A C-helix Residue, Arg-123, Has Important Roles in Both the Active and Inactive Forms of the cAMP Receptor Protein

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The cAMP receptor protein (CRP) of Escherichia coli exists in an equilibrium between active and inactive forms, and the effector, cAMP, shifts that equilibrium to the active form, thereby allowing DNA binding. For this equilibrium shift, a C-helix repositioning around the C-helix residues Thr-127 and Ser-128 has been reported as a critical local event along with proper β4/β5 positioning. Here we show that another C-helix residue, Arg-123, has a unique role in cAMP-dependent CRP activation in two different ways. First, Arg-123 is important for proper cAMP affinity, although it is not critical for the conformational change with saturating amounts of cAMP. Second, Arg-123 is optimal for stabilizing the inactive conformation of CRP when cAMP is absent, thereby allowing a maximal range of regulation by cAMP. However, Arg-123 does not appear to be critical for a functional response to cAMP, as has been proposed previously (Berman, H. M., Ten Eyck, L. F., Goodsell, D. S., Haste, N. M., Korney, A., and Taylor, S. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 45–50). Based on mutagenic evidence, we also propose the basis for the stabilization of the inactive form to be through a salt interaction between Asp-68 and Arg-123.

The cAMP receptor protein (CRP)2 of Escherichia coli is one of the best-studied transcriptional activators (1–3). It is a homodimeric protein that binds specific DNA sequences with high affinity in response to binding a molecule of cAMP to each monomer. In cases where it activates gene expression, the DNA-bound CRP interacts with RNA polymerase to stabilize polymerase binding and, thus, stimulate transcription. CRP is a classic allosteric protein, each subunit of which is composed of two distinct domains connected by a hinge region; this is, an N-terminal effector binding domain and a C-terminal DNA binding domain (4). cAMP binding to the effector binding domain triggers a conformational change in the protein that leads to a precise repositioning of the DNA binding domains that promotes binding to its target DNA sequence. CRP exists in equilibrium between an active form that can bind specific DNA target sequences and an inactive form that cannot. In this view, cAMP binding to the protein shifts the equilibrium toward the active form either by destabilizing the inactive form or by stabilizing the active form.

Although an enormous amount is known about the behavior of the DNA binding form of CRP, relatively less is known about the process of activation by cAMP binding. The critical obstacle has been the absence of a crystal structure of the cAMP-free form, although several structures of active CRP are known both in the presence and absence of DNA (4–6). A number of research groups have used other methods to better understand the cAMP-dependent activation of CRP protein (3). We have also contributed to this field by identifying two distinctive local effects of cAMP binding in the activation process (7); (i) C-helix repositioning through direct interaction of cAMP with two C-helix residues Thr-127 and Ser-128 and (ii) the concomitant reorientation of the β4/β5 loop.

Recently another group compared the structures of a large number of cAMP-binding proteins of quite diverse function (8). They showed that despite large functional differences there were underlying structural similarities in both cAMP sensing and cAMP response. One of these conserved functions concerns a residue that they proposed should serve as an allosteric trigger of cAMP sensing. The homologous residue in CRP of E. coli is Arg-123. More specifically, these authors noted that a hydrophobic sandwich for cAMP is formed by Arg-123 and Val-49, and they posited that Arg-123 would be critical for cAMP response. Not surprisingly, this residue has been examined before. Moore et al. (9) made several substitutions at those positions and showed that some of these variants were unable to function in vivo, whereas others had a weak response in the presence of cAMP (9). Their conclusion was that Arg-123 provides an important but not essential contact for CRP activation. Both of these claims are consistent with the high degree of conservation of this residue in all known CRP homologs.

Because Arg-123 is a C-helix residue relatively close to Thr-127 and Ser-128, which are important for CRP activation, we were intrigued by its suggested role and high sequence conservation and, therefore, examined it more closely. Our results do indicate a set of important roles for the residue, but these roles are different from those proposed by Berman et al. (8). Instead, Arg-123 appears to be important for determining a biologically proper affinity for cAMP and stabilizing the inactive form of CRP when cAMP is absent.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Recombinant DNA Methodology—For the isolation and manipulation of DNA, standard methods were
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Cloning of crp and Generation of Mutants—E. coli crp was cloned into pEXT20, and then six histidine codons were subsequently added between the last sense codon and stop codon of the crp gene as previously described (7). Site-directed mutagenesis involved PCR amplification of crp-containing pEXT20 with primers designed to incorporate the desired nucleotide changes as described elsewhere (11). The method used for codon randomization was essentially identical to that used for site-directed mutagenesis, except that the primers contained completely randomized codons for the desired positions.

