Dual Roles of an E-Helix Residue, Glu167, in the Transcriptional Activator Function of CooA

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CooA is a transcriptional activator that mediates CO-dependent expression of the genes responsible for CO oxidation in Rhodospirillum rubrum. In this study, we suggest in vitro and in vivo models explaining an unusual requirement of CooA for millimolar levels of divalent cations for high-affinity DNA binding. Several lines of evidence indicate that an E-helix residue, Glu167, plays a central role in this requirement by inhibiting sequence-specific DNA binding via charge repulsion in the absence of any divalent cation and that divalent cations relieve such repulsion in the process of DNA binding by CooA. Unexpectedly, the Glu167 residue is the optimal residue for in vivo transcriptional activity of CooA. We present a model in which the Glu167 from the downstream subunit of CooA helps the protein to interact with RNA polymerase, probably through an interaction between activating region 3 and σ subunit. The study was further extended to a homologous protein, cyclic AMP receptor protein (CRP), which revealed similar, but not identical, roles of the residue in this protein as well. The results show a unique mechanism of CooA modulating its DNA binding and transcriptional activation in response to divalent cations among the CRP/FNR (fumarate and nitrate reductase activator protein) superfamily of regulators.

The CO sensor CooA regulates the expression of the genes required for CO-dependent growth in Rhodospirillum rubrum (23, 24). Upon binding CO to its hemes, CooA activates the transcription of two operons encoding the CO oxidation system in R. rubrum (11). CooA belongs to a superfamily of transcriptional activator proteins that includes the cyclic AMP (cAMP) receptor protein (CRP) and the fumarate and nitrate reductase activator protein (FNR) (23, 24). CooA, like CRP, acts as a homodimer in which each subunit consists of an effector-binding domain and a DNA-binding domain that are connected by a hinge. The dimerization interface between the effector-binding domains in both CooA and CRP is a long helix, termed the C helix (20, 23). Within this family, the structures of only effector-free CooA and effector-bound CRP have been determined thus far. Although the proteins are not identical, a structural comparison is informative for the following reason. As revealed by structural analysis, the two proteins have similar secondary structures and even tertiary topology within each domain (15). The relative positions of the two domains are obviously different in the structures: in active CRP, both DNA-binding domains are positioned to interact with DNA, while in effector-free CooA, these domains are dramatically reoriented (15, 20). The other difference was found in the relative positions of the C helices that serve as the dimer interface. It was suggested that the C helices might be a signaling tool for each protein (15, 20). In CooA, the crucial role of the C helices in signaling the required conformational change for DNA binding has been confirmed (13, 38, 41).

The effector-binding domain of each CooA monomer contains a b-type heme, the recognition site for CO (29). The heme can undergo oxidation-reduction, with CooA existing in three physiological states: Fe(III), Fe(II), and Fe(II)-CO forms. The structure of the Fe(II) form has been elucidated, and it contains a six-coordinate low-spin heme with the axial ligands of His77 and Pro2, where the N-terminal Pro2 ligand is derived from the opposite subunit of the CooA homodimer (15). The incoming CO displaces the Pro2 ligand (37) and shifts the equilibrium from the inactive form to the active form. The essential part of this conformational change involves the proper reorientation of the DNA-binding domains to support high-affinity DNA binding. Among the three forms of CooA, only the Fe(II)-CO form is competent to bind DNA with a high affinity in vitro and activate transcription in vivo. A focus of research has been the mechanism by which CO binding to the heme triggers the appropriate conformational change. When CooA binds to DNA, it functions as a transcriptional activator by recruiting RNA polymerase to appropriate promoters that make specific contacts with RNA polymerase. By analogy to the CRP and FNR homologs that have been studied more, there are three activating regions (ARs) that serve as determinants of interaction with RNA polymerase: activating region 1 (AR1) and AR2 interact with the σ subunit, while AR3 interacts with the σ subunit. CooA apparently possesses all three ARs (12, 16).

To this point, CO binding to the heme of Fe(II) CooA has been thought to be both necessary and sufficient to create the DNA-binding form of the protein. However, in the course of analyzing the conditions of the DNA-binding assay, it became apparent that the presence of divalent cations was an absolute requirement for high-affinity DNA binding by CooA. This was surprising, because CRP has not been reported to require divalent cations, and in fact, the presence of such ions typically inhibits, rather than supports, DNA binding by proteins (17, 30). This report demonstrates that the basis of this require-
ment for CooA is the Glu167 residue. We found that Glu167 inhibits DNA binding through charge repulsion between itself and the DNA, and divalent cations mask and prevent the repulsion. In addition, we show that Glu167 enhances the interaction of CooA with RNA polymerase to maximize its transcriptional activity, apparently acting as a part of AR3 to enhance the interaction with the σ subunit. The study was extended to the homologous residue of CRP; therefore, the results have implications for the same functional properties of other proteins related to CooA, including members of the large CRP/FNR superfamily.

