Mapping CooA-RNA Polymerase Interactions

IDENTIFICATION OF ACTIVATING REGIONS 2 AND 3 IN CooA, THE CO-SENSING TRANSCRIPTIONAL ACTIVATOR*

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CooA is a CO-sensing protein that activates the transcription of genes encoding the CO-oxidation (coo) regulon, whose polypeptide products are required for utilizing CO as an energy source in *Rhodospirillum rubrum*. CooA binds to a position overlapping the −35 element of the P_{cooA} promoter, similar to the arrangement of class II CRP (cAMP receptor protein)- and FNR (fumarate and nitrate reductase activator protein)-dependent promoters when expressed in *Escherichia coli*. Gain-of-function CooA variants were isolated in *E. coli* following mutagenesis of the portion of *cooA* encoding the effector-binding domain. Some of the mutations affect regions of CooA that are homologous to the activating regions (AR2 and AR3) previously identified in CRP and FNR, whereas others affect residues that lie in a region of CooA between AR2 and AR3. These CooA variants are comparable to wild-type (WT) CooA in DNA binding affinity in response to CO but differ in transcription activation, presumably because of altered interactions with *E. coli* RNA polymerase. Based on predictions of similarity to CRP and FNR, loss-of-function CooA variants were obtained in the AR2 and AR3 regions that have minimal transcriptional activity, yet have WT-like DNA binding affinities in response to CO. This study demonstrates that WT CooA contains AR2- and AR3-like surfaces that are required for optimal transcription activation.

*Rhodospirillum rubrum* is a photosynthetic bacterium capable of oxidizing carbon monoxide (CO) to CO₂ with concomitant evolution of H₂, which is coupled to energy generation (1). The components of the CO-oxidizing (coo) regulon, which are produced only in the presence of exogenous CO, are encoded by two operons, and their expression is controlled by CooA, a transcriptional activator that binds CO under reducing conditions and is itself expressed constitutively from an adjacent operon (2). The structural components of the CO-oxidizing system include CooS (CO dehydrogenase), which oxidizes CO to CO₂, CooF, a CooS-associated iron-sulfur protein that donates reducing equivalents to CooH, a CO-tolerant hydrogenase (3–5).

CooA is a heme-containing protein that belongs to the cAMP receptor protein (CRP) (6)) and the fumarate and nitrate reductase activator protein (FNR (7)) superfamily of transcriptional activator proteins. All three proteins are homodimers when competent to bind DNA, and the structures of CooA and CRP reveal effector-binding and DNA-binding domains in each monomer. The structure of CooA in the reduced (Fe²⁺) form has been solved recently (8) and revealed that the general folding topology of CO-free CooA and cAMP-bound CRP (9) were similar (see Fig. 1 below). In the case of CRP, only the effector (cAMP)-bound structure has been solved (9), and for CooA, only the effector (CO)-free structure is known (8). Significant differences were observed in the positions of the DNA-binding domains of the two proteins. However, because both proteins bind to similar DNA sequences (2), it is assumed that the DNA-binding regions of the effector (CO)-bound form of CooA should adopt a roughly similar orientation compared with that of effector (cAMP)-bound CRP (8). This suggests that a rotation of ~180° of the recognition helices occurs in CooA upon effector binding (see Fig. 1). In CooA, CO binds to the heme in the effector-binding domain and causes a conformational change that subsequently positions the DNA-binding domain to interact effectively with target DNA. Some CooA variants have been described with varying levels of effector-independent activity (10). These have been referred to as "CooA*" by analogy to the variants of CRP (denoted CRP⁺ (11)) with cAMP-independent activity. In CooA, some of the substitutions with this phenotype affect the heme region directly (10), whereas others affect residues in and around the long "C helices" (Fig. 1) that form the dimer interface.2

Members of the CRP/FNR/CooA family respond to specific intracellular signals, bind specifically to one or more promoter regions, and activate transcription through contacts with RNA polymerase (RNAP (11–13)). RNAP (Fig. 2) consists of two molecules of the α subunit, one molecule each of the β and β' subunits, and one of several σ subunits. The α subunit can be further divided into an N-terminal domain (α-NTD) and a C-terminal domain (α-CTD), which are joined by a flexible linker. These domains, along with the σ subunit, have distinct functions in transcription activation through specific interactions with activator proteins. CRP is known to activate transcription in distinct ways at three different classes of promoters (classes I, II, and III (14)).