For screening the cAMP-dependent in vivo function of CRP variants, we used an E. coli strain, UQ4249, a crp::cam derivative of UQ3741 (M182 carrying λ prophage with CC(61.5::lacZ fusion) (7)). The assay monitors the ability of the CRP variants to cause β-galactosidase accumulation in colonies on agar plates. The crp genes for selected variants were sequenced to determine the causative residue changes.

Overexpression and Purification of CRP Proteins—Overexpression of wild-type (WT) CRP and CRP variants used in this study were all His- tagged in E. coli CRP and CRP variants used in this study were all His- tagged in E. coli. The strain, UQ4249, a crp::cam derivative of UQ3741 (M182 carrying λ prophage with CC(61.5::lacZ fusion) (7)). The assay monitors the ability of the CRP variants to cause β-galactosidase accumulation in colonies on agar plates. The crp genes for selected variants were sequenced to determine the causative residue changes.

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Cultures of UQ3811 cells transformed with plasmids encoding appropriate CRP variants were grown aerobically (220 rpm) in 2 × LC medium (2% tryptone, 1% yeast extract, and 1% NaCl) containing 50 μg/ml ampicillin at 37 °C to an A600 = 0.5, at which point the synthesis of CRP was induced by the addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside. Each culture volume was 40 ml in a 250-ml Erlenmeyer flask. Cultures were grown for another 5 h before harvesting by centrifugation, and the cells were stored at −80 °C. The purification of CRP proteins was done using a nickel-nitrilotriacetate column (Novagen, Madison, WI) because WT CRP and CRP variants used in this study were all His- tagged. Briefly, cell pellets were resuspended in 0.5 M KCl, 25 mM MOPS, pH 7.4, and broken using a French press. The lysates were cleared by centrifugation at 10,000 × g for 10 min, and then the supernatants were loaded into Novagen nickel-nitrilotriacetate column. After washing with 50 mM imidazole, 0.5 M KCl, 25 mM MOPS, pH 7.4, the CRP proteins were eluted with 250 mM imidazole, 0.5 M KCl, 25 mM MOPS, pH 7.4, and then precipitated with 42.3% ammonium sulfate and stored at −80 °C until use. All protein preparations were >95% homogeneous.

Measurement of In Vitro DNA Binding Activity of CRP Proteins—In vitro DNA binding assays were performed using the fluorescence polarization technique with a Beacon 2000 fluorescence polarization detector (Panvera Corp., Madison, WI). A fluorescent DNA probe was generated in which a 26-base pair target DNA containing CCpmeR (5′-GTAATGGTATCATCACTACATGAT-3′) was labeled with Texas Red on one end of the duplex (12). Binding assays were performed in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA. The probe was used at a concentration of 5 nM in the presence of 6.4 μM salmon sperm DNA. Dissociation constants (Kd) were calculated by fitting the binding data to the equation,

$$r = r_1 + \Delta r$$

where, $r$, $r_1$, and $\Delta r$ are the observed anisotropy, the anisotropy of free DNA, and the total change in anisotropy, respectively, $[D]_T$ and $[P]_T$ are the total concentrations of DNA and CRP protein, and $K_d$ is a dissociation constant.

