centrifuged at 100,000×g for 90 min. The clarified extract was diluted with 3 volumes of PC + 0.3 M KCl. CRP was isolated as described by Harman *et al.* (15). CRP was greater than 95% pure as judged by electrophoresis on 12.5% polyacrylamide-SDS gels stained with coomassie blue. CRP concentration was determined using the extinction coefficient 3.5×10^{-4} $M^{-1}cm^{-1}$ at A_{280} nm (3).

cAMP binding assay

During the course of CRP purification, cAMP binding activity was assayed by the ammonium sulfate precipitation procedure of Anderson *et al.* (3) as modified by Puskas *et al.* (25). For

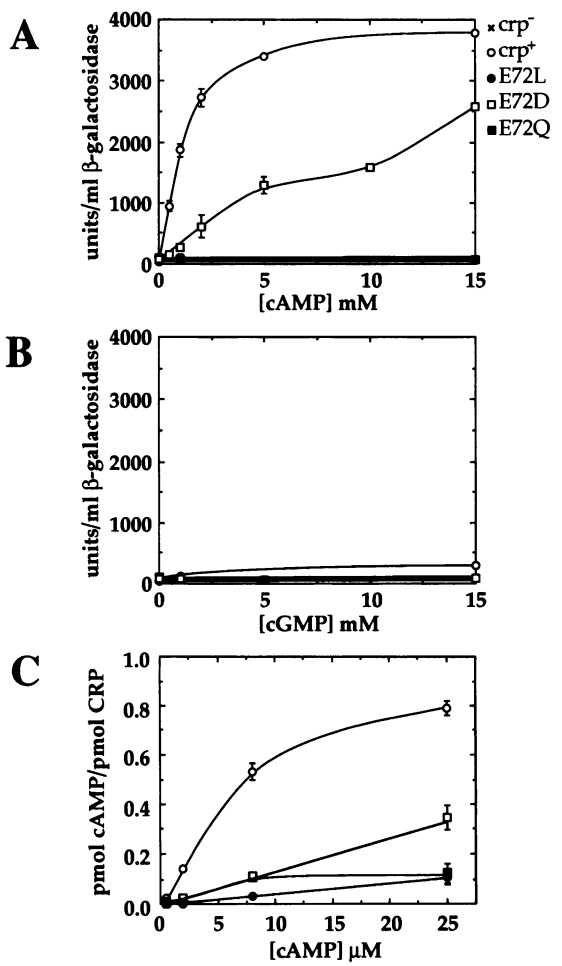

Figure 1. β-galactosidase activity in cells containing wild-type CRP and position 72 CRP mutants. CRP was expressed from pRE2*crp* plasmids in *E.coli* strain CA8445/pRK248. *crp*⁻ represents the control culture of *E.coli* CA8445/pRK248 that contained pRE2 vector DNA alone. Each value is the mean of 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 indicate 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.** cAMP binding on partially purified cell free extracts of D1210HP that contained the pRE2*crp* plasmid DNA expressing either the wild-type CRP or position 72 CRP mutants. An equivalent amount of D1210HP/pRE2 extract did not bind cAMP above background levels. *crp* allele contained on the pRE2*crp* plasmids (*crp*⁻, x; *crp*⁺, open circle) and the position 72 amino acid substitutions represented by the one letter designation: L, leucine (filled circle); D, aspartate (open square); Q, glutamine (filled square).

cAMP binding studies we utilized a solid phase partition assay adopted from Santa-Coloma *et al.* (26) and modified for CRP. 20 μg CRP and ^{3}H-cAMP were preincubated in 0.44 ml PC + 0.1 mM KCl on ice for 1 hr. 10 mg of phosphocellulose resin equilibrated in PC + 0.1 M KCl was added followed by incubation on ice. The samples were mixed by gentle vortexing every 15 min for period of one hour. The tubes were centrifuged at 12,000×g for 5 min and the supernatant fractions were discarded. The pellets were suspended in 0.4 ml PC + 2.0 M KCl and vortexed for 15 seconds every 5 min for 15 min. The tubes were centrifuged at 6500 RPM for 5 min. The radioactivity contained in 0.3 ml of sample was determined by conventional liquid scintillation counting. The results were adjusted to 0.4 ml and expressed as pmol cAMP. Control tubes containing reaction mixture and phosphocellulose but lacking CRP were used as blanks to determine the amount of ^{3}H-cAMP trapped in the phosphocellulose resin pellets.

