Interaction of CRP L124 with cAMP Affects CRP cAMP Binding Constants, cAMP Binding Cooperativity, and CRP Allostery†

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ABSTRACT: A cyclic nucleotide-binding pocket of the CRP dimer is composed of amino acid residues contributed by both subunits. Leucine (L) 124 of one subunit packs against the adenine ring of cAMP bound to the opposing subunit. We have undertaken a study designed to evaluate the role of L124 in CRP allostery. Wild-type (WT) apo-CRP is a 47 kDa protease-resistant dimer composed of identical subunits that exhibits a biphasic isotherm in cAMP titration studies. The WT CRP−cAMP complex is a protease-sensitive dimer degraded by protease to a dimer core that ranges between 26.5 and 30.5 kDa. Substitution of L124 with isoleucine (I), valine (V), cysteine (C), or alanine (A) generated a series of CRP variants that exhibited unique differences in apo-CRP resistance to protease, the mass of the core fragments generated in protease digestion reactions, cAMP-mediated allostery, and CRP−cAMP complex functionality. Differences in the affinity of the position 124 CRP variants for cAMP were observed. The binding constants that drive the formation of the WT and L124I CRP−cAMP complexes deviated by not more than a factor of 1.5. In contrast, the L124V, L124A, and L124C forms of CRP exhibited both a decreased K_{app}^{cAMP1} and an increased K_{app}^{cAMP2} to produce 2.4-, 55-, and 204-fold reductions, respectively, in the difference between these two parameters compared to that observed for WT CRP. The data indicate that the van der Waals volume and/or the hyrophobicity of the L124 side chain are important determinants of CRP cAMP binding properties and affect, either directly or indirectly, cAMP-mediated conformation changes in CRP.

CRP[1] is a 47 238 Da dimer made up of identical 209-amino acid residue subunits (*1*, *2*). Cyclic AMP interacts with amino acids located in the cyclic nucleotide-binding pocket of CRP and affects changes in CRP conformation (*3*). These conformation changes produce a protein that functions, along with DNA and RNA polymerase, in establishing active ternary transcription complexes at CRP-dependent promoters (*3*, *4*).

Seventeen amino acids lie within 4.5 Å of at least one atom of cAMP bound to either CRP subunit (*3*). Nine hydrophobic and six hydrophilic residues are contributed by the cAMP-bound subunit, whereas amino acid residues leucine (L) 124 and serine (S) 128 are contributed by the opposing subunit (Figure 1). Several studies have reported the structure and function effects of amino acid substitutions at cAMP-binding pocket positions occupied by polar or charged residues in CRP. The effects of amino acid substitutions at positions that contain hydrophobic amino acid residues in CRP have not been reported.

L124 packs against one face of the adenine ring of cAMP (*3*, *5*). During the course of a separate study, L124 was substituted with cysteine (S. R. Tomlinson and J. G. Harman, unpublished observations). Cells that contained the L124C CRP−cAMP complex weakly fermented ribose and lactose. L124C was found to be cross-linked through a L124C positional pair disulfide bond, to be insensitive to protease in both the absence and presence of cAMP, and to exhibit unique cAMP association constant values. These results led us to evaluate the role of L124 in CRP activation. To establish if the introduction of a disulfide bond or the loss of side chain volume gave rise to the unique characteristics of L124C CRP, we substituted L124 with isoleucine (I), valine (V), or alanine (A). These amino acid substitutions provided side chain volumes that ranged from 140 Å^3 for isoleucine to 67 Å^3 for alanine (*6*). The results of this study show that amino acid substitution at position 124 alters CRP cAMP binding parameters and affects the allosteric response of CRP to cAMP binding and CRP−cAMP complex efficacy as a transcription factor.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Escherichia coli CA8445/pRK248 (*7*) were used as host strains for recombinant *crp* plasmids. Plasmid pRK248 (λcI^{ts}, tet^r) (*8*) encodes a thermolabile λcI repressor used to control the λP_L promoter. Plasmid pLEX (Invitrogen) was used for high-level wild-type (WT), L124I, L124V, L124A, and L124C CRP expression. Plasmid pHA7 (*1*) and its *crp*L124I, *crp*L124V, *crp*L124A, or *crp*L124C derivative were used to assess the

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[1] Abbreviations: cyclic AMP or cAMP, cyclic 3′:5′-adenosine monophosphate; CRP, cAMP receptor protein; *lac*P, lactose operon promoter; IPTG, β-D-galactopyranoside; RNAP, RNA polymerase; PMSF, phenylmethanesulfonyl fluoride; ANS, 8-anilino-1-naphthalenesulfonic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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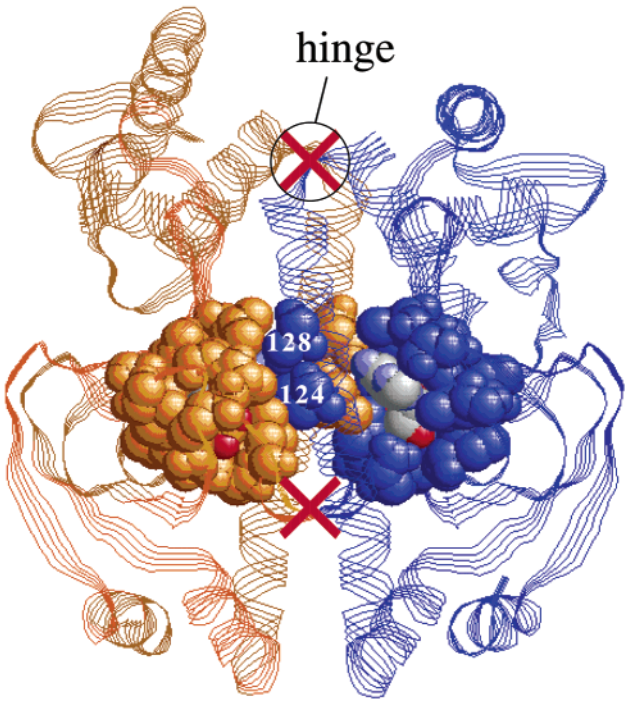