Measurement of Apparent cAMP Binding Affinity Coupled with DNA Binding Measurement—cAMP binding affinity of WT CRP and various Arg-123 CRP variants were calculated by measuring DNA binding activity of each protein with various amounts of cAMP up to 1 mM. Concentrations of CRP and DNA were 200 and 5 nM. Otherwise the method is identical to the one described above. In principle, there are 5 possible conformers of CRP (with 0 ~ 4 cAMP bound) that can interact with DNA (13). However, the apoCRP form has been reported to have very low DNA affinity that may not contribute to specific binding equilibrium (14). In addition, the population of CRP with 3 or 4 cAMP bound can be neglected because the relatively low [cAMP] does not allow binding to the poor-affinity secondary site. Therefore, the multiple equilibria we consider are,

$$P + L \rightleftharpoons PL + L \rightleftharpoons PL_2 \rightleftharpoons PL_2D$$

REACTION 1

where $P$, $L$, and $D$ represent CRP, cAMP, and DNA, respectively. The system is completely described by intrinsic binding constant of cAMP to a binding site in CRP ($k$), cooperativity between two cAMP binding sites ($w$), DNA binding constant of CRP with one cAMP bound ($K_1$), and DNA binding constant of CRP with two cAMP bound ($K_2$) (2k = [PL]/[PL][L], $\frac{1}{2}kw = [PL_2]/[PL][L]$, $K_1 = [PLD]/[PD][D]$, $K_2 = [PL_2D]/[PL_2][D]$). The equations for total concentrations of all species in system are

$$[P]_T = [P] + 2k[P][L] + k^2w[P][L]^2$$

+ $2kK_1[P][L][D] + K_2k^2w[P][L]^2[D]$ (Eq. 2)

$$[L]_T = 2k[P][L] + k^2w[P][L]^2 + 2kK_1[P][L][D]$$

+ $2kK_2k^2w[P][L]^2[D]$ (Eq. 3)

$$[D]_T = [D] + 2kK_1[P][L][D] + K_2k^2w[P][L]^2[D]$ (Eq. 4)

Once $[P]$, $[L]$, and $[D]$ are solved for the three equations, the concentrations of all components in the system can be calculated. First, $[D]$ can be expressed in terms of other unknowns,
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\[ [P] = \frac{[D_{\text{tot}}]}{1 + 2K_k [P][L] + K_k w [P][L]^2} \quad (\text{Eq. 5}) \]

Equation 2 can be rearranged into a quadratic equation of \([P]\) after substituting Equation 5 for \([D]\). Therefore, \([P]\) is obtained from a root of the quadratic equation in terms of \([L]\),

\[ [P] = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad (\text{Eq. 6}) \]

where \(a = (2K_k [L] + K_k w [L]^2)(1 + 2K_k [L] + K_k w [L]^2), b = 1 + 2K_k [L] + K_k w [L]^2 + ([D_{\text{tot}}] - [P_{\text{tot}}])(2K_k [L] + K_k w [L]^2),\) and \(c = -[P_{\text{tot}}].\) Finally, after substituting Equation 5 and Equation 6 for \([D]\) and \([L]\), Equation 3 is numerically solved for \([L]\) \((0 \leq [L] \leq [L]_{\text{tot}})\) using the bisection algorithm. Binding isotherms for WT CRP and the Arg-123 variants were analyzed using Equation 7 by assuming that the anisotropy signal of singly cAMP-bound CRP is the same as that of doubly cAMP-bound form,

\[ r = r_1 + \Delta r \frac{[PLD] + [PL_2D]}{[D_{\text{tot}}]} \quad (\text{Eq. 7}) \]

where \(r_1\) is the anisotropy of free DNA, \(\Delta r\) is the anisotropy change relative to free DNA upon binding of CRP with one or two cAMP bound, \([PLD] = 2K_k [P][L][D],\) and \([PL_2D] = K_k w [P][L]^2[D].\)

Although this general scheme was used for a comprehensive model (case 2), we also considered a simpler model (case 1). In case 1, we assumed that (i) the two cAMP binding sites are identical and non-cooperative \((w = 1)\) and that (ii) only doubly cAMP-bound CRP is active in DNA binding (consequently, there is no equilibrium of PL + D \(\leftrightarrow PLD,\) and \(K_1 = 0).\)

Implicit fitting was performed in the nonlinear regression program NONLIN (15) combined with the numerical algorithm described above. The anisotropy of free DNA \((r_1)\) was independent of [cAMP], and the average of \(\sim 5\) independent measurements was used in fitting other parameters. The binding constant of CRP with two cAMP bound to DNA \((K_2)\) was independently measured at a saturating concentration of cAMP \((1 \text{ mM})\). We obtained \(1 \times 10^8 \text{ M}^{-1}\) of \(K_2\) consistently for WT CRP and all of the variants except for R123I variant.