MATERIALS AND METHODS

Strains and plasmids. Wild-type (WT) CooA and CooA variants were constructed using a pLEX20-based expression plasmid, which provides tight control of cooA expression (6, 33). Escherichia coli crp was cloned into Ecol-RhindIII-digested pET20 after PCR amplification of the chromosomal DNA from E. coli DH5α using 5′ (EcoRI-containing) and 3′ ( HindIII-containing) primers designed as previously described (13). The CRP-CooA chimera contains the effector-binding domain of CRP (positions 1 to 136) and the DNA-binding domain of CooA (position 132 to the end), in which the junction is at the conserved Phe residue in both proteins. The chimera was constructed through two successive PCR amplifications and cloning of the PCR product into Ecol-RhindIII-digested pET20. Site-directed mutagenesis was performed by PCR amplification with primers designed to incorporate the desired nucleotide changes, as described elsewhere (5). CRP and CRP-CooA chimeras were then tagged with six or seven histidines at the C terminus. E. coli strains UQ3809 and UQ3811 are the cya cpr mutant versions of E. coli strains RLG4649 and RLG4650, respectively. To create strains UQ3809 and UQ3811, cya and cpr mutant alleles were successively introduced into the parental strains by P1 transduction. For purification, CRP and the chimera proteins were expressed in strains UQ3809. The 7D CooA variant lacks the nine residues at the C terminus, which includes seven Asp residues. This variant proved to be unstable, probably because the deletion of the nine residues exposes a new C-terminal signal sequence (−Ala Ala Ala) that is sensitive to the OmpX protease of E. coli (7). To overcome this problem, we randomized the nucleotide sequences for terminal residues Ala212 and Ala213 in the ΔTD CooA background and selected for colonies with high in vivo activity in the presence of CO, assuming that highly active CooA variants would have reasonable protein stability. Several mutants were identified, and two CooA variants, termed 7D(LY) and 7D(TR), were constructed using a reduced pyridine-hemochromogen method (35). CRP-related proteins were purified by using a Novagen Ni2+-nitrilotriacetic acid column according to the manufacturer's instructions. Protein concentration was measured by using the bicinchoninic acid assay kit (Pierce).

Preparation of hydroxylapatite batch-treated CooA samples. Hydroxylapatite batch-treated CooA samples were prepared using the procedure described previously (40). By this method, heme-containing CooA was enriched to ~10% of total protein in the case of WT CooA. These preparations were used only for the initial measurement of the Mg2+ dependence of in vitro DNA-binding activities of CooA variants (see Table 1).

In vivo transcriptional activity assays, in vivo DNA-binding assays, and in vitro DNA-binding assays. In vivo DNA-binding and transcriptional activities of WT CooA and CooA variants were measured by transforming each plasmid into E. coli strain UQ3519, which was developed specifically for this in vivo DNA-binding assay. UQ3519 is a derivative of UQ1639, our standard reporter strain (28), which contains the chromosomally inserted-PcooF promoterless lacZ operon sequence fused to a motpromoterless lacZ encoding β-galactosidase. In vivo transcriptional activities of WT CooA and CooA variants were measured by monitoring in vivo β-galactosidase activity in the UQ3519 background using a standard protocol (1). The in vivo DNA-binding assay uses a separate transcriptional fusion in which the car gene is fused to a promoter containing a CooA binding site, PcooA, overlapping the +1 position, such that active CooA acts as a repressor of car expression, making cells sensitive to chloramphenicol. An unexpected substitution of the last six codons of car was found in this construct, but the substitution appeared unimportant with regard to the assay; in the absence of active CooA, cells are resistant to chloramphenicol, while under conditions where CooA is expected to be active, cells are chloramphenicol sensitive. In the assay, tubes (18 by 150 mm) containing 9 ml of morpholino propane sulfonic acid (MOPS) medium (31) supplemented with 100 μg of ampicillin per ml and 50 μM IPTG were sealed with stoppers and made anaerobic on a manifold by evacuating and flushing with argon three times. CO (350 μl) was injected in tubes for CO-induced cultures, creating a 2% CO headspace. The tubes were inoculated with 500-μl samples of cultures of E. coli strains UQ3520 (WT CooA) or UQ3522 (E167L CooA) grown overnight and then diluted to an optical density at 600 nm (OD600) of 0.06 to 0.07. The tubes were incubated on their sides at 37°C with gentle shaking. When cultures reached an OD600 of ~0.2, after ~6 h of incubation, an anaerobic solution of chloramphenicol was injected to a final concentration of 60 μg/ml, and incubation was continued for 3.5 to 5 h. OD600 measurements were monitored using a Spectronic 20D+ spectrometer (ThermoSpectronic). Doubling times were calculated from putting the growth data into the following equation: 

\[ N(t) = N_0 \times e^{kt} \]

where \( N_t \) is the population at time \( t \), \( N_0 \) is the population at time zero, \( k \) is the normalizing constant, and \( t \) is time.