At class II CRP-dependent promoters, CRP binds to a site centered around −41.5 in which the CRP-binding site overlaps the −35 promoter element. At these promoters (Fig. 2), the

FNR, *E. coli* fumarate and nitrate reductase activator protein; AR1, -2, -3, activating regions 1–3; P_{cooA}, the promoter of cooF; P_{cooM}, the promoter of cooM; RNAP, RNA polymerase; WT, wild-type; α-CTD, C-terminal domain of the α-subunit of RNA polymerase; α-NTD, N-terminal domain of the α-subunit of RNA polymerase; MOPS, 4-morpholinepropanesulfonic acid.

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1 The abbreviations used are: CRP, cAMP receptor protein;
are typical of class II-dependent promoters, comparison of potential CooA-RNAP interaction sites with those that are known for CRP and FNR will suggest general properties of the CRP/FNR/CooA family in terms of RNAP interactions. This work characterizes the AR2 and AR3 regions of CooA, with implications for the properties of the CRP/FNR superfamily of activator proteins.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The construction of *E. coli* strains expressing WT CooA and CooA variants and strains containing a chromosomal *β-galactosidase* reporter system was described previously (21). Site-directed variants were constructed in a pKK223-3-based plasmid expression system as described previously (21).

**Random Mutagenesis and Screen for Suppressors**—Random mutations affecting the effector-binding domain (residues 1–131) of CooA variants H77E and H77K were created using error-prone polymerase chain reaction in two separate mutagenesis reactions that were adapted from Vogel and Das (23) and from Fromant et al. (24). The mutagenized segments were then cut with EcoRI and Clal (Promega) and gel-purified. The resulting mutagenized fragment was ligated into a digested pKK223-3 vector encoding the wild-type (WT) C terminus of CooA (residues 132–222). The resulting pool of plasmids, encoding a randomly mutagenized CooA effector-binding domain fused to a WT CooA DNA-binding domain, was transformed into the *E. coli* strain in which β-galactosidase is expressed from *P* _cooF_, a CooA-dependent promoter. Clones were screened for significant activity in the presence of CO, using MOPS-buffered agar plates (10) containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 450 μg/ml ampicillin. Plates were incubated anaerobically in the presence of CO for 2 days at 30 °C. After storing the plates aerobically at 4 °C to allow color development, blue colonies were isolated and corresponding plasmids containing *cooA* were sequenced to determine the mutations that were responsible for the gain-of-function phenotype.

Because of the mutagenesis method, most sequenced mutants contained several mutations. These were then individually recreated by site-directed mutagenesis in a WT *cooA* background (i.e. encoding the normal His at position 77), and all further analyses were carried out with those strains. Putative loss-of-function variants were similarly created by site-directed mutagenesis in a WT *cooA* background and verified by sequencing.

**Quantitative in Vivo β-Galactosidase Activity Assay**—Strains containing the *P* _cooF_-lucZ reporter fusion and plasmid-borne cooA variants were measured, in the absence or presence of CO, for β-galactosidase activity as described previously (25).

**Purification of WT CooA and CooA Variants**—Purification of WT CooA and CooA variants (to >95% homogeneity) was performed using standard procedures as described previously (21). The heme content of CooA preparations was measured using either the Soret peak absorbance from UV-visible absorption spectroscopy (extinction coefficient of FeIII CooA = 105 M⁻¹ cm⁻¹ at 423 nm (26)) or the reduced pyridine hemochromogen method (27).

**Fluorescence Polarization Assay for DNA Binding**—*In vitro* DNA binding of CooA preparations was measured using a fluorescence polarization assay as described (28, 29).