FIGURE 1: CRP−$(cAMP)_2$ structure. The CRP dimer is presented as a strand model. Subunit A is gold. Subunit B is blue. The amino acid residues that make up the cAMP-binding pockets are drawn as space-filled molecules; residues 124 and 128 of subunit B are labeled. The hinge-proximal (circled X) and subunit interface (X) protease cleavage sites are indicated. Cyclic AMP is space-filled and shown in CPK colors. This figure was generated from the coordinates of 1G6N (*21*) using SwissPdbViewer (3.6b2).

Table 1: Oligonucleotide Primers Used for *crp* Mutagenesis

CRP	nucleotide sequence
L124I	CAGATGGCGCGTCGTATTCAAGTCACTTCAGAG
L124I RC	CTCTGAAGTGACTTGAATACGACGCGCCATCTG
L124V	CAGATGGCGCGTCGTGTGCAAGTCACTTCAGAG
L124V RC	CTCTGAAGTGACTTGCACACGACGCGCCATCTG
L124C	CAGATGGCGCGTCGTTGCCAAGTCACTTCAGAG
L124C RC	CTCTGAAGTGACTTGGCAACGACGCGCCATCTG
L124A	CAGATGGCGCGTCGTGCGCAAGTCACTTCAGAG
L124A RC	CTCTGAAGTGACTTGCGCACGACGCGCCATCTG

effect of amino acid substitutions on CRP efficacy in stimulating *lac*P activity *in vitro*.

Enzymes and Other Materials. Restriction enzymes were obtained from New England Biolabs. Shrimp alkaline phosphatase, phenylmethanesulfonyl fluoride (PMSF), and isopropyl thio-β-D-galactopyranoside (IPTG) were purchased from United States Biochemical Corp. Cyclic AMP and subtilisin BPN′ (type XXVII, 7.9 units/mg) were purchased from Sigma Chemical Co. Precast 10 to 20% polyacrylamide gels were purchased from Bio-Rad Laboratories. DNA isolation kits were purchased from Qiagen and from Promega. Synthetic oligonucleotides were synthesized by the Texas Tech University Biotechnology Institute Core Facility. Common salts and buffers were reagent grade or better.

Site-Directed Mutagenesis of crp. Double-stranded plasmid DNA was mutagenized using the Quick-Change PCR protocol (Stratagene) coupled with the mutagenic oligonuceotide pairs listed in Table 1.

Assay of β-Galactosidase. CA8445/pRK248 cells harboring pHA7, pHA7*crp*L124I, pHA7*crp*L124V, pHA7*crp*L124A, or pHA7*crp*L124C were cultured as described by Belduz et al. (*9*). Samples were assayed for β-galactosidase activity according to the method of Miller (*10*).

CRP Isolation. CRP was prepared using standard ion-exchange chromatography techniques. CRP induction, extract preparation, and purification over BioRex 70 resin were carried out as described by Harman et al. (*7*).

Protease Probe of CRP Structure. Protease digestion reactions were carried out at 23 °C in a volume of 40 μL in NP buffer that was 100 mM in NaCl (*11*). Reaction mixtures contained 20 μg of CRP and, where indicated, cAMP. Reactions, initiated by the addition of protease, ran for 1 h and were terminated by the addition of PMSF to a concentration of 5 mM. Peptides were resolved on 10 to 20% gradient polyacrylamide−SDS gels, visualized by staining with acidic Coomassie blue R-250, and quantitated using a Molecular Dynamics 300B laser densitometer. Samples were prepared for N-terminal amino acid sequence analysis by equilibrating the gels in a 10 mM CAPS buffer (pH 11) solution that contained 10% methanol and 2 mg/mL dithiothreitol. The peptides were electrophoretically transferred to a PVDF membrane. PVDF membranes were stained for 1 min in a 0.1% solution of Coomassie blue R-250 with 40% methanol and 1% acetic acid and destained in a 50% methanol solution. The CRP core fragments were excised and analyzed in a Porton model 2020 peptide sequencer.

CRP Core Fragment Mass Determinations. WT, L124I, L124V, and L124A CRP (50 μg) were digested with subtilisin as described above. The reaction mixtures were diluted in 100 volumes of water and concentrated to a final volume of 20 μL using Centricon centrifugal filters (molecular weight cutoff of 10 000). The samples were then brought to 40 μL with water and diluted with the addition of 2 volumes of an ionizing matrix solution that contained 10 mg/mL sinapinic acid, 10% trifluoroacetic acid, and 50% acetonitrile. The samples were analyzed on a PerSeptive Biosystem Voyager DE matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. Spectra were calibrated to cytochrome *c* and lysozyme.