In vitro DNA-binding assays were performed using the fluorescence polarization technique with a Beacon 2000 fluorescence polarization detector (Panvera Corp., Madison, Wis.) as described previously (33). The effects of a divalent cation on DNA binding by CooA and CRP-CooA chimera proteins were analyzed in the following assay buffer: 40 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM (v/vol) glycerol, 5 mM dithiothreitol, 6.4 μM Texas Red-labeled PcooF, and 6.4 μM salmon sperm DNA (with or without 10 mM MgCl2). In some cases, 1 mM EDTA was also included in the assay mixture to ensure the absence of any available divalent cation. For the measurement of in vitro DNA binding of CRP proteins, a probe based on the consensus sequence was generated. A 26-bp DNA fragment containing CGCn (5′-GTTAAATGTGATCTACATCAGTAT-3′) was labeled with Texas Red on one end of the duplex and used at the concentration of 5 mM in the presence of 6.4 μM salmon sperm DNA. Binding assays for CRP variants were performed in a mixture of 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1 mM EDTA with or without cAMP. Equilibrium dissociation constants (KD) were calculated by putting the binding data into an equation that

| TABLE 1. The Mg2+ dependence of in vitro DNA binding of the CO-bound form of CooA variants |
|---------------------------------|---------------------------------|
| CooA | DNA-binding activity of CO-bound forma |
| None (control)b | – | – |
| Wild-type | – | + |
| E59K | + | + |
| E60L | + | + |
| E60K | + | + |
| E60A | + | + |
| E62K | + | + |
| D128K | + | + |
| D128A | + | + |
| D145K | + | + |
| D145K | + | + |
| E167K | + | + |
| E167L | + | + |
| E168K | + | + |
| 7D (LY) E60K | + | + |
| 7D (LY) E60A | + | + |
| 7D (LY) E128K | + | + |

a For this assay, hydroxylapatite batch-treated CooA samples were used. By this method, heme-containing CooA was enriched to ~10% of total protein in the case of WT CooA. The affinity for the 26-mer target DNA in the presence or absence of Mg2+ is indicated as follows: ++, KD < 10 nM; +++, 10 nM < KD < 50 nM; +++, 50 nM < KD < 250 nM; +++ ≥ 250 nM.
b The inclusion of 1 mM EDTA did not change the range of KD values.
c Control indicates an E. coli strain harboring only vector plasmid (pET20).

d The C-terminus sequence (−AADGDDDDEDDD) was changed to −AKK RKKKKKK.

e The C-terminus sequence (−AADGRDDDDDEDDD) was changed to −LY.

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incorporated a fluorescence-quenching factor upon DNA-protein interaction as described elsewhere (18).

**RESULTS AND DISCUSSION**

CooA needs both CO and a divalent metal cation for in vitro DNA binding. For the development of a fluorescence anisotropy assay for CooA for DNA binding, we originally employed a buffer that was used successfully for in vitro transcription assays with the protein. Because of the requirements of RNA polymerase in that assay, the buffer contained 10 mM MgCl$_2$ (12). After the successful development of the anisotropy assay, it was pointed out that the presence of a divalent cation, such as Mg$^{2+}$, typically inhibits DNA binding by proteins (17, 30). Therefore, we were surprised that millimolar levels of Mg$^{2+}$ were actually necessary for high-affinity binding of CooA to its target DNA. In the presence of 10 mM Mg$^{2+}$, CO-bound CooA exhibited a $K_D$ value of 8 nM for DNA (Fig. 1A). In the absence of Mg$^{2+}$, CO-bound CooA showed a $K_D$ value for DNA of $\sim$3,100 nM, which is $\sim$400 times lower than that in the presence of Mg$^{2+}$ (Fig. 1A). DNA binding was not detected in CooA in the absence of CO, irrespective of Mg$^{2+}$ (Fig. 1A). This stimulating effect on DNA binding of CooA was not specific to Mg$^{2+}$, as both Ca$^{2+}$ and Mn$^{2+}$ effectively replaced it with surprisingly similar $K_D$ values: 0.62 mM for Mn$^{2+}$, 0.98 mM for Ca$^{2+}$, and 1.7 mM for Mg$^{2+}$. Though the interaction of CooA and these divalent cations is clearly not very specific, monovalent cations (Na$^+$ or K$^+$) were ineffective up to 200 mM (data not shown). Such a divalent cation requirement for the DNA-binding activity of CRP has not been reported, and such a requirement was not revealed by direct examination (see below). Therefore, we wished to understand the basis for this unusual property of CooA.