Protease digestion assay of protein structure

Protease digestion reactions were carried out as described by Harman *et al.* (15) in a volume of 0.1 ml at 37°C in buffer that was 30 mM in Tris−HCl, pH 8.0 at 25°C, 2.5 mM in $MgCl_2$, 0.1 mM in EDTA and 100 mM in NaCl (in place of KCl to avoid SDS precipitation during SDS-PAGE experiments). The ratio of CRP to subtilisin BPN' was 300:1 by weight. Peptides were resolved by electrophoresis on 20% polyacrylamide-SDS gels and visualized by staining with coomassie blue. The gels were scanned with either a Molecular Dynamics Computing densitometer (Sunnyvale, CA) or a Bioimage Visage 2000 image analysis system (Ann Arbor, MI).

Analytical procedures

Protein assay. Protein determinations were performed using a protein assay kit based on the method of Bradford (27) using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. Electrophoresis on 12.5% or 20% polyacrylamide−SDS gels was carried out as described by Laemmli (28). 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. Gels were destained in a solution that was 5% in methanol and 7% in acetic acid.

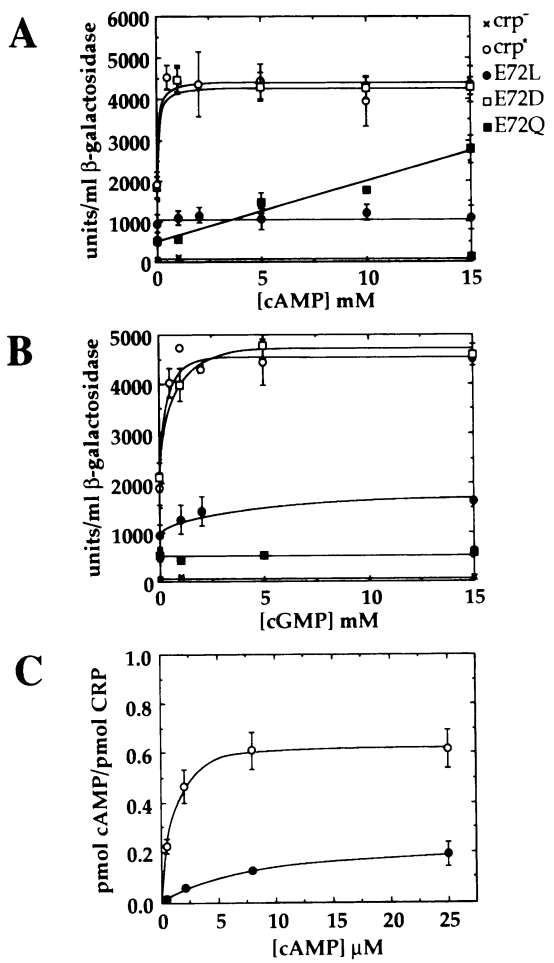

Figure 2. β-galactosidase activity in cells containing CRP*[A144T] or CRP*[A144T] position 72 mutants. Panel **A.** β-galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B.** β-galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C.** cAMP binding on partially purified cell free extracts of D1210HP that contained the pRE2*crp* plasmid DNA expressing either the CRP*[A144T] or CRP*[A144T] position 72 mutants. *crp**allele contained on the pRE2*crp* plasmids (*crp*⁻, x; *crp**, open circle) and the position 72 amino acid substitutions represented by the one letter designation: L, leucine (filled circle); D, aspartate (open square); Q, glutamine (filled square).

RESULTS

Assay of β-galactosidase and cAMP binding of position 72 mutants

β-galactosidase expression was measured to quantitate the effects of mutations on CRP function. The results presented in Figure 1, panel A illustrate that in *E.coli* CA8445/pRK248 high level β-galactosidase synthesis is strictly dependent upon the expression of CRP and, in cells that contain wild-type CRP, cAMP. cGMP does not substitute for cAMP in mediating β-galactosidase synthesis in cells that contained wild-type CRP (Figure 1, panel B). The substitution of leucine or glutamine for glutamate 72 produced forms of CRP that failed to promote β-galactosidase synthesis above background levels in the presence or absence of cAMP and cGMP (Figure 1, panels A and B). The substitution of aspartate for glutamate 72 produced a form of CRP that promoted cAMP-dependent β-galactosidase synthesis (Figure 1, panel A). Cells that contained E72D CRP required ten- to twelve-fold higher concentrations of cAMP to promote the level of β-galactosidase synthesis observed for cells that contained wild-type CRP.