**In Vitro Transcription Assays**—The ability of isolated CooA to activate *in vitro* transcription under anaerobic conditions was determined by procedures described previously (25). The cooA variants in the starting strains (either H77K or H77E substitutions) have a very low level of CO-independent activity and, although they bind CO with an affinity roughly similar to that of WT CooA, CO binding does not result in increased activation of transcription (10). We assume that the current study in space-filled display; the significant reorientation of the DNA-binding domain in effector-bound CRP compared with effector-free CooA is apparent. The approximate location of the proposed activating regions are circled, and the C helices that form the dimerization interface in each protein is noted. The β sheets that join at the AR3 are termed the 4/5 loop. Left panel, a similar display of effector (cAMP)-bound CRP (Protein Data Bank no. 1g6n). Right panel, the “B” monomer of effector (CO)-free CooA in the FeII state (PDB no. 1f9). The exact locations of activating regions (AR) will certainly be altered upon CO binding. The CRP residue Glu399, referred to in the text, lies immediately in front of His399 in this view but is not shown for reasons of clarity.

**Fluorescence Polarization Assay for DNA Binding**—*In vitro* DNA binding of CooA preparations was measured using a fluorescence polarization assay as described (28, 29).

**RESULTS AND DISCUSSION**

**Isolation of Gain-of-function Variants Altered at the Surface of CooA**—This study began as a screen for mutations in cooA that would suppress mutations that alter His77, one of the normal heme ligands in the FeIII (8) and FeIII + CO forms (28, 30). The CooA variants in the starting strains (either H77K or H77E substitutions) have a very low level of CO-independent activity and, although they bind CO with an affinity roughly similar to that of WT CooA, CO binding does not result in increased activation of transcription (10). We assume that the current study in space-filled display; the significant reorientation of the DNA-binding domain in effector-bound CRP compared with effector-free CooA is apparent. The approximate location of the proposed activating regions are circled, and the C helices that form the dimerization interface in each protein is noted. The β sheets that join at the AR3 are termed the 4/5 loop. Left panel, a similar display of effector (cAMP)-bound CRP (Protein Data Bank no. 1g6n). Right panel, the “B” monomer of effector (CO)-free CooA in the FeII state (PDB no. 1f9). The exact locations of activating regions (AR) will certainly be altered upon CO binding. The CRP residue Glu399, referred to in the text, lies immediately in front of His399 in this view but is not shown for reasons of clarity.
that the forms of WT CooA that are active and inactive for DNA binding exist in an equilibrium and that CO binding shifts the equilibrium toward the active form. The replacement of His77 in CooA apparently shifts that equilibrium slightly toward the active form in the absence of CO, but CO binding does not further change that equilibrium, so that the low level of activation in vivo is not increased by the presence of CO. Using an in vivo \( \beta \)-galactosidase assay system in \( E. coli \), we screened a pool of cooA-bearing clones in which the region encoding the effector-binding domain (residues 1–131) of the two His77 variants had been randomly mutagenized. Colonies displaying significantly higher \( \beta \)-galactosidase activity than did the starting strains were chosen, and the cooA region was amplified and sequenced.

Because of the nature of the mutagenesis, many of the strains contained multiple substitutions. Each mutation was recreated by site-directed mutagenesis in a WT cooA background (i.e. with the WT Fe\(^{III} \) heme-ligand His77). This allowed us to identify those mutations that were causative of the increased \( \beta \)-galactosidase activity and to analyze them without the complication of the mutation affecting the His77 position. From each original strain with multiple mutations, only one mutation displayed a clearly mutant phenotype in the WT background, and all subsequent analyses were limited to these mutations in that background.

Two general classes of suppressor mutations were found: (i) those that render CooA active in the absence of CO and affect residues in its interior (CooA\(^{A} \) (10)) and (ii) those that remain CO-dependent for activity and affect surfaces of CooA that might contact RNA polymerase.

The first class of suppressor mutations encoded the following changes in CooA: S78L, P98L, E128K, and D134N. Because of their effector independence, as well as the positions of the affected residues in the CooA structure, they may perturb the pathway by which CO binding to the heme is transmitted to the DNA binding regions. It appears that a repositioning of the C helices (Fig. 1) with respect to each other plays a role in signal transmission within CooA (8). We assume that these substitutions mimic CO binding by stimulating that repositioning in some fashion.