The requirement for divalent cations is independent of CO and the heme and localizes to residue Glu167 in the DNA-binding domain. The divalent cation requirement was demonstrated to be independent of the presence of CO by showing that two CooA variants, M124R and L116K CooA, that are active without CO, retain a similar divalent cation requirement for their DNA-binding activities (Fig. 1B). M124R CooA is spectrally and functionally unusual in its Fe(III) form, displaying high activity both in vivo and in vitro (32). Likewise, L116K CooA is highly active in vivo and in vitro in its Fe(II) form, and it has been proposed that Lys116 ligation to the heme iron, replacing the normal Pro2 ligand, is the basis for this activation without CO (41). The divalent cation requirement is even independent of the heme itself, since a novel hemeless CooA variant, $^{2}$CK$^{10}$/$^{75}$YYT$^{77}$, which lacks all of the residues that serve as heme ligands in the various ligation states of WT CooA (M. Conrad et al., unpublished results), displays a similar requirement (Fig. 1B).

The divalent cation requirement was then shown to localize to the DNA-binding domain using a CRP-CooA chimera. This chimera is composed of the effector-binding domain of CRP (ending at CRP position Phe136) and the DNA-binding domain of CooA (starting at CooA position Phe132) (Fig. 1C). It recognizes CooA target DNA in a CAMP-dependent manner. Importantly for this study, the chimera also has a requirement for divalent cations for DNA binding that is similar to that of CooA (Fig. 1D). The CAMP-bound chimera exhibited $K_D$ values for DNA of 32 and $\sim$4,000 nM in the presence and absence of 10 mM Mg$^{2+}$, respectively. These results clearly show that the critical feature conferring divalent dependence lies in the DNA-binding domain of CooA.

At the same time as the chimeras were being tested, we made a number of specific substitutions within CooA on the basis of the hypothesis that Asp and Glu residues would be excellent candidates for serving as divalent cation-binding sites. We further reasoned that the role of divalent cations...
might be to bridge such residues to stabilize the active form of CooA. Specific Asp and Glu residues were therefore chosen where it appeared that such interactions might occur on the basis of findings from modeling the CooA sequence on the active CRP structure. Lys substitutions were initially tested, under the hypothesis that a positive charge might replace a hypothesized divalent salt bridge between the two negative charges, but neutral substitutions were also examined. The in vitro DNA-binding assay was performed on proteins partially purified using hydroxylapatite resin, as described in Materials and Methods. The results demonstrate that Glu167 is central for the divalent cation requirement (Table 1). No other single substitution at other positions alleviates this requirement, and the basis for the moderate effects seen with other substitutions is discussed later in the paper. To provide a context for the following results, we will briefly introduce our working hypothesis, though it is expanded later. As shown in Fig. 2, we imagine that Glu167 is rather near the DNA and in the absence of divalent ions, is forced toward the DNA by other negative charges in CooA. In the presence of divalent ions, however, a salt bridge is formed to move Glu167 away from DNA, facilitating binding. 

**Glu167 directly affects DNA binding by its proximity to DNA.** E167K CooA displays little divalent cation requirement for DNA binding in the presence of CO, and E167L CooA behaved similarly (Table 1), so the effect is due to the elimination of the Glu residue and not to the hypothesized creation of a salt bridge. Purified CO-bound E167L CooA variant shows high DNA-binding activity even without a divalent cation. Data were obtained with 10 mM MgCl2 (triangles) and without MgCl2 (circles). Data collected in the absence of CO (open symbols) and data measured with 250 mM KCl in the reaction mixture (gray symbols) are indicated. (B) Gin170 is near target DNA in the solved structure of the CRP-DNA complex (PDB accession no. 1CGP). The nearest distance between the hydrogen atom of Gin170 and the phosphate oxygen of G23 is 2.1 Å. This figure was generated by the SwissPro structure-viewing program (http://www.expasy.org/spdbv). (C) Purified Q170E CRP variant (triangles) is highly perturbed in DNA binding compared to WT CRP (circles). Samples with cAMP (solid symbols) and samples without cAMP (open symbols) are indicated. (D) Predicted positions of CooA Glu59, Glu60, and Glu128, and Glu167 residues in the known active CRP context (PDB accession no. 1CGP): CRP residues Gly132 and Gin170 were changed to CooA residues Glu128 and Glu167, respectively (CRP Glu54 and Glu55 residues correspond to CooA Glu59 and Glu60, respectively), using the SwissPro structure-viewing program. For each residue, the most probable conformer was chosen. The inset shows the tip of β4 and β5 sheets containing Glu59 and Glu60.