RESULTS

Arg-123 Has an in Vivo Role in cAMP-dependent CRP Activation—To systematically analyze the role of Arg-123 in cAMP-dependent CRP activation, we randomized the codon for Arg-123 in a library of crp-bearing plasmids and transformed the resulting pool of plasmids into UQ4249, a cya+ crp– E. coli reporter strain that contains the lacZ gene under the control of the class I (CC-61.5) promoter (7, 16). Although the expression of crp from the vector, pEXT20, is strictly regulated by isopropyl 1-thio-β-β-d-galactopyranoside, we screened without isopropyl 1-thio-β-β-d-galactopyranoside, which yielded a low but sufficient level of CRP accumulation that allowed us to differentiate CRP variants based on the range of blue color. We chose a cya– strain rather than using external cAMP with a cya+ strain for two reasons. First, the cAMP level in a cya+ strain directly reflects a reasonable intracellular level in an E. coli cell. Second, we have shown that externally added cAMP does not efficiently elevate intracellular cAMP concentration (7).

More than 400 colonies were examined, which ensured that all possible residues at position 123 were examined. Less than 5% of the colonies were white, similar to background levels, suggesting a low population of inactive CRP variants altered at this position. Consistent with this percentage, sequence analysis identified the inactive variants as having Pro, stop codons, or secondary mutations (Table 1). The other colonies showed a spectrum of blue colors that was above the background. To differentiate among those variants, we picked colonies from the top 10% (more active than the bulk) and the bottom 10% (less active than the bulk) in terms of blue color. As shown in Table 1, Arg, Ser, Val, or Asn substitution led to high activity, and Trp, Ile, or Thr substitution resulted in relatively low activity. The variety of residues identified in colonies with activity along with the high frequency of blue colonies suggests that Arg-123 is not essential for a significant response to the intracellular level of cAMP.

Arg-123 Is Not Important for the cAMP-induced Conformational Change of CRP—The results obtained in vivo are highly suggestive, but there are a variety of complicating factors, such as interaction with RNA polymerase, that make it impossible to directly interpret those results simply in terms of the formation of the active form of CRP by cAMP binding. We, therefore, chose (or created) five Arg-123 variants for further analysis: R123K, R123N, R123S, R123E, and R123I CRP. These variants were His-tagged at the C terminus, since we have already shown that His-tagged WT CRP has an affinity for target DNA in the presence of cAMP that is comparable with untagged WT CRP (7).

The proteins were purified and tested for DNA affinity using fluorescent anisotropy, as we have done previously (7). As shown in the top portion of Table 2, R123K, R123S, R123N, and R123E CRP had affinities for target DNA in the presence of 1 mM (also of 0.1 mM) cAMP that were indistinguishable from W, white colonies; B, colonies from the subpopulation that was darkest blue (high activity); LB, colonies from the subpopulation that was lightest blue (relatively less activity).

| R123X | Amino acid (DNA codon) (sequence at 123 position) |
|-------|-----------------------------------------------|
| Wild type | Arg (CGT) |
| W-1* | Pro (CCC) |
| W-2 | Pro (CCG) |
| W-3 | Stop (TAG) |
| W-4 | Thr (ACC) + R185H (CAC) |
| W-5 | Phe (TGT) + P194L (CTA) |
| B-1 | Arg (AGA) |
| B-2 | Ser (AGT) |
| B-3 | Arg (CGG) |
| B-4 | Arg (CCG) |
| B-5 | Val (GTT) |
| B-6 | Arg (AGA) |
| B-7 | Asn (AAT) |
| LB-1 | Trp (TGG) |
| LB-2 | Ile (ATC) |
| LB-3 | Ile (ATA) |
| LB-4 | Thr (ACT) |