Effects of mutations in the cyclic nucleotide binding pocket of CRP that limit cAMP-mediated effects on β-galactosidase synthesis could result from several causes including, but not limited to, decreased affinity of CRP for cAMP. To determine the effects of mutations on cAMP binding, cAMP binding affinity was measured using partially purified CRP prepared as described in Materials and Methods. The data presented in Figure 1, panel C demonstrate that substitution of either leucine, glutamine or aspartate for glutamate 72 resulted in decreases in CRP cAMP binding activity that ranged from moderate (E72D) to large (E72L and E72Q).

The results presented in Figure 2, panel A demonstrate that β-galactosidase synthesis in CA8445/pRK248/pRE2*crp* that

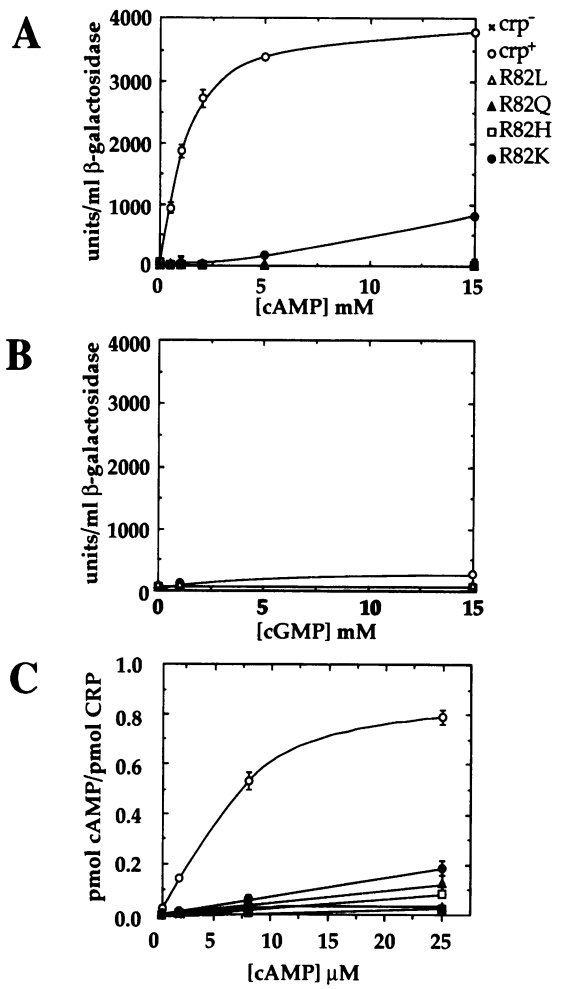

Figure 3. β-galactosidase activity in cells containing wild-type CRP and position 82 CRP mutants. Panel **A.** β-galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B.** β-galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C.** cAMP binding activity in partially purified cell free extracts of D1210HP that contained the pRE2*crp* plasmid DNA expressing either the wild-type CRP or position 82 CRP mutants. *crp* allele contained on the pRE2*crp* plasmids crp^-, x; crp^+, open circle) and the position 82 amino acid substitutions represented by the one letter designation: L, leucine (open triangle); Q, glutamine (closed triangle); H, histidine (open square); and K, lysine (closed circle).

contains CRP*[A144T] is both independent of cyclic nucleotide and stimulated to approximately 2-fold higher levels upon the addition of either cAMP or cGMP. Measurement of the activity of CRP*[A144T] in mediating *lac* gene expression provides a means to assess the effects of cyclic nucleotide binding pocket substitutions on CRP*[A144T] structure.

The substitution of aspartate for glutamate 72 had no apparent effect on the inherent activity of CRP*[A144T] (Figure 2, panel A). We believe this indicates that the aspartate substitution had little if any global effect on CRP*[A144T] structure. In contrast, the substitution of either leucine or glutamine for glutamate 72 resulted in a 2- to 3-fold decrease in the inherent activity of CRP*[A144T] (Figure 2, panel A). This indicates that in addition to local effects on CRP structure, E72L and E72Q substitutions introduce global structural changes that limit CRP*[A144T] function as a positive control element.