**FIG. 2.** Model showing the role of a divalent cation (here Mg2+ in DNA binding by CO-bound WT CooA. Glu167, without any divalent cation, experiences charge repulsion (indicated by arrows) from Glu60 and/or the poly(Asp) tail, which forces Glu167 close to the target DNA and prevents high-affinity DNA binding. A divalent cation affords high-affinity DNA binding by coordinating Glu167 to Glu60 and/or the poly(Asp) tail and thereby keeping away Glu167 from the target DNA.

**FIG. 3.** Effect of an E-helix residue substitution in CooA (Glu167) and CRP (Gln170) on DNA binding. (A) Purified E167L CooA variant shows high DNA-binding activity even without a divalent cation. Data were obtained with 10 mM MgCl2 (triangles) and without MgCl2 (circles). Data collected in the absence of CO (open symbols) and data measured with 250 mM KCl in the reaction mixture (gray symbols) are indicated. (B) Gin170 is near target DNA in the solved structure of the CRP-DNA complex (PDB accession no. 1CGP). The nearest distance between the hydrogen atom of Gin170 and the phosphate oxygen of G23 is 2.1 Å. This figure was generated by the SwissPro structure-viewing program (http://www.expasy.org/spdbv). (C) Purified Q170E CRP variant (triangles) is highly perturbed in DNA binding compared to WT CRP (circles). Samples with cAMP (solid symbols) and samples without cAMP (open symbols) are indicated. (D) Predicted positions of CooA Glu59, Glu60, and Glu128, and Glu167 residues in the known active CRP context (PDB accession no. 1CGP): CRP residues Gly132 and Gin170 were changed to CooA residues Glu128 and Glu167, respectively (CRP Glu54 and Glu55 residues correspond to CooA Glu59 and Glu60, respectively), using the SwissPro structure-viewing program. For each residue, the most probable conformer was chosen. The inset shows the tip of β4 and β5 sheets containing Glu59 and Glu60.
repulsion of the DNA and that divalent cations mitigate that effect.

To test the notion of charge repulsion between the Glu residue and DNA, we made the analogous substitution (Q170E) in CRP, along with a His tag for easy purification. In in vitro DNA-binding assays, His-tagged cAMP-bound Q170E CRP displayed an affinity for the consensus CRP sequence that was 20-fold lower than that of His-tagged cAMP-bound WT CRP (Fig. 3C). This significantly lower DNA affinity is consistent with the proposed proximity of the residue to DNA as a source of repulsion. Surprisingly, there was no improvement in DNA binding of cAMP-bound Q170E CRP upon the addition of divalent cations (data not shown), which is different from CooA. We believe that this is because Q170E CRP lacks appropriately positioned residues in the effector-binding domain to mitigate that effect, as explained later.

Other CooA residues are involved in divalent cation dependence, and analysis of these residues leads to a consistent model for the effect. While most other examined single substitutions showed little or no effect on Mg2+ -independent DNA binding (data not shown), there were several interesting exceptions (Table 1). The most striking exceptions were when the Asp-rich C terminus of CooA was removed, termed Δ7D(LY) CooA, or replaced with Lys residues, termed 7D–7K CooA. Both CooA variants showed modest affinity for DNA in the absence of Mg2+, indicating that it is the absence of the Asp-rich tail that provides this effect. Minor effects were also seen with single substitutions at residues E59, E60, and E128. The Lys substitutions at Glu59 and Glu128 positions were responsible for this property, since E59L and E128A did not display this effect. These findings suggest that a salt bridge is the basis for the effects at these positions, as discussed below. With Glu60, the effect was not specific to Lys, indicating that the elimination of Glu was the critical factor. To understand these data in a structural sense, we considered the structure of DNA-bound CRP (Fig. 3D), because the structure of the DNA-bound form of CooA remains unknown. CRP should serve as an excellent model, because of the general similarity of the two proteins as well as the similarity of the DNA sequences that each protein binds. The exception to this is the Asp-rich C terminus of CooA, which was not resolved in the CooA structure, and there is not an analogous portion in CRP.

Our working hypothesis for the phenomena described above is based on the CRP model and presented in Fig. 2. We imagine that the primary issue is repulsion between Glu167 of CooA and the target DNA in the absence of any divalent cation and that the role of a divalent cation, such as Mg2+, is to serve as a bridge between Glu167 and nearby negative charges in the active protein, thereby obviating the Glu167-DNA repulsion. This hypothesis explains the Mg2+ independence of CooA variants that eliminate the negative charge at residue 167 (E167K or E167L), since the critical repulsion is absent. We further suppose that the repulsion is also affected by the degree to which the flexible Glu167 is itself repulsed from other negative charges in the direction opposite that of the DNA (Fig. 2). This notion then explains the basis for the modest Mg2+ independence caused by the E60K substitution, since Glu60 is very likely to be positioned so as to repel Glu167 toward the DNA on the basis of the CRP structure (Fig. 3D). E60A CooA is modestly Mg2+ independent, consistent with this hypothesis in that it is the absence of charge repulsion at this position that is important. We also propose, simply on the basis of these data, that the Asp-rich C terminus might also lie in this region and have a similar effect, though of course this is highly speculative. The fact that a CooA variant lacking both Glu60 and the Asp-rich C terminus [see Δ7D(LY) E60K and Δ7D(LY) E60A CooA variants in Table 1] is almost completely Mg2+ independent supports this notion.