* In the UQ4249 background (no isopropyl 1-thio-β-β-d-galactopyranoside).
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In vitro DNA binding activities of various Arg-123 variants in the wild-type CRP background, in the T127L/S128V CRP background, or in the T127L/S128I CRP background

| CRP          | Sequence       | In vitro DNA binding activity (Kd) |
|--------------|----------------|-----------------------------------|
|              | 123  | 127  | 128  | No cAMP | 0.1 mM cAMP | 1 mM cAMP | 1 mM cGMP |
| Wild type    | Arg  | Thr  | Ser  | —      | 10  | 9   | —     |
| R123K        | Lys  | Thr  | Ser  | —      | 10  | 9   | —     |
| R123N        | Asn  | Thr  | Ser  | —      | 9   | 10 | 54 |
| R123S        | Ser  | Thr  | Ser  | —      | 13  | 12 | 10 |
| R123E        | Glu  | Thr  | Ser  | —      | 12  | 10 | — |
| R123I        | Ile  | Thr  | Ser  | —      | 45  | 25 | — |
| (R)LV        | Arg  | Leu  | Val  | 792   | 18  | 19 | ND |
| KLV          | Lys  | Leu  | Val  | 19  | 9   | 12 | ND |
| NLV          | Asn  | Leu  | Val  | 46  | 14  | 15 | ND |
| SLV          | Ser  | Leu  | Val  | 69  | 16  | 17 | ND |
| ELV          | Glu  | Leu  | Val  | 102 | 118 | 52 | ND |
| ILV          | Ile  | Leu  | Val  | 120 | 63  | 50 | ND |
| DLV          | Asp  | Leu  | Val  | 55  | 74  | 57 | ND |
| (R)L1        | Arg  | Leu  | Ile  | 35  | 14  | 16 | ND |
| KLI          | Lys  | Leu  | Ile  | 14  | 16  | 15 | ND |
| NLI          | Asn  | Leu  | Ile  | 12  | 13  | 13 | ND |
| SLI          | Ser  | Leu  | Ile  | 14  | 15  | 16 | ND |

* a — Samples where the Kd > 5000 nM.

* b Weak response at high protein concentration (>1 μM).

* c ND, not determined.

that of WT CRP, although R123I CRP had slightly lower (~2-fold) DNA affinity. This result indicates that the cAMP-bound form of each CRP variant is functionally similar to that of WT CRP. Furthermore, the fact that a variety of dissimilar residues are fully capable of supporting normal activation of CRP by cAMP binding disproves an essential role of Arg at this position for the proper conformational change of CRP in response to cAMP as has been proposed (8).

**Arg-123 Is Important for Apparent cAMP Affinity** — If Arg-123 is not important for a response to saturating levels of cAMP, it might still be important for appropriate cAMP affinity. To test this, we performed a coupled assay with each CRP protein that measures the DNA affinity at various concentrations of cAMP. As shown in Fig. 1, WT CRP showed the highest affinity isotherm, and R123I CRP showed with lowest among the variants tested. Because all Arg-123 variants displayed comparable DNA affinities at a saturating levels of cAMP (Table 2), the likely basis for the differences in binding isotherms would appear to be differential cAMP affinity.

To estimate the different cAMP affinities of WT and the Arg-123 variants, binding isotherms were further analyzed by a simple model (case 1) described under “Experimental Procedures.” In this model, the two cAMP binding sites are assumed to be identical and non-cooperative (w = 1), and only doubly cAMP-bound CRP is assumed to be active in DNA binding. This simple model provided reasonable fits for all isotherms (see Fig. 1) with the cAMP affinity of WT CRP being the highest and that of R123I CRP being the lowest (see k of case 1 in Table 3). Compared with WT CRP, R123N CRP is 2-fold poorer, and the other variants are 4–44-fold poorer.

Although this result suggests that the difference in binding isotherms reflects the effects of Arg-123 mutation on apparent cAMP affinity, we were concerned that the calculated cAMP affinity of WT CRP by case 1 (1.2 × 10^6 M^-1) was significantly higher than values reported previously (1.4 × 10^5 to 2.8 × 10^4 M^-1) (17–19). A more sophisticated model was devised to reconcile this discrepancy. In the new model (case 2) we introduced two additional parameters (K1 and w); K1 is the DNA affinity of singly cAMP-bound CRP, and w is the cooperativity between the two cAMP binding sites. We reasoned that the interaction of singly cAMP bound CRP with DNA and cooperativity between cAMP binding sites, which have not been explicitly taken account into in simple model, may explain the apparent discrepancy.