The second class of suppressors, which is the focus of this paper, consisted of four substitutions: E60G, D94N, E38K, and E41K. The first two affect residues homologous to the AR2 and AR3 regions of CRP (Fig. 1), whereas the latter two fall in an intermediate position. We used the position of these residues as an indicator of the regions of CooA involved in activation and compared these positions to those of residues affecting AR2 and AR3 of CRP and FNR. We verified that the behavior of this second class was not a result of altered protein accumulation or differential heme content, because they all displayed WT levels of heme-containing CooA in extracts (data not shown). It is our working hypothesis that this class was detected in the screen, because they have a significant increase in their ability to interact with \( E. coli \) RNAP and that this interaction “traps” the small fraction of these CooA variants that happens to be transiently arranged in the DNA binding conformation.

The AR2 Region of CooA—In \( \beta \)-galactosidase assays, D94N CooA showed activity that was greater than that of WT both with and without CO (Table I). Increases in CO responsiveness have been reported for a D94A variant of CooA in another study (31), but no further analysis of the basis of its behavior was performed. An examination of the local structures of both CooA and CRP suggested that a gain-of-function substitution in CRP (E96N) maps to the same surface region and apparently acts by removing an unfavorable charge-charge interaction between CRP and \( \alpha \)-NTD. This residue lies near the AR2 of CRP, a set of basic residues (His\(^{19} \), His\(^{21} \), and Lys\(^{101} \)) that contact an acidic patch on \( \alpha \)-NTD (15). To further test whether WT CooA actually possesses an AR2 region, we attempted to construct loss-of-function mutants in this region on the basis of structural comparisons with CRP. Both T97E and K26E CooA substitutions were constructed (the former is analogous to CRP Lys\(^{101} \) and the latter was chosen because of its charge and position), because we anticipated that they would create unfavorable charge-charge interactions at the AR2-\( \alpha \)-NTD interface. Both mutants have severely reduced activity in \( \beta \)-galactosidase assays (Table I), consistent with our hypothesis that this region is important for activation.

For a more direct analysis of the basis for the altered behavior of these CooA variants, T97E and D94N CooA proteins were purified as described under “Experimental Procedures” and tested for DNA binding capacity using a fluorescence polarization assay and for interactions with RNAP using an in vitro transcription assay. Consistent with the suggestion that the alterations affect interactions with RNAP but not with DNA, both variants bound DNA normally in the fluorescence polar-

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**Table I**

- **In vivo \( \beta \)-galactosidase and in vitro fluorescence polarization assays of selected CooA variants**

| Genotype * | Activity \(-\)CO | Activity \(+\)CO | \(K_{D}^{I} \) | In vivo function |
|------------|-----------------|-----------------|----------------|----------------|
| pKK223–3   | 2 (0.62 ± 0.16) | 2 (0.79 ± 0.09) | ND | Negative control |
| WT         | 4 (1.4 ± 0.14)  | 100 (52 ± 1.3)  | 9.0 ± 2.5 | Positive control |
| D94N       | 11 (3.4 ± 0.42) | 300 (98 ± 7.2)  | 12 ± 2.5 | +AR2 |
| K26E       | 3 (0.98 ± 0.60) | 8 (2.4 ± 0.12)  | ND | -AR2 |
| T97E       | 3 (1.1 ± 0.35)  | 16 (5.2 ± 1.6)  | 8.6 ± 2.6 | -AR2 |
| E38K       | 34 (11 ± 0.62)  | 270 (87 ± 9.8)  | ND | +AR2/3 |
| E38A       | 53 (17 ± 0.62)  | 650 (210 ± 21)  | 14 ± 1.2 | +AR2/3 |
| E41K       | 15 (4.8 ± 1.2)  | 180 (58 ± 4.1)  | ND | +AR2/3 |
| E41A       | 12 (3.7 ± 0.87) | 240 (76 ± 3.9)  | ND | +AR2/3 |
| E60G       | 90 (29 ± 5.2)   | 310 (99 ± 13)   | 13 ± 3.3 | -AR3 |
| E60A       | 13 (4.2 ± 0.71) | 140 (45 ± 2.6)  | ND | -AR3 |
| V57K       | 1 (0.44 ± 0.02) | 2 (0.69 ± 0.18) | 5.3 ± 0.90 | -AR3 |
| E62A       | 1 (0.35 ± 0.03) | 30 (9.5 ± 2.0)  | ND | -AR3 |

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*Strains that are underlined were isolated during the initial screens for gain-of-function CooA variants.