The results with Glu59 and Glu128 are somewhat more complicated to explain, since Glu128 should not be near Glu167 and Glu59 is apparently oriented away from Glu167 (Fig. 3D). Rather, these two residues are predicted to be directed toward each other. Because only the Lys substitutions at residues 59 and 128 caused small, but reproducible, effects on Mg2+ independence, it seems that charge attraction between these two residues is important for this effect. One possibility would be that this attraction draws Glu60 away from Glu167, producing the context similar to that of E60A or E60K substitution. However, this hypothesis cannot explain why the double mutant E60A E128K CooA variant is much better than either the E60A or E128K CooA variant in terms of divalent cation independence for DNA binding (Table 1). It is plausible that residue 59 or 128 directly or indirectly reduces the distribution of negative charges composed of Glu60 and/or the Asp-rich C terminus. Finally, we cannot rule out the possibility of a secondary divergent cation site, independent of Glu167, such that divergent cation coordination to this site moderately shifts CooA toward the active conformation. In this scenario, the interaction between Glu59 and Glu128 either by divalent cations or Lys substitution of one residue contributes to the conformational change during CooA activation.

We wished to test these hypotheses for the divalent cation effect by introducing analogous substitutions in the CRP background but realized that it would be complicated. In the case of the poly(Asp) tail, it would be impossible to guess what portion to attach to CRP to place it in a similar position, because CooA and CRP are so different in this region. A similar challenge exists with Glu60, where CooA is missing a residue near the tip of the loop formed by the β4 and β5 sheets with respect to CRP and FNR, making it hard to know what substitutions would be appropriate. Finally, the homologous position to Glu128 in CRP is near the cAMP-binding site, which might complicate analyses. Nevertheless, two observations make it clear that the effect of Glu at this position (position 167 in CooA and position 170 in CRP) depends in part on the rest of the protein context. (i) The loss of DNA-binding activity by Q170E substitution in CRP is not as striking as the gain of DNA-binding activity by E167K or E167L substitution in CooA in the absence of divalent cations. (ii) As noted above, Q170E CRP shows little or no improvement in DNA-binding affinity in the presence of divalent cations (data not shown).

The proposed model (Fig. 2) can explain these differences. First, CRP lacks the Asp tail, and the model predicts that this alone should significantly reduce the charge repulsion experienced by the substituted Glu170 in Q170E CRP. Consistent with this model, the CRP-CooA chimera described above has this Asp tail and more closely resembles CooA in terms of its divergent cation requirement. Second, it seems likely that the position of Glu60 in CooA might be subtly but importantly different from that of its analogous residue in CRP, Glu55.
This notion is based on the fact that the β4/β5 loop of CooA, which contains Glu59 and Glu60, has one fewer residue than does the same loop in CRP, which must have some structural implications for the Glu55 residue of CRP. These two differences between CRP and CooA should affect the charge repulsion between the Glu167 or Glu170 residue (Glu167 in CooA and Glu170 in Q170E CRP) and the rest of the protein. Therefore, we hypothesize that these differences also change the abilities of these various residues to be bridged by a divalent cation, explaining the absence of a divalent cation effect in CRP.

**Glu167 of CooA is the optimal residue for in vivo transcriptional activation.** The in vitro DNA-binding assay used above demands only an interaction with a specific DNA sequence. In contrast, the in vivo assay we typically employ measures the ability of CooA to cause the synthesis of β-galactosidase, which requires proper interaction with RNA polymerase as well (23, 24). While E167L CooA (indeed all the CooA variants listed in Table 1) has apparently sufficient levels of CooA accumulation to saturate the CooA binding site of the reporter system under the assay condition (data not shown), the protein displays poor transcription activation in vivo in the presence of CO compared to that of WT CooA (Fig. 4A). Because purified E167L CooA displays WT CooA-like in vitro DNA-binding affinity in the presence of CO (Fig. 3A), the obvious implication is that E167L CooA is defective in its interaction with RNA polymerase. E167K CooA was similar to E167L CooA in these properties (data not shown) and presumably has the same defect. However, this conclusion rests on the assumption that these variants bind DNA in vivo at least as well as WT CooA does, so we tested this assumption directly.