Unfortunately, fitting these four parameters (Δr, K1, k, and w) does not yield a unique set of the solutions because of the intrinsic correlations among K1, k, and w. We, therefore, relied on the reported values for WT CRP, with k = 4 × 10^4 M^-1 and w = 2 (17). When the WT isotherm in Fig. 1 was fitted with these
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values, we obtained a value of $K_1$ of $2.7 \times 10^8$ (M$^{-1}$) with the fitting quality being statistically and visually indistinguishable from that of case 1 (data not shown). For analysis of the Arg-123 fit, we used the data shown in Table 2. The resultant triple variants changed the equilibrium. We chose T127L/S128I CRP toward the active form in the absence of cAMP and asked how the inactive CRP form by the following argument. We reasoned that Arg-123 is unimportant in stabilizing the inactive form of cAMP-free CRP, which would in turn affect the pre-existing equilibrium between the two forms and eventually the effectiveness of ligand binding. As indicated in Table 2, no Arg-123 substitution resulted in detectable DNA affinity in the absence of cAMP. However, this does not necessarily mean that Arg-123 is unimportant in stabilizing the inactive CRP form by the following argument. We reasoned that cAMP-free WT CRP had extremely low DNA binding activity, and therefore, even a significant shift of the equilibrium might not be experimentally measurable. We, therefore, started with a CRP variant that is already partially shifted toward the active form in the absence of cAMP and asked how the additional substitutions at position 123 in that background changed that equilibrium. We chose T127L/S128I CRP because we have already shown that it has modest activity in the absence of cAMP (Ref. 7, Fig. 2). The resultant triple variants (R123X/T127L/S128I variants) were monitored for their DNA affinity in the absence of cAMP. The striking result is that all the Arg-123 substitutions tested resulted in substantially higher DNA affinity in the absence of cAMP (7–42-fold) than did the starting variant with Arg-123 (Fig. 2 and Table 2). Such a change in affinity could result from either stabilization of the active form or destabilization of the inactive form or both. The fact that a variety of dissimilar residues had a fundamentally similar effect shows that it is the absence of Arg-123 that is causative of this improved affinity and not the presence of any specific replacement residue. Similarly, the fact that it is the absence of Arg-123 that is causative is consistent with the proposal that a new specific contact stabilizes the active form and much more easily rationalized by a destabilization of the inactive form. Furthermore, the R123K CRP, which is expected to be most similar to the WT residue, is the one variant that showed the most dissimilar property in this particular experiment, implying that this role is highly specific to Arg. We speculate that Lys-123 not only lacks the necessary property for the stabilization of the inactive form but also has additional effect of unclear nature.

We wondered if the substitutions at position 123 would also have an effect on the cAMP-independent activity on a different CRP variant that was even more shifted toward the active form. We tested T127L/S128I CRP because it has only an ~2-fold lower DNA affinity in the absence of cAMP than does cAMP-bound WT CRP, and cAMP addition increases its DNA affinity to the WT CRP level (Ref. 7, Table 2). As shown in Table 2, the additional substitution at position 123, such as Ser, Lys, and Asn, afforded the T127L/S128I CRP full DNA binding activity in the absence of cAMP, and such activity was not further increased by cAMP. This result indicates that the hypothesized role of Arg-123 is not specific to T127L/S128I CRP back-

| CRP      | Case 1 (k ($\times$10$^3$) | Case 2 (k ($\times$10$^3$)) | Relative affinity | Relative affinity |
|----------|-----------------------------|-----------------------------|-------------------|-------------------|
| Wild type| 6.3 $\times$ 10$^3$          | 5.8 $\times$ 10$^3$         | 1.00              | 1.00              |
| R123N    | 2.7 $\times$ 10$^3$         | 2.0 $\times$ 10$^3$         | 0.53              | 0.50              |
| R123S    | 1.5 $\times$ 10$^3$         | 1.0 $\times$ 10$^3$         | 0.13              | 0.09              |
| R123K    | 1.3 $\times$ 10$^3$         | 1.0 $\times$ 10$^3$         | 0.11              | 0.10              |
| R123E    | 6.0 $\times$ 10$^3$         | 5.0 $\times$ 10$^3$         | 0.10              | 0.10              |