Cyclic AMP Binding Assay. Fluorescence titration experiments were conducted at room temperature on protein samples that had been dialyzed against 50 mM Tris-HCl (pH 7.8), 0.1 M KCl, and 1 mM EDTA. Fluorescence measurements were performed and the data analyzed as described previously (*11*).

RESULTS

Changes in the fluorescence intensity of ANS−CRP complexes measured as a function of cAMP concentration generate a biphasic titration curve that is typically fitted to a two-site model. The apparent association constants for cAMP binding to WT CRP exhibit values that range from 3.0 to 30 $\times$ 10^4 M^{-1} for K_{app}^{cAMP1} and from 2.0 to 10 $\times$ 10^2 M^{-1} for K_{app}^{cAMP2} (*11*−*13*). The interpretation of these binding isotherms has been that apo-CRP contains two identical cAMP binding sites that develop negative cooperativity upon the binding of one cAMP molecule to either site. To determine the effects of position 124 amino acid substitutions on cAMP binding to CRP, we measured ANS−CRP complex fluorescence intensity changes as a function of cAMP concentration and fitted the data to the two-site model.

Table 2: Cyclic AMP Binding Constants for the WT and Amino Acid-Substituted Forms of CRP

CRP	K_{app}^{cAMP1}	K_{app}^{cAMP2}	$K_{app}^{cAMP1}/K_{app}^{cAMP2}$
L124I	4.8×10^5	1.3×10^2	3700
WT	3.1×10^5	1.9×10^2	1600
L124V	2.0×10^5	3.0×10^2	670
L124A	4.2×10^4	1.4×10^3	30
L124C	2.3×10^4	2.8×10^3	8.2

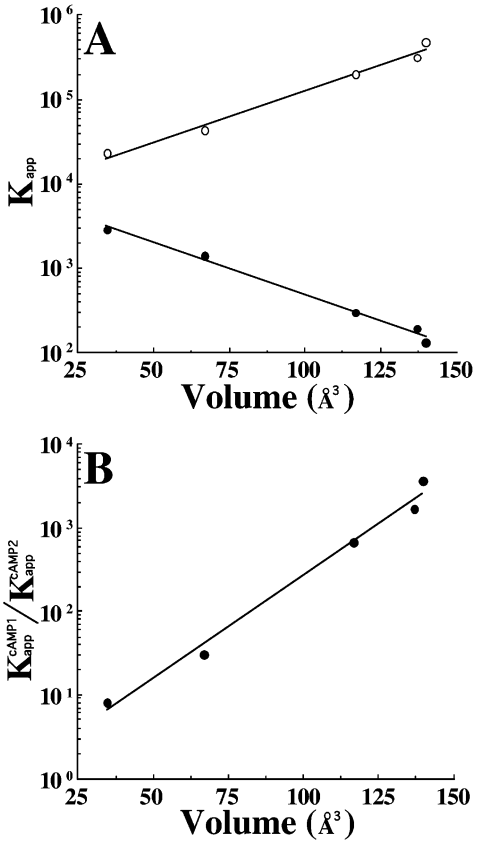

FIGURE 2: Effects of position 124 nonpolar side chain atom volumes on CRP association constants for cAMP binding. (A) The association constants K_{app}^{cAMP1} (○) and K_{app}^{cAMP2} (●) are plotted against nonpolar side chain volumes; the data fit to exponential equations having R^2 values of 0.98 and 0.99, respectively. (B) The ratio of K_{app}^{cAMP1} to K_{app}^{cAMP2} are plotted against position 124 nonpolar side chain volume. The data fit to an exponential equation having an R^2 value of 0.99.

Calculated K_{app}^{cAMP1} values for WT, L124I, L124V, L124A, and L124C CRP were 3.1×10^5, 4.8×10^5, 2.0×10^5, 4.2×10^4, and 2.3×10^4 M^{-1}, respectively (Table 2). The calculated K_{app}^{cAMP2} values for WT, L124I, L124V, L124A, and L124C CRP were 1.9×10^2, 1.3×10^2, 3.0×10^2, 1.4×10^3, and 2.8×10^3 M^{-1}, respectively. A comparison of K_{app}^{cAMP1} and K_{app}^{cAMP2} values to the volume of the position 124 side chain clearly demonstrates that the side chain hydrophobic atom volume directly correlates with K_{app}^{cAMP1} ($R^2 = 0.98$) and inversely correlates with K_{app}^{cAMP2} ($R^2 = 0.99$) (Figure 2A). Cooperativity between cAMP binding sites was assessed by calculating the ratio of K_{app}^{cAMP1} and K_{app}^{cAMP2}. Data presented in Figure 2B illustrate that the decreased side chain volume at position 124 was accompanied by decreased negative cooperativity in cAMP binding. The magnitude of the decrease in cooperativity was 461-fold when comparing isoleucine to cysteine. Figure 2A demonstrates that this decrease in negative cooperativity resulted from reciprocal effects of the position 124 amino acid side chain on K_{app}^{cAMP1} and K_{app}^{cAMP2}.