As described in Materials and Methods, we created a system in which the *cat* gene is expressed from a constitutive promoter but then created a CooA-binding site that overlaps that promoter. In this system, CooA binding prevents *cat* transcription. The expression of *cat* causes chloramphenicol resistance, so the ability of CooA to bind that promoter correlates with the inability of the strain to grow in the presence of chloramphenicol. This assay is fundamentally different from the one in which CooA activates *lacZ* transcription, since the latter requires not only DNA binding but also precise positive interactions of CooA with RNA polymerase. In the *cat* system, the only requirement for a CooA effect is DNA binding, which indirectly blocks transcription. In this way, we can assay the relative affinities of different CooA variants to bind DNA in vivo.

The result in Fig. 4B clearly shows that E167L CooA causes a level of chloramphenicol sensitivity (as measured by the reduction in growth) that is at least as severe as that caused by WT CooA. This experiment was performed in the identical strain background as the β-galactosidase assay that reports CooA transcriptional activation, so the result demonstrates that the lower transcription activation in the same strain background must reflect a lower ability to interact with RNA polymerase. This strongly suggests that the presence of Glu167 in CooA has a positive effect on the interaction with RNA polymerase and therefore serves as an important residue in an AR of CooA.

Which AR is Glu167 part of? The two known *R. rubrum* promoters activated by CooA are of the class II type (8, 11, 23, 24), and in vivo transcriptional activity of CooA is routinely measured in an *E. coli* strain at a promoter of the same type (28). Therefore, the above result applies to class II type activation. Since class II type activation uses at least three different regions for contact with RNA polymerase, Glu167 could influence any of the three ARs. On the basis of the structure of active CRP, it was tempting to propose that Glu167 is a part of AR3, which is known to interact with the σ subunit of RNA polymerase (Fig. 5). However, because AR1 residues are primarily localized to the DNA-binding domain in FNR and CRP (2, 4, 36) and AR3 residues have not been demonstrated in that domain in these proteins, AR1 could not be discounted.

To suggest which AR might be affected by Glu167, we used CRP, because its ARs have been much better defined than have those of CooA, and we measured in vivo transcriptional activity of Q170E CRP at both class I (CC-61.5) and class II (CC-41.5) promoters along with that of WT CRP. As shown in Table 2, Q170E CRP is clearly defective in vivo activation from the class I promoter, while it is comparable to WT CRP for in vivo activation at the class II promoter. We interpret this to mean that the poor transcription activation at the class I promoter reflects the defect of Q170E CRP in DNA binding, and therefore the approximately normal transcription activation at the class II promoter represents a gain-of-function AR as a compensatory mechanism. In CRP, transcription activation requires the AR of only one subunit (42). Therefore, Glu170 in the CRP variant from the other subunit still experiences DNA repulsion, which may explain why the in vivo transcriptional activation of the Q170E CRP variant at the class II promoter is not better than that of WT CRP under the assay condition (Fig. 5). This improved AR cannot be AR1, because it would have affected the class I site as well; therefore, it must be either AR2 or AR3. However, AR3 is a far more
likely candidate, because the known AR2 surfaces lie in the DNA-distal portion of CRP and FNR (3). Also consistent with an AR3 effect of a Glu at this position is the fact that all other DNA-distal portion of CRP and FNR (3). Also consistent with the peptide chain from the N to C terminus is indicated by the arrows. For the interaction of the upstream subunit of CooA with DNA, which involves a divalent cation, see Fig. 2.

![Diagram](image)

**FIG. 5.** Model showing that the Glu167 residue of CooA is either coordinated by Mg$^{2+}$ (upstream subunit) or interacts with σ$^{70}$ (downstream subunit) in the CooA-RNA polymerase transcription complex. The CooA interaction with RNA polymerase at a class II type promoter is shown at the top of the figure. There are three activating regions in CooA termed AR1, AR2, and AR3. AR1 interacts with the α subunit of the C-terminal domain (α-CTD) of RNA polymerase, whereas AR2 makes contacts with the α subunit of the N-terminal domain (α-NTD) of RNA polymerase. AR3 interacts with the σ subunit of RNA polymerase. The proposed region of interaction between Glu167 in the downstream CooA subunit and the σ$^{70}$ subunit is enlarged and shown to the lower right of the figure. Val57, Glu60, and Glu62 are also included in the figure, because they have been already identified as AR3 residues (16). The portion of σ$^{70}$ participating in the interaction (region 4.2; + indicates positively charged) is based on the study with CRP (21). The direction of the peptide chain from the N to C terminus is indicated by the arrows. For the interaction of the upstream subunit of CooA with DNA, which involves a divalent cation, see Fig. 2.

there is an electrostatic interaction between the negative charges of AR3 and positively charged regions on the σ subunit (region 4.2) (14, 16, 22). Last, the position of this residue in the active CRP structure shows that it is reasonably positioned to interact with the σ subunit. This evidence strongly supports the notion that Glu167 represents the first identification of a residue in the DNA-binding domain serving in AR3. By obvious extension, a role of Glu167 in CooA is to enhance interaction with RNA polymerase by an improved AR3 region. The results above with CRP also show that Glu170 can provide an AR effect in that protein as well. We considered testing Q170E in vitro as a gain-of-function AR in CRP, but such a substitution would simultaneously decrease DNA affinity (Fig. 3C), so that the result would not be readily interpretable.