FIGURE 2. The substitutions at Arg-123 commonly improved the cAMP-independent DNA affinity of T127L/S128I CRP variant. Data are shown for T127L/S128I ( ), R123N/T127L/S128I ( ), R123S/T127L/S128I ( ), R123K/T127L/S128I ( ), R123D/T127L/S128I ( ), R123E/T127L/S128I ( ), R123K/T127L/S128I ( ), R123D/T127L/S128I ( ), R123E/T127L/S128I ( ), and R123K/T127L/S128I ( ). Solid lines show the best fit of the data to Equation 1 under “Experimental Procedures.”
The introduction of Arg-68 counteracts cAMP-independent DNA binding improvement of R123D/T127L/S128V CRP variant. DLV and SLV stand for the R123D/T127L/S128V and R123S/T127L/S128V CRP variants, respectively. Solid lines show the best fit of the data Equation 1 under “Experimental Procedures.”

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Asp-123 and Arg-68 and, therefore, interaction between Arg-123 and Asp-68 as a stabilizing factor of inactive conformation of cAMP-free CRP. Given the striking difference in effect between Arg-123 and Lys-123, we hypothesize that Lys-123 is too short to reach the Asp-68 residue. When we pursued a similar approach to test the possible Glu-72–Arg-123 interaction, the activities were destroyed with the Arg-72 substitution not only in the R123E/T127L/S128V CRP background but also in the R123S/T127L/S128V CRP background, so no conclusion could be drawn (data not shown).

Why might the Arg-123–Asp-68 salt bridge have this effect? It is difficult to answer this question because the relevant structure, that of inactive CRP, has not been structurally solved. Because C-helix repositioning is important for cAMP signal transmission and Arg-123 is one of the C-helix residues, the interaction might further stabilize the inactive form of C-helix conformation. On the other hand, we note that a comparison between the active structure of CRP and the inactive structure of CooA (21), a heme-containing CRP homolog, has revealed a substantial difference in the positioning of the \(\beta_4/\beta_5\) loop with respect to the rest of the effector binding domain. This loop is particularly interesting because it makes contacts with the DNA binding domain in the active form of CRP so that its repositioning must have an effect on the equilibrium poise between active and inactive forms. Asp-68 falls at the base of the \(\beta_4/\beta_5\) loop, and it is also possible that the Arg-123–Asp-68 salt bridge helps to maintain the position of the \(\beta_4/\beta_5\) loop relative to the rest of the effector binding domain in a position appropriate to the inactive form.

In short, we propose that in WT CRP, the interaction between Arg-123 and Asp-68 stabilizes the inactive form of cAMP-free CRP. A priori, this proposal implies another role of cAMP binding for the required conformational change in WT CRP, disruption of the salt bridge.

**R123N CRP Responds to cGMP as well as to cAMP**—A number of CRP variants have been shown previously to respond to cGMP (22–25). However, the underlying mechanisms are elusive, in part because the substitutions that cause this behavior are typically far from the effector binding site. In fact, many cGMP-responding CRP variants display a significant degree of effector-independent DNA binding activity as well, and therefore, one would reason that the cGMP response is indirect. The following results show that R123N CRP variant probably responds to cGMP through a specific interaction.

A number of variants altered at residue 123 were tested for their DNA affinity in response to cGMP, and only R123N CRP variant displayed a substantial response (Table 2). R123S CRP and R123E CRP showed a very weak response that was only detectable at high protein concentration (above 1 \(\mu\)M) (Table 2). Fig. 4 shows the binding isotherm of the R123N CRP variant in response to either 0.1 or 1 mM cGMP. As evident in the figure, the DNA affinity at 1 mM cGMP is slightly better than that at 0.1 mM cGMP, indicating that this variant is not saturated at the 0.1 mM cGMP concentration and implying it binds cGMP less well than cAMP. The \(K_d\) value of R123N CRP for DNA in the presence of 1 mM cGMP was calculated to be 54 nM, which is also poorer than the variant’s DNA affinity in response to cAMP (10 nM). This might indicate either that (i) the variant...
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![Graph showing anisotropy (r) vs. [CRP] (nM) for 1 mM cGMP and 0.1 mM cGMP]

FIGURE 4. R123N CRP variant responds to cGMP. In vitro DNA binding activity of the R123N CRP variant was measured with no effector (open circle), 0.1 mM cGMP (gray circle), and 1 mM cGMP (closed circle). Solid lines show the best fit of the data to Equation 1 under “Experimental Procedures.”