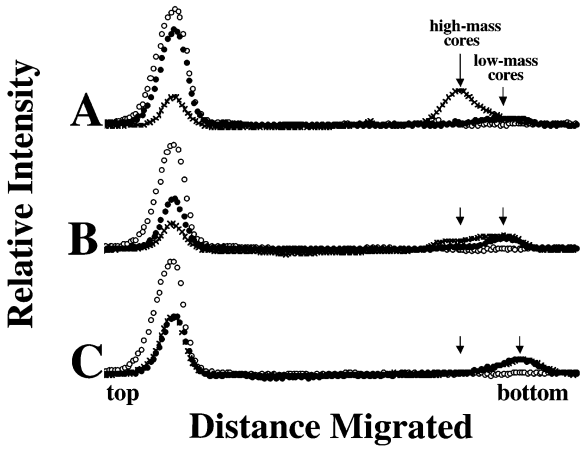

FIGURE 3: Densitometer tracings of the protease reaction products separated on SDS−polyacrylamide gels. The relative intensity of Coomassie blue staining material is displayed against the migration distance through the gel. Lanes contained L124I CRP (A), L124V CRP (B), and L124A CRP (C) in the absence of protease (○), in the presence of protease (●), or in the presence of protease and cAMP (×). The baseline values for three data sets were shifted vertically for clarity of presentation. Arrows highlight the approximate mass center of the CRP core populations described in the text as either high-mass or low-mass cores.

Apo-CRP and the CRP−cAMP complex are distinguished by their sensitivity to protease (*12*). Apo-CRP is resistant to protease, whereas the CRP−cAMP complex is protease-sensitive. Subtilisin cleaves the CRP−cAMP complex to yield a core fragment population composed of proteins whose N-terminus maps to CRP residue 2 or 4 and whose C-terminus is located either in the CRP hinge (positions 133−141) or in the middle of the C helix (positions 116−121) depending on the reaction conditions and stability of the CRP dimer (*14*, *15*; Figure 1). Subtilisin hydrolysis of peptide bonds in the CRP hinge yields high-mass (∼15 kDa) core fragments. Subtilisin hydrolysis of peptide bonds in the central portion of the CRP C helix, bonds inaccessible to protease in the CRP dimer, yields low-mass (∼13 kDa) core fragments.

The effects of position 124 amino acid substitutions on CRP structure and the response to cAMP allostery were investigated in protease digestion reactions. The protease digestion characteristics of WT CRP and the position 124-substituted forms of CRP were unique in most cases. The apo forms of WT CRP (not shown), L124I CRP (Figure 3), and L124C CRP (not shown) were all refractory to subtilisin digestion. Incubation of apo-L124I CRP with subtilisin led to a small decrease in the CRP monomer population and the appearance of a small amount of low-mass core fragments (Figure 3). We interpret these results to reflect the degree to which L124I CRP subunits exchanged during the course of the exposure of L124I CRP to protease. The incubation of apo-L124V CRP or apo-L124A CRP with subtilisin led to a greater loss of the CRP monomer and greater production of low-mass CRP core populations (Figure 3). To verify the point of CRP hydrolysis in these reactions, N-terminal amino acid sequence and MALDI-TOF analysis was conducted. The results show that the L124V CRP and L124A CRP core populations contained two predominant N-terminal amino

Table 3: Summary of N-Terminal Amino Acid and MALDI-TOF Mass Spectral Analysis of the Core Fragments Generated from the WT and Position 124 Mutant Forms of CRP

CRP	N-terminus	MALDI-TOF mass (Da)	predicted mass (Da)	predicted sequence
WT	not done	not digested		
L124I	not done	not digested		
L124V	VL/GKPQTD	13 173	13 171	G4−S117
		13 379	13 384	V2−S117
L124A	VL/GKPQTD	unstable, not done		
L124C	not done	not digested		

Table 4: Summary of N-Terminal Amino Acid and MALDI-TOF Mass Spectral Analysis of the Core Fragments Generated from the WT and Position 124 Mutant Forms of CRP Digested in the Presence of 90 μM cAMP

CRP	N-terminus	MALDI-TOF mass (Da)	predicted mass (Da)	predicted sequence
WT	VL/GKPQTD	15 050	15 054	G4−L134
		15 264	15 267	V2−L134
L124I	VL/GKPQTD	15 057	15 054	G4−L134
		15 269	15 267	V2−L134
		14 330	14 327	G4−T127
		14 543	14 539	V2−T127
L124V	VL/GKPQTD	15 049	15 041	G4−L134
		15 264	15 253	V2−L134
		14 319	14 313	G4−T127
		14 532	14 525	V2−T127
		13 380	13 371	G4−Q119
		13 585	13 583	V2−Q119
		13 178	13 171	G4−S117
		13 380	13 384	V2−S117
L124A	VL/GKPQTD	unstable, not done		
L124C	not done		not digested	

acid sequences: that predicted for CRP after removal of the N-terminal formylmethionine (VLGKPQTD) and that predicted for CRP after removal of the N-terminal formylmethionine and residues V1 and L2 (GKPQTD) (Table 3). This coupled with values for the L124V CRP core fragments masses enabled us to deduce that this core population was generated by subtilisin cleavage at position 117 (Table 3). We were unable to determine the masses of the L124A CRP cores; however, knowing the N-terminal sequence of this core population and observing the position to which the cores migrated in SDS−polyacrylamide gels (Figure 3), we conclude that the L124A CRP was cleaved at a position located in the C helix.