β-galactosidase synthesis in cells that contained E72D CRP*[A144T] was indistinguishable from that observed in cells that contained CRP*[A144T]. The substitution of aspartate for glutamate 72 had no apparent effect on the response of these cells to either cAMP or cGMP (Figure 2, panel A and B). The substitution of either leucine or glutamine for glutamate 72 severely limited the ability of CRP*[A144T] to promote effector-mediated stimulation of β-galactosidase synthesis (Figure 2, panels A and B). cAMP binding measurements show that decreased cAMP affinity accounts for this result for the E72L form of CRP*[A144T] (Figure 2, panel C).

Assay of β-galactosidase and cAMP binding of position 82 mutants

The data presented in Figure 3 show that the substitution of glutamine, leucine, isoleucine, or histidine for arginine 82 produced forms of CRP that failed to promote cAMP-mediated β-galactosidase synthesis. With the exception of R82K CRP, none of the position 82 substituted forms of CRP were observed to promote β-galactosidase synthesis above background levels (Figure 3, panels A and B). R82K CRP promoted significant β-galactosidase synthesis at high cAMP concentrations, requiring approximately 30-fold higher concentrations of cAMP to promote the level of β-galactosidase synthesis in cells that contained wild-type CRP (Figure 3, panel A). As was the case for wild-type CRP, cGMP did not substitute for cAMP in eliciting R82K CRP-dependent β-galactosidase synthesis over the range of concentrations tested. The data presented in Figure 3 panel C clearly demonstrate that all substitutions for arginine 82 resulted in large decreases in CRP affinity for cAMP.

The substitution of glutamine, histidine, or lysine for arginine had either no effect or altered the inherent activity of CRP*[A144T] by less that 25%, suggesting that none of these substitutions had significant affects on CRP*[A144T] structure (Figure 4, panel A). The substitution of leucine for arginine at this position had less than a 2-fold effect (Figure 4, panel A). The substitution of either leucine or glutamine for arginine 82 gave rise to forms of CRP*[A144T] that were severely limited in their ability to promote effector-mediated stimulation of β-galactosidase synthesis (Figure 4, panels A and B) and had decreased affinity for cAMP (Figure 4, panel C).

Cells that contained the R82K CRP*[A144T] required approximately 5- to 10-fold higher concentrations of cAMP to promote the level of β-galactosidase synthesis observed in cells that contained CRP*[A144T]. Approximately 20-fold higher concentrations of cAMP were required in cells that contained R82H CRP*[A144T] to promote the level of β-galactosidase synthesis observed in cells that contained CRP*[A144T] (Figure 4, panel A). Both the R82K and R82H CRP*[A144T] were capable of promoting β-galactosidase synthesis in a cGMP-dependent manner; however, approximately 30 to 60-fold higher concentrations of cGMP were required in cells that contained the R82K and R82H forms of CRP*[A144T] to promote the same level of β-galactosidase synthesis observed in cells that contained CRP*[A144T] (Figure 4, panel B). cAMP binding data demonstrate that the affinity of CRP*[A144T] for cAMP was affected very little by the substitution of lysine for arginine 82, and was substantially decreased by histidine substitution (Figure 4, panel C).

The data derived from position 82 mutants illustrate the importance of the ionic interaction in CRP binding to cyclic