May not be saturated even by 1 mM cGMP or (ii) the fully cGMP-bound form of the variant is functionally different from cAMP-bound form. The result that this property is found only in R123N CRP (among those tested) suggests that it is due to the introduction of Asn-123 and not the absence of Arg-123. The close proximity of the Asn-123 residue to the effector binding site suggests that the effect of R123N substitution is direct, unlike the other cGMP-responding CRP variants reported earlier (22–25). We, therefore, performed structural modeling using Swiss-PdbViewer 3.7 with the known cAMP-bound CRP structure (Protein Data Bank code 1CGP) and assumed that the orientation of the cGMP bound to R123N CRP would be similar to that of cAMP bound to WT CRP. Because of the relative discrimination of R123N CRP between cAMP and cGMP, we then looked for possible discriminatory interactions. The result was a plausible H-bonding interaction between N2 of cGMP and the O of the Asn side chain. We suppose that this interaction would allow cGMP binding in a functional way leading to a proper conformational change of the protein for DNA binding.

DISCUSSION

We have here shown that Arg-123 has two roles in CRP function. It provides optimal cAMP affinity, and it helps to set the proper equilibrium between active and inactive forms by the stabilization of the inactive cAMP-free CRP form.

The current study also helps to explain an earlier finding about Arg-123 variants. Moore et al. (9) reported that the in vivo activity defects of several Arg-123 CRP variants could be corrected by an additional A144T substitution; the properties of R123A/A144T, R123E/A144T, or R123Q/A144T CRP are very similar to those of WT CRP in terms of the requirement of cAMP for activation in vivo (9). Based on the results in this paper, we favor the interpretation that the Arg-123 substitutions examined by Moore et al. (9) simply lower cAMP affinity, but that this effect is compensated by the A144T substitution, which by itself is able to afford a higher affinity for cAMP (24).

How does Arg-123 afford an optimal context for cAMP affinity? The first possibility is that Arg is particularly good at its interaction with the adenine ring of cAMP as Berman et al. (8) proposed. The second possibility is that, in the presence of cAMP, Arg might have unique ability to hydrogen-bond with Glu-72, the residue that has been reported to be crucial for cAMP affinity (26). It may be that both roles are important for cAMP affinity and are satisfied only by Arg.

It is worth noting that the equilibrium-shift effect of the Arg-123 substitutions also has an effect on cAMP affinity. Marvin and Hellinga (27) have shown that ligand binding affinities can be manipulated indirectly by altering protein equilibrium rather than the intrinsic affinity of the site for the ligand, which they termed “conformational coupling” (27). In our case, the fact that some Arg-123 substitutions destabilize inactive conformation means that their apparent cAMP affinity would be increased because they stabilize the active form with a higher cAMP affinity. Therefore, we assume these Arg-123 substitutions must decrease cAMP affinity even more substantially than is apparent from our analysis.

The crystal structures of CRP suggest several important CAMP-contacting residues including Glu-72, Arg-82, Ser-83, Thr-127, Ser-128, and Arg-123. Our current hypothesis is that they might be classified into two groups in terms of their roles in cAMP-dependent CRP activation; one group has a role in the conformational change and the other for cAMP affinity. For the conformational change, we have shown that CAMP interaction with Thr-127 and Ser-128 provides two locally critical events, C-helix repositioning and proper β4/β5 positioning (7). Based on the current study of Arg-123 and our unpublished preliminary results on Glu-72, Arg-82, and Ser-83, we propose that these residues are generally important for CAMP affinity. This view is consistent with the fact that the latter residues are highly conserved among all CAMP-binding proteins (8) but that Thr-127 and Ser-128 are only conserved in closely related CRP-like proteins.

In conclusion, we have shown that Arg-123 also has a role in stabilizing the inactive CRP conformation in the absence of CAMP and then demonstrated that an Arg-123→Asp-68 interaction is the underlying mechanism for this property. Not surprisingly, residue Asp-68 is highly conserved among the closely related CRP-like proteins, as is Arg-123. This might suggest a general role of this interaction in the closely related CRP family proteins.

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