When complexed with cAMP, the WT (not shown), L124I, L124V, and L124A (Figure 3) forms of CRP were all hydrolyzed by subtilisin, whereas the L124C CRP was not (not shown). The subtilisin cleavage site for the WT CRP−cAMP complex was mapped to hinge position L134 (Table 4). Similarly, subtilisin cleaved the L127I CRP−cAMP complex in or near the hinge region at hinge position L134 and C-proximal C helix residue T127 located near the hinge (Table 4). This indicates that, as with WT CRP (*12*), cAMP binding affects the conformation of the L124I CRP hinge. In contrast to these results, the L124V CRP−cAMP complex yielded a mixed population of high- and low-mass core fragments, whereas the L124A CRP−cAMP complex yielded only a population of low-mass core fragments. Subtilisin cleaved the L124V CRP−cAMP complex to a core population that terminated at hinge or hinge-proximal positions 134 and 127 and at C helix positions 119 and 117 (Table 4). The positions in SDS−polyacrylamide gels to which the

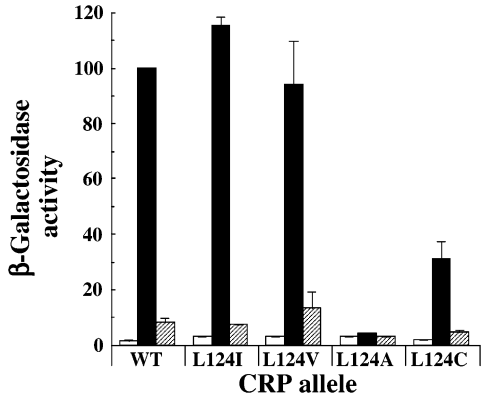

FIGURE 4: β-Galactosidase activity in cells containing the WT or position 124 mutant forms of CRP. CRP was expressed from pHA7 derivatives contained in *E. coli* strain CA8445/pRK248. Each value is the mean of at least two independent experiments. Cells were cultured in the absence of cAMP (white bars), in the presence of cAMP at 5 mM (black bars), or in the presence of cGMP at 5 mM (striped bars). The activity measured in cultures of cells transformed with pHA7 grown in the presence of cAMP was set at 100. The vertical error bars show one standard deviation.

cores generated in L124A CRP−cAMP complex digestion reactions migrate are again consistent with hydrolysis of the L124A CRP at C helix positions (Figure 3 and Table 4). The data presented in Figure 3 and Tables 3 and 4 show that decreasing the side chain hydrophobic atom volume at position 124 affected the exposure of C helix subtilisin cleavage sites in CRP. In addition, the protease data show that cAMP binding to the L124V CRP produced, to a degree, hinge conformation changes similar to those observed for WT CRP. This was not the case for the L124A CRP where we saw no evidence to suggest the generation of high-mass core fragments in reactions that included the L124A CRP−cAMP complex. Unlike WT CRP, the L124A CRP does not adopt a protease-sensitive hinge conformation upon binding cAMP. This interpretation is consistent with the observation that the L124C CRP−cAMP complex was not digested by subtilisin. L124C CRP clearly binds cAMP yet did not develop a protease-sensitive hinge in response to cAMP binding. This property is unique to the L124C CRP inasmuch as the mutant with the cysteine substitution at position 127, which generates a disulfide-cross-linked CRP, shares several characteristics with WT CRP, including the development of a protease-sensitive hinge in response to cAMP binding (*11*, *16*).

The effects of position 124 amino acid substitutions on CRP activity were assessed *in vivo* by monitoring the production of β-galactosidase activity in cells (CA8445/pRK248, $cya_{\Delta}crp_{\Delta}cI^{ts}$) that contained the WT CRP and position 124 CRP variants (Figure 4). Cells that contained WT CRP or mutant CRP produced basal levels of β-galactosidase when cultured in the absence of cAMP. These cells failed to produce elevated levels of β-galactosidase in the presence of cGMP, indicating that amino acid substitution at position 124 did not affect CRP ligand specificity. Cells that contained the WT, L124I, or L124V form of CRP synthesized similar amounts of β-galactosidase when cultured in the presence of cAMP. Cells that contained either the L124A or L124C CRP and cultured in the presence of cAMP produced 4 or 30%, respectively, of the β-galactosidase produced by cells that contained WT CRP cultured under the same conditions. The reduced activity of the L124A and

L124C forms of CRP did not result from reduced production of CRP; roughly equivalent amounts of the CRP monomer were observed in Coomassie blue-stained SDS−polyacrylamide gels loaded with extract prepared from cells that contained any one of the five forms of CRP. We did observe that the overall yield of L124A CRP was lower than that of the other forms of CRP after purification. Loss of L124A CRP occurred during a 100000*g* centrifugation and subsequent chromatography step, suggesting that this form of the protein was less soluble in aqueous solution than WT CRP. The lack of a cAMP-mediated response of β-galactosidase in cells that contained the L124A CRP suggests either the presence of a preponderance of CRP monomers in cells, the formation of inactive L124A CRP dimers in the presence of cAMP and DNA, or the formation of protein aggregates that effectively reduced the cellular concentration of the L124A CRP−cAMP complex. The relatively robust response of cells that contained the L124C CRP to cAMP, a dimer form of the protein that did not develop a protease-sensitive hinge upon binding cAMP, indicates that this variant can adopt in the presence of cAMP and DNA a conformation that shows significant activity.