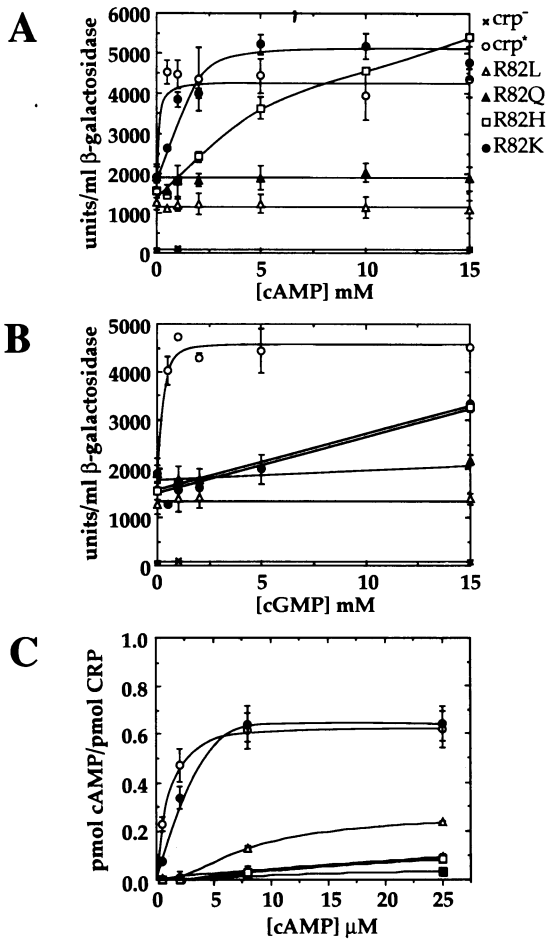

Figure 4. β-galactosidase activity in cells containing CRP*[A144T] or CRP*[A144T] position 82 mutants. Panel **A**. β-galactosidase activity measured in the presence or absence of cAMP at the indicated concentration. Panel **B**. β-galactosidase activity measured in the presence or absence of cGMP at the indicated concentration. Panel **C**. cAMP binding on partially purified cell free extracts of D1210HP that contained the pRE2*crp* plasmid DNA expressing either the CRP*[A144T] or CRP*[A144T] position 82 mutants. *crp**allele contained on the pRE2*crp* plasmids (*crp*⁻, x; *crp**, open circle) and the position 82 amino acid substitutions represented by the one letter designation: L, leucine (open triangle); Q, glutamine (closed triangle); H, histidine (open square); and K, lysine (closed circle).

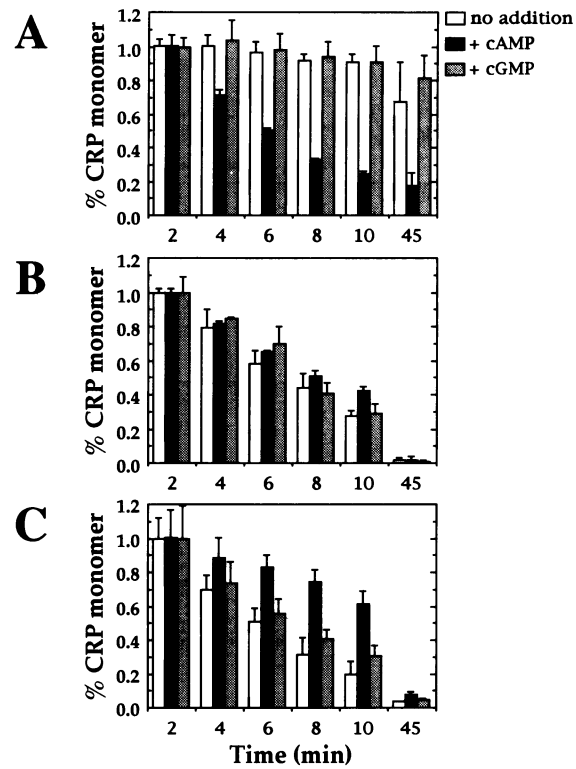

Figure 5. Protease digestion of CRP. The wild-type CRP (panel **A**), CRP*[A144T] (panel **B**) and R82K CRP*[A144T] (panel **C**) were incubated with protease in the absence of cyclic nucleotide (white bars), in the presence of 100 μM cAMP (black bars), or in the presence of 100 μM cGMP (gray bars). Protease digestion was terminated at the indicated time and 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 percent of the CRP monomer present at two minutes after the addition of protease. Data represent the mean and standard deviation of three independent experiments.

nucleotide. The distance and position of the positively charged residue [either permanent (lysine) or pH-induced (histidine)] proved to be an important factor in both cAMP binding and cAMP-mediated activation of CRP*[A144T] as well as establishing the response of the protein to an alternative effector, cGMP.

In vitro characterization of CRP and CRP*

Wild-type CRP can exist *in vitro* in at least two conformations that are differentiated in protease digestion reactions (29). CRP is relatively resistant to proteolysis in the absence of cAMP and is rapidly degraded by the protease in the presence of cAMP (Figure 5, panel A). CRP* forms of CRP are more sensitive to proteases in the absence of cAMP than is wild-type CRP (15, 17, 30; Figure 5, panel B). The R82K form of CRP*[A144T] displays protease sensitivity similar to that of CRP*[A144T]. These data support the prediction that the substitution of lysine for arginine 82 has minimal effects on the overall structure of CRP.