Figure 5 illustrates a model for the in vivo transcriptional activation by CooA that also incorporates divalent cations. We hypothesize that in the cell, Glu167 from the downstream subunit of CooA interacts with the σ subunit of RNA polymerase, while the residue from the other subunit coordinates a divalent cation with neighboring residues. We also hypothesize that Glu167 probably does not coordinate Mg$^{2+}$ when interacting with the σ subunit of RNA polymerase, because the contact between AR3 and the σ subunit uses electrostatic interaction, at least in the case of CRP (21). In such scenario, the low affinity of CooA for divalent cations may ensure the displacement of a Glu167-coordinating divalent cation by the σ subunit of RNA polymerase in the formation of the transcription complex.

It is interesting that this residue is common among CooA homologs (39) but is otherwise somewhat unusual within the CRP/FNR superfamily (Fig. 6). If it provides a useful interaction with RNA polymerase, why is it not more common? We do not know the answer to this but suggest the following possibility. Physiologically appropriate transcription activation requires an affinity of the activator for RNA polymerase that is high enough to cause promoter binding in the presence of effector but not so high that the activator-polymerase complex leads to significant activation without effector. This affinity is based on the sum of interactions of the activator (at regions termed AR1, AR2, and AR3) with three different regions of RNA polymerase. Therefore, it is possible that the CooA family has evolved in such a way that some of the surfaces important for interaction with RNA polymerase in most other members of the CRP family are absent and that the Glu167 position happened to evolve as a compensatory feature.

Finally, we considered the possibility that the divalent cation requirement could serve as a physiological sensor for CooA function in vivo and tested this by comparing the transcriptional activation of WT and E167L CooA in vivo under external conditions with either limiting or excess divalent cations, but no difference was seen (data not shown). Obviously, a negative result can be explained in many ways, but it suggests that divalent sensing is not a part of a physiological response of CooA. Since Mg$^{2+}$ is thought to be available at millimolar levels in vivo (10), but Ca$^{2+}$ or Mn$^{2+}$ are not, we assume that Mg$^{2+}$ is the relevant cation that satisfies this requirement in vivo. Quite possibly the levels of available divalent cations are high enough in the cell to support DNA binding by CooA in any event. By this model, Glu167 was selected through evolution to stimulate interaction with RNA polymerase, while the

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**TABLE 2.** In vivo transcriptional activities of Q170E CRP variant at both class I (CC-61.5) and class II (CC-41.5) promoters compared with those of WT CRP

<table>
<thead>
<tr>
<th>CRP variants</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class I</td>
</tr>
<tr>
<td></td>
<td>−cAMP</td>
</tr>
<tr>
<td>None (control)$^c$</td>
<td>54</td>
</tr>
<tr>
<td>WT</td>
<td>49</td>
</tr>
<tr>
<td>Q170E</td>
<td>49</td>
</tr>
</tbody>
</table>

$^a$ WT CRP and Q170E variant were tagged at the C terminus with six histidines.

$^b$ The activity was measured using aerobically grown cells (to an OD$_{600}$ between 1 and 2) cultured in LC medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) in the presence (+) or absence (−) of cAMP at 37°C. To express the proteins, 25 μM IPTG was used. The values are the means of two independent measurements.

$^c$ Cells containing only vector, pEXT20, without cooA were used as a control.
negative effect on DNA binding would be functionally irrelevant in vivo.

**Important conclusions derived from these results.** The results in this paper demonstrate the following important points.

(i) Glu167 is critical for the unusual divergent cation requirement for DNA binding by CooA. In addition to being biochemically interesting, this result calls into question the results of analyses in which supposed CooA-DNA interactions have been analyzed in the absence of divergent cations (34). (ii) Glu167 serves as an AR residue in CooA and CRP, and it is highly likely that it belongs to AR3. Though the functional importance of these has not been shown in CRP or FNR, it establishes the important possibility of such contacts for the very large family of homologous proteins. (iii) Finally, these results highlight the complexity of analyzing proteins that undergo substantial conformational changes and that have multiple functions involved in biological activity. A single specific residue can be involved in multiple forms and functions of the protein, and each of these functions might have different biochemical constraints for acceptable residues.

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**REFERENCES**