DISCUSSION

Amino acid substitutions at CRP positions 124 and 128, residues separated by one turn of the C helix, affect both cAMP binding affinity and binding cooperativity. S128 lies along the C6−N7 edge of cAMP and forms a hydrogen bonding interaction with the N^6-amino group of cAMP (Figure 1; *3*). The role of the hydrogen bond formed between the N^6-amino group of cAMP and CRP S128 has been investigated (*16*, *17*). Threonine (T) substitutes for S128, and with the exception of relaxed effector specificity, S128T CRP is indistinguishable from WT CRP (*16*). Alanine (A) does not functionally substitute for serine at position 128, producing instead a form of CRP that is unable to undergo proper cAMP-mediated conformation transitions and is limited in its ability to activate *lac*P in the presence of cAMP (*16*). Alanine substitution for serine 128, while having little effect on K_{app}^{cAMP1}, decreases K_{app}^{cAMP2} (*17*).

In contrast to the results obtained with position 128 substitutions, position 124 substitutions affect both K_{app}^{cAMP1} and K_{app}^{cAMP2}. L124 interacts with the adenine face of cAMP (*18*, *19*). In addition, the methyl proton resonance of CRP L124 has been shown to undergo a chemical shift in response to cAMP binding to CRP (*20*). We have shown in this study that with respect to functionality CRP will tolerate at position 124 leucine, isoleucine, or valine, amino acid residues having nonpolar side chain atom volumes that range between 140 and 117 Å^3. A 2% (L → I) increase in the nonpolar side chain atom volume resulted in the approximate doubling of the ratio of K_{app}^{cAMP1} to K_{app}^{cAMP2}. A 15% (L → V) decrease in nonpolar side chain atom volume resulted in the halving of the ratio of K_{app}^{cAMP1} to K_{app}^{cAMP2}. Neither of these changes had an apparent effect on CRP efficacy in stimulating the expression of β-galactosidase in *E. coli*. A 50% (L → A) to 75% (L → C) decrease in nonpolar side chain atom volume resulted in larger effects on both K_{app}^{cAMP1} and K_{app}^{cAMP2} and were shown to limit CRP function in stimulating the expression of β-galactosidase. The results of this study lead us to three conclusions.

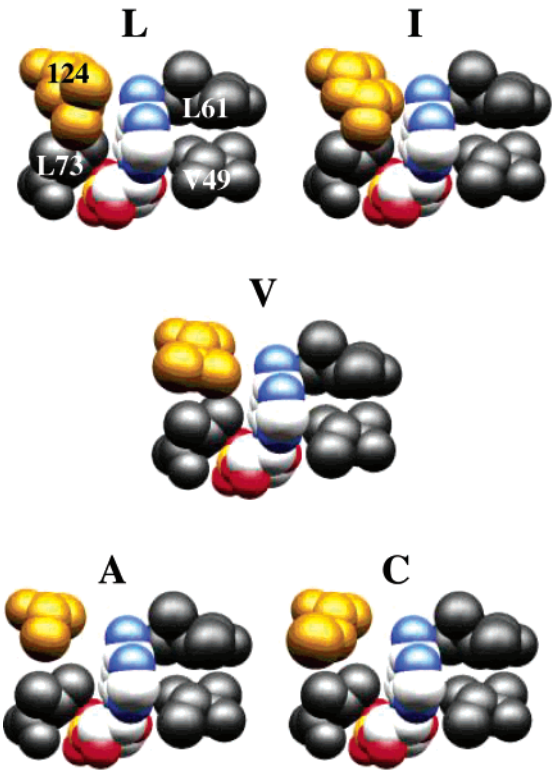

FIGURE 5: Packing of amino acid side chains and cAMP bound to the CRP B subunit. Cyclic AMP bound to subunit B, in CPK colors, and residues V49, L61, and L73 of subunit B are illustrated as space-filled molecules. The residue at position 124, contributed by CRP subunit A, is gold and illustrated as a space-filled molecule. The one-letter designation is used to identify the amino acid residue at position 124. This figure was generated from the coordinates of 1G6N (*21*) using SwissPdbViewer (3.6b2).

Conclusion 1. The Position 124 Amino Acid Residue Plays a Role in Communicating cAMP Binding Site Occupancy. Amino acid substitution at position 124 resulted in remarkably equivalent and opposite effects on K_{app}^{cAMP1} and K_{app}^{cAMP2} (Table 2 and Figure 2B). The magnitude of the effects on K_{app}^{cAMP1} and K_{app}^{cAMP2} showed a strong correlation with the nonpolar side chain atom volume of the amino acid at position 124. To address the mechanism underlying the effects of amino acid substitution at position 124 on the cAMP binding parameters K_{app}^{cAMP1} and K_{app}^{cAMP2}, it is important to consider the characteristics of cAMP binding to CRP.