The effect of substitutions at position 82 on the binding constant of CRP to cAMP was examined by the cAMP binding assay described in Materials and Methods (Figure 6). Takahashi *et al.* (31) determined the intrinsic association constant of CRP for cAMP in the absence ($K = 4.8\times10^4\ M^{-1}$) and in the presence ($K' = 3.3\times10^5\ M^{-1}$) of non-specific DNA. The cAMP association constant of wild-type CRP (Figure 6, $K = 1.8\times10^5\ M^{-1}$) determined in this study is in reasonably good agreement with Takahashi *et al.* (31). CRP*[A144T] (Figure 6, $K = 1.2\times10^6\ M^{-1}$) has approximately 7-fold higher affinity for cAMP than wild-type CRP. This result is comparable to the 20-fold increase in K for a CRP* that contains the A144T substitution present in CRP*[A144T] (CRP*) and an R142H substitution (32). The substitution of lysine for arginine 82 resulted in decreased affinity of CRP*[A144T] for cAMP by a factor of 4.5 (Figure 7, $K = 2.7\times10^5\ M^{-1}$).

cGMP is both a competitive inhibitor of cAMP binding to wild-type CRP (33) and an activator of the CRP*[A144T]. The data presented in Figure 4 show that the R82K CRP*[A144T] has regained, to a large degree, the capacity to descriminate between cAMP and cGMP as effector signals. To confirm that a R82K CRP*[A144T]:cGMP complex does indeed form, the effectiveness

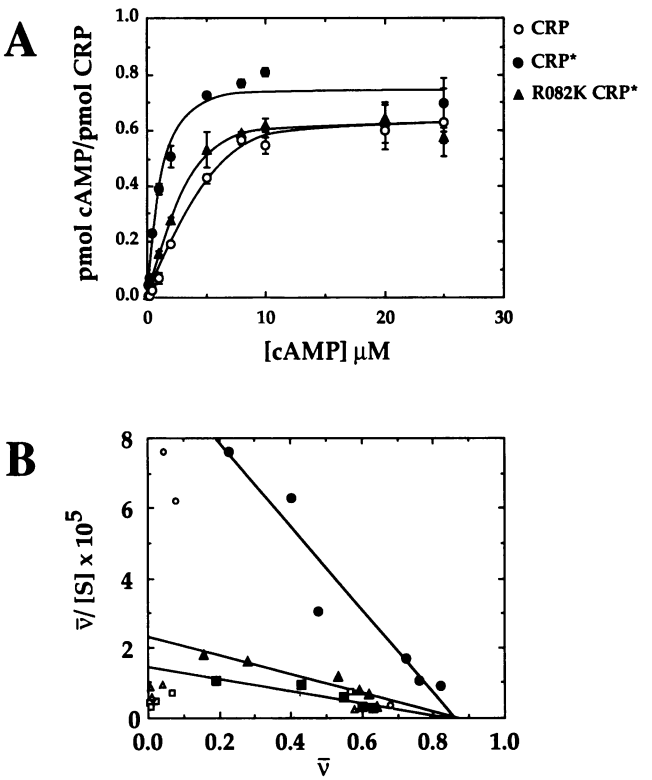

Figure 6. cAMP binding studies conducted using purified CRP preparations. Panel **A**. Wild-type CRP (open circles), CRP*[A144T] (closed circles), and R82K CRP*[A144T] (closed triangles) affinities for cAMP was measured using the solid phase partition assay as outlined in Materials and Methods. Each data point represents the average of two independent experiments. Panel **B**. Scatchard analysis (40) of the data presented in panel A; wild-type CRP (squares), CRP*[A144T] (circles), and R82K CRP*[A144T] (triangles). Filled data points were utilized in a linear regression analysis for determining the intrinsic affinity constants.

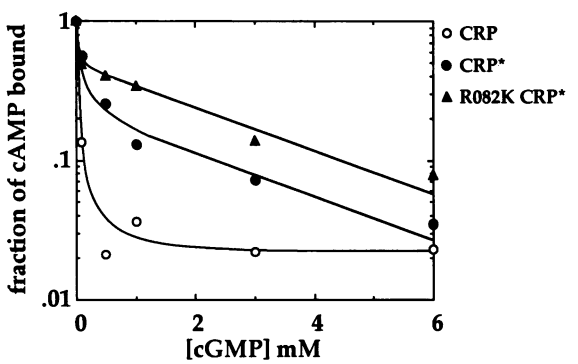

Figure 7. cGMP competition assay. The ability of cGMP to compete with cAMP in binding the wild-type CRP (open circles), CRP*[A144T] (closed circles), and R82K CRP*[A144T] (closed triangles) was measured as outlined in Materials and Methods. The initial ^{3}H-cAMP concentrations employed were 5.0 μM for wild-type CRP ($0.9\times K_d$), 0.5 μM for CRP*[A144T] ($0.6\times K_d$) and 2.0 μM for R82K CRP*[A144T] ($0.54\times K_d$).