Reported activity values are displayed as a percentage compared to that of WT CooA. Miller unit values are included in parentheses. Standard errors of measurement represent a minimum of three replicate determinations.

\( K_{D}^{I} \) refers to the calculated dissociation constant of DNA binding by selected CooA variant according to the in vitro fluorescence polarization assay. Standard errors of measurement represent a minimum of three replicate determinations.

ND, not determined.
Neither loss- nor gain-of-function variants in CooA in vivo are affected in their ability to interact with target DNA in vitro. In vitro fluorescence polarization assays of DNA binding by WT CooA and CooA variants in response to CO binding. In this particular assay, the target DNA was 6.4 nt. Lines represent the best fit of the binding data using a non-linear curve fitting equation described by Lundblad (29). A, WT CooA and loss-of-function CooA variants; B, gain-of-function CooA variants. Quantitative $K_D$ values from these curves are listed in Table I. As a negative control, WT CooA in the absence of CO resulted in anisotropy values no higher than 5% of the baseline anisotropy value (data not shown).

The identification and characterization of both gain- and loss-of-function variants of CooA in this region demonstrate that CooA contains an activating region in the same approximate position as AR2 of CRP and that negatively charged residues on this surface region are deleterious to its activation function. This is consistent with the hypothesis that the AR2-α-NTD interaction is electrostatic with a negatively charged surface on the α-NTD of RNAP interacting with a positively charged surface of CooA. Because CooA is only the second member of this large protein superfamily for which the x-ray crystal structure is known, the similarity in positioning and function of these regions is striking and suggests that the hypothesis might extend generally to other divergent members of the CRP family.

The AR3 Region of CooA—Another CooA variant that increased β-galactosidase activity with and without CO contained an E60G substitution (Table I), which lies on the exposed β-turn region as shown by the d/b loop (Fig. 1). Because CooA, unlike CRP and FNR, lacks a Gly residue in this “turn” region, we wondered if the phenotype caused by the E60G substitution was due to the loss of the Glu or the acquisition of the Gly. E60A CooA was constructed and also exhibited slightly higher than WT activity in vivo (Table I), suggesting that both the flexibility contributed by the introduction of the Gly as well as the removal of the Glu side chain are responsible for the increased activity of E60G CooA.

Although the WT AR3 of CRP is thought to be uninvolved in transcription activation, the AR3 of FNR has been identified (16) as an important activation determinant, presumably at the region indicated in Fig. 1 (the structure of FNR has not been solved). Specific acidic residues in the AR3 of FNR have been hypothesized to contact a basic patch on the RNAP $\sigma^70$ subunit, which is important for interaction (16). The structure of the region is also important, because G58A FNR (Gly58 of FNR is adjacent to the homolog of Glu55 of CooA) is impaired for AR3 function (18), which is consistent with the usefulness of Gly at this position for AR3 function in FNR.

To test the hypothesis that this region is serving as an AR3 in WT CooA, substitutions in that region were created that should result in a loss of function by analogy with FNR and CRP. Lys52 is the residue in CRP that must be removed for its AR3 function (18), which is consistent with the usefulness of Gly at this position for AR3 function in FNR.

As the case of the AR2 variants, in vitro analysis was performed with purified proteins. Both isolated E60G and V57K CooA proteins bind DNA with similar affinity as does WT CooA in our in vitro assay (Fig. 3, A and B, and Table I), further suggesting that their differences in vivo are the result of altered interactions with RNAP. Consistent with this hypothesis, V57K CooA was impaired in transcriptional activation in vitro (Fig. 4, upper panel), whereas E60G, the gain-of-function variant found in our initial screen, supported a increase in AR activity to 30% of WT FNR, suggesting an important role for this hydrophobic residue. We therefore replaced Val57 of CooA with Lys because the equivalent residue in CRP is unfavorable for AR3 function. We also changed residue Glu62, the acidic residue in CooA homologous to the most important charged residues for AR3 function in CRP (Glu68 (16)). V57K and E62A CooA both yielded reduced levels of β-galactosidase activity under all conditions, with V57K showing almost no activity even under inducing conditions (Table I).

Although WT CooA does not have a Gly residue in the AR3 β-turn, it is clear from the structure of the effector-free protein, that a turn is nevertheless made in