One interpretation of the biphasic cAMP binding isotherm coupled with crystal structure information led to a proposal of a two-site binding model that predicted the existence of identical cAMP-binding sites that develop, upon binding the first molecule of cAMP, negative cooperativity (*12*, *13*, *18*; Figure 1). The biphasic isotherm was interpreted to show, at micromolar cAMP concentrations, the filling of the first site followed, at millimolar cAMP concentrations, by the filling of the second site. Figure 5 shows the cAMP-binding pocket of one CRP subunit. If the two-site binding model correctly represents the manner in which CRP binds cAMP, then the interpretation of reciprocal effects of the position 124 substitutions on the cAMP binding parameters K_{app}^{cAMP1} and K_{app}^{cAMP2} may be explained as follows. In the absence of cAMP, the hydrophobic amino acid residues of the cAMP-binding pocket arrange to maximize van der Waals interactions. Once cAMP with defined affinity K_{app}^{cAMP1} binds, L124−cAMP packing interactions in the filled subunit

translate directly to the adjacent subunit stabilizing the empty cAMP-binding pocket and decreasing K_{app}^{cAMP2}. Such a mechanism would result in an apparent negative cooperativity between otherwise identical binding sites in dimer CRP. Decreasing (L124V, L124A, or L124C) the position 124 nonpolar side chain atom volume is expected to decrease K_{app}^{cAMP1} due to a loss of hydrophobic packing energy and to increase K_{app}^{cAMP2} due to weaker cAMP-binding pocket constriction in the adjacent subunit. Conversely, increasing (L124I) the position 124 nonpolar side chain atom volume is expected to increase K_{app}^{cAMP1} by increasing the hydrophobic packing energy and to decrease K_{app}^{cAMP2} by further constricting the cAMP-binding pocket in the adjacent subunit.

In developing a rationale to explain the behavior of the position 124 mutants, we recognize that uncertainty exists in interpreting the biphasic cAMP binding isotherms. The two-site cAMP binding model has come into question with the discovery that CRP can bind up to four molecules of cAMP (*19*, *21*−*23*). In CRP crystals, two molecules of cAMP are buried in what have been characterized as high-affinity sites located in the N-proximal portion of CRP (Figure 1). A second pair of cAMP molecules is bound to low-affinity cAMP binding sites located on the surface of the CRP C-proximal domain (*19*; not shown in Figure 1). Reinterpretation of the cAMP binding data in light of this discovery led to the proposal that, at micromolar cAMP concentrations, the first inflection in the cAMP binding data results from cAMP filling the high-affinity sites almost simultaneously and in a positively cooperative manner (*19*). The two low-affinity sites subsequently fill at millimolar cAMP concentrations, completing the second phase of the cAMP binding isotherm. One piece of evidence supporting this model comes from calorimetry studies, the results of which have been interpreted to indicate positive cooperativity in cAMP binding to the two high-affinity cAMP binding sites (*24*, *25*). Lin and Lee (*25*) recently reported that cAMP titration data may be fit to either a two-site or a three-site binding model. While the three-site model was found to fit the titration data obtained from the WT CRP and some CRP mutant forms, the cAMP binding properties of other mutant forms of CRP could only be fit using a two-site model. In our laboratory, cAMP titration data can be fit using a two-site binding model only. If in fact a three- or four-site model more accurately represents the mode of cAMP binding to CRP, then our data clearly show that the low-affinity cAMP binding sites are not independent of the high-affinity cAMP binding sites.

How might signal from the N-proximal high-affinity cAMP binding sites translate to the low-affinity sites? Cyclic AMP binding to CRP has been shown to affect not only the methyl proton resonance of L124 but also the methyl proton resonances of residues V49, L61, and L73 (*20*). It is possible that the residues critical to communicating cAMP binding site occupancy lie on the adenine face opposite that contacted by L124. A reduction of the hydrophobic amino acid residue volume on one face of the adenine ring could manifest its effects by reducing the number of amino acid interactions that take place on the opposite face of the adenine ring. V49 and L61 occupy positions in β-strands 4 and 5, respectively. These β-strands form a loop structure that serves as a flap covering the N-proximal cAMP-binding pockets and has been implicated as being important in transmitting signal from the N-proximal to the C-proximal domain in cAMP-mediated allosteric changes in CRP conformation (*19*).

Conclusion 2. Leucine at Position 124 Plays a Role in Establishing the Strength of Subunit−*Subunit Interactions in CRP.* Previous results from our laboratory showed that the rate of CRP C helix peptide bond hydrolysis was directly proportional to the rate of CRP subunit exchange (ref *15* and Figure 1). For apo-WT CRP, subunit exchange, and hence C helix peptide bond hydrolysis, are negligible during a 1 h incubation with protease. For those forms of CRP (i.e., Y99A CRP) that exhibit unusually high rates of subunit exchange, C helix peptide bond hydrolysis is extensive (*15*). The extent of C helix peptide bond hydrolysis in protease digestion reactions that include the Y99A CRP was reduced in the presence of cAMP, a condition known to strengthen subunit−subunit interaction (*26*), and eliminated by introducing a covalent cross-link between Y99A CRP subunits. The results of the protease assay clearly show that both the L124V and L124A apo forms of CRP yielded low-mass core fragment populations, consistent with protease access to C helix peptide bonds. We do not know at this time if the valine and alanine substitutions at position 124 act to lower the CRP subunit equilibrium dimerization constant or to increase the rate of CRP subunit exchange. The net result of either would be to decrease the stability of the CRP dimer and increase the extent to which C helix peptide residues are exposed to solvent and hence protease.