of cGMP in competing with cAMP in the R82K CRP*[A144T]:cAMP complex was assessed. The data presented in Figure 7 confirm that cGMP is competitive with cAMP binding to wild-type CRP, to the CRP*[A144T] and to the R82K CRP*[A144T]. At cAMP concentrations near their respective K_d value ($0.5-0.9\times K_d$) a 90% decrease in cAMP binding was observed at approximately 0.1 mM cGMP for wild-type CRP, 2.5 mM cGMP for CRP*[A144T], and 4.5 mM for R82K CRP*[A144T]. Clearly cGMP forms a complex with all three forms of CRP. The CRP*[A144T]:cAMP and R82K CRP*[A144T]:cAMP complexes have lower affinity for cGMP than does the wild-type CRP:cAMP complex. The lysine for arginine substitution in the CRP*[A144T] background had less than a 2-fold effect on cGMP binding yet the R82K CRP*[A144T]:cGMP complex was not as effective an activator of *lac* as the CRP*[A144T]:cGMP complex (Figure 4, panel A).

DISCUSSION

The 2′ hydroxyl group of cAMP is an important determinant in mediating CRP-cAMP interactions; 2′ deoxy cAMP is a poor competitor of ^{3}HcAMP prebound to wild-type CRP (3−5) and of ^{3}HcGMP prebound to a CRP* (32). In CRP:$(cAMP)_2$ crystals the 2′ hydroxyl of cAMP participates in hydrogen bonding interactions with glutamate 72 and with the position 70−71 amide (13). The results presented here confirm the importance of the amino acid residue at position 72 in cAMP binding to CRP. CRP shows an absolute requirement for an acidic residue at position 72 in maintaining cAMP binding and responsiveness. Substitution of glutamine or leucine for glutamate 72 produced forms of CRP that were inactive in the absence of cAMP, unresponsive to cAMP and had low affinity for the nucleotide (Figure 1). The substitution of aspartate for glutamate 72, residues that differ by a methylene group, produced a form of CRP that was activated by cAMP and had a 10-fold decrease cAMP affinity. Both the affinity of CRP for cAMP and cAMP-mediated activation of CRP apparently have stringent requirements for the position of the position 72 carboxyl group within the cyclic nucleotide binding pocket.

Conclusions regarding the importance of position 72 in mediating the allosteric activation of CRP are less certain. Eschenlauer and Reznikoff (34) described the *in vivo* characteristics of a CRP mutant with valine at position 72. This is a weak CRP^{pc} (positive control) mutant, characterized as having affinity for CRP target sequences located in the *lac* and *gal* control regions yet failing to activate transcription initiation at *lac*P and *gal*P2. Our analysis of E72L CRP, which we predict is similar to E72V CRP, shows that this form of CRP fails to bind cAMP at physiologically relevant concentrations (0−10 μM). Its status with regard to the CRP^{pc} phenotype is unknown. A hydrophobic amino acid residue at position 72 apparently affects CRP in a manner that precludes high affinity cAMP binding. Our data do not directly confirm that the conformation of E72L CRP*[A144T] differs from that of wild-type CRP; however, this substitution decreased the inherent activity of CRP* by a factor of two. This result is consistent with the idea that position 72 substitutions can effect the overall conformation of CRP.

The activity of CRP*[A144T], a mutation-activated form of the protein, is stimulated to higher levels with the addition of either cAMP or cGMP (15, Figures 2 and 4). The conformation of the cyclic nucleotide binding pocket of CRP*[A144T] is thought to differ from that of unliganded wild-type CRP (17, 32). Wild-type CRP both binds and discriminates between cAMP and cGMP; CRP*[A144T] binds both cyclic nucleotides yet fails to discriminate between them. Substitution of aspartate for glutamate

72 had no apparent effect on either the inherent activity of CRP*[A144T] or on the ability of E72D CRP*[A144T] to bind and respond to both cAMP and cGMP (Figure 2). This suggests that the cyclic nucleotide binding pocket of CRP*[A144T] is more flexible than that of wild-type CRP. These data also show that position 72 does not play a major role in CRP cyclic nucleotide discrimination.