Conclusion 3. Decreased Side Chain Hydrophobic Atom Volume at Position 124 Interferes with cAMP Allostery. The third conclusion, drawn from the results of both the protease data and *in vivo* β-galactosidase measurements, is that decreased side chain hydrophobic atom volume at position 124 interferes with cAMP allostery. This is most evident from the results obtained for the L124A and L124C forms of CRP. Alanine substitution of leucine at position 124 precluded high-mass core fragment production from the L124C CRP−cAMP complex; only low-mass cores were observed for the L124A CRP in the absence and presence of cAMP. In addition, cysteine at position 124, while stabilizing the CRP dimer through a disulfide cross-link, failed to support the development of a protease-sensitive hinge in the L124C CRP−cAMP complex.

This report characterizes, for the first time, the effects of amino acid substitution in the cAMP-binding pocket at a position occupied by a hydrophobic amino acid residue in CRP and demonstrates the importance of hydrophobic packing within the cyclic nucleotide-binding pocket on CRP structure and function. In addition to effects on CRP cAMP binding parameters and activity, there is a consequence of position 124 amino acid substitution on CRP subunit stability. That the L124V CRP and L124A CRP yield low-mass cores when digested by subtilisin in the presence or absence of cAMP indicates that either the dimerization equilibrium constant or the rate of subunit exchange of these two mutant forms of CRP differs from that of WT CRP. Stabilizing the dimer with a disulfide bond at position 124 produced a form of CRP that activated *lac*P to a level one-third of that of WT CRP. This suggests that at least some of the problems associated with decreased nonpolar side chain atom volume at position 124 can be overcome by forced, disulfide-mediated, dimerization.

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REFERENCES

1. Aiba, H., Fujimoto, S., and Ozaki, N. (1982) *Nucleic Acids Res. 10*, 1345−1362.
2. Cossart, P., and Gicquel-Sanzey, B. (1982) *Nucleic Acids Res. 10*, 1363−1378.
3. Harman, J. G. (2001) *Biochim. Biophys. Acta 1547*, 1−17.
4. Busby, S., and Ebright, R. H. (1999) *J. Mol. Biol. 293*, 199−213.
5. Passner, J. M., Schultz, S. C., and Steitz, T. A. (2000) *J. Mol. Biol. 304*, 847−859.
6. Miller, S., Janin, J., Lesk, A. M., and Chothia, C. (1987) *J. Mol. Biol. 196*, 641−656.
7. Harman, J. G., Peterkofsky, A., and McKenney, K. (1986) *J. Biol. Chem. 261*, 16332−16339.
8. Bernard, H.-U., and Helinski, D. R. (1979) *Methods Enzymol. 68*, 482−493.
9. Belduz, A. O., Lee, E. J., and Harman, J. G. (1993) *Nucleic Acids Res. 21*, 1827−1835.
10. Miller, J. H. (1972) *Experiments in Molecular Genetics*; Cold Spring Harbor Laboratory Press, Plainview, NY.
11. Leu, S.-F., Baker, C. H., Lee, E. J., and Harman, J. G. (1999) *Biochemistry 38*, 6222−6230.
12. Heyduk, T., and Lee, J. C. (1989) *Biochemistry 28*, 6914−6924.
13. Takahashi, M., Blazy, B., Baudras, A., and Hillen, W. (1989) *J. Mol. Biol. 207*, 783−796.
14. Tsugita, A., Blazy, B., Takahashi, M., and Baudras, A. (1982) *FEBS Lett. 144*, 304−308.
15. Baker, C. H., Tomlinson, S. R., García, A. E., and Harman, J. G. (2001) *Biochemistry 40*, 12329−12338.
16. Lee, E. J., Glasgow, J., Leu, S.-F., Belduz, A. O., and Harman, J. G. (1994) *Nucleic Acids Res. 22*, 2894−2901.
17. Cheng, X., Kovac, L., and Lee, J. C. (1995) *Biochemistry 34*, 10816−10826.
18. Weber, I. T., and Steitz, T. A. (1987) *J. Mol. Biol. 198*, 311−326.
19. Passner, J. M., Schultz, S. C., and Steitz, T. A. (2000) *J. Mol. Biol. 304*, 847−859.
20. Lee, B.-J., Aiba, H., and Kyogoku, Y. (1991) *Biochemistry 30*, 9047−9054.
21. Passner, J. M., and Steitz, T. A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2843−2847.
22. Chu, S. Y., Tordova, M., Gilliland, G. L., Gorshkova, I., Shi, Y., Wang, S., and Schwarz, F. P. (2001) *J. Biol. Chem. 276*, 11230−11236.
23. Won, H.-S., Lee, T. W., Park, S.-H., and Lee, B. J. (2002) *J. Biol. Chem. 277*, 11450−11455.
24. Gorshkova, I. L., Moore, J. L., McKenney, K. H., and Schwarz, F. P. (1995) *J. Biol. Chem. 270*, 21679−21683.
25. Lin, S.-H., and Lee, J. C. (2002) *Biochemistry 41*, 11857−11867.
26. Brown, A. M., and Crothers, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7387−7391.

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