The E72L and the E72Q forms of CRP*[A144T] showed both decreased inherent activity and either a poor response to cAMP and no response to cGMP (E72Q), or no response to either cyclic nucleotide (E72L). The ionic interaction between glutamate 72 and arginine 123 observed in CRP:cAMP crystals does not play a *critical* role in maintaining either the structure or activity of CRP*[A144T]. This interaction may, however, be important in promoting cyclic nucleotide stimulated activity of CRP*[A144T]. Alternatively, hydrogen bond formation between the 2′ hydroxyl of cAMP and *both* the carboxyl group of glutamate and the position 070–071 amide may provide a set of interactions required for cAMP activation of the protein. The E72L and E72Q forms of CRP affect the formation of both position 72 and position 070–071 amide hydrogen bonds.

The CRP* phenotype can result from the substitution of serine for glycine 141 (12). A variant of this CRP* that contains both the position 141 substitution and a substitution of alanine for glutamate 72 showed no difference in inherent activity from the parental CRP* and, in contrast to the results obtained with CRP*[A144T], was activated by both cAMP and cGMP. Hydrogen bonding interactions between the glutamate side chain and 2′ hydroxyl of either cAMP or cGMP are apparently unimportant in cyclic nucleotide-mediated activation of the CRP* resulting from the position 141 substitution. Cyclic nucleotide activation of different CRP* forms apparently emphasize different portions of the ribose 3′, 5′ cyclic phosphate moiety.

Gronenborn *et al.* (35) demonstrated that the substitution of leucine for arginine 82 produced a form of CRP which failed to bind cAMP. The present study confirms and extends this result. Position 82 substitutions in the wild-type CRP background were found to dramatically affect CRP:cAMP complex-mediated β-galactosidase synthesis and CRP affinity for cAMP (Figure 3). β-galactosidase synthesis in cells that contained the conservative amino acid substitution R82K required approximately 30-fold higher concentrations of cAMP than cells that contained wild-type CRP. As was the case for position 72, what appears to be a small change in charge location at position 82 decreased the affinity of CRP for cAMP. Similar substitutions have been reported in a type I cAMP dependent protein kinase. A position homolog to arginine 82 in CRP was substituted with lysine in site A and with either tryptophan or glutamine in site B. Paralleling the results of the present study, all three mutants failed to bind cAMP (36, 37).

The activity of CRP*[A144T] was only modestly effected by amino acid subtitutions at position 82. Effector-mediated stimulation of CRP*[A144T] was greatly decreased by the substitution of either leucine or glutamine at this position and influenced in various ways by other substitutions. β-galactosidase synthesis in cells that contained R82K CRP*[A144T] was responsive to the addition of cAMP but failed to respond to cGMP (Figure 4). The binding constant of R82K CRP*[A144T] for cAMP is 4.5-fold lower than that of CRP*[A144T]. cGMP, while failing to stimulate the activity of R82K CRP*[A144T], is a competitive inhibitor of cAMP binding for both CRP*[A144T] and R82K CRP*[A144T] (Figure 7). Taken together, these data indicate that position 82 plays a role both in cyclic nucleotide binding and in effector discrimination (or lack thereof) in CRP*[A144T]. The fused furanose/phosphodiester ring system of 3′, 5′ cyclic nucleotides is similar in both cAMP and cGMP (38). Effector discrimination in the R82K variant of CRP*[A144T] must occur as the result of differences in the positioning of the invariant portions of cAMP and cGMP within the cyclic nucleotide binding pocket. Position differences for cAMP and cGMP are expected to arise through differences in the interactions between the purine base and amino acids 127 and 128 of CRP (12). The effect of amino acid substitutions at positions 127 and 128 on the ability of CRP and CRP*[A144T] to bind cAMP, to respond to cAMP and cGMP, and to descriminate between cAMP and cGMP is currently under investigation.

During revision of this manuscript we became aware of the report of Moore et al. (39) that presents *in vivo* characterization of position 72, 82, 83 and 123 amino acid substitutions in CRP and CRP*[A144T]. The results presented in the present study confirm and extend the results of Moore et al. (39) for position 72 and position 82 substitutions in CRP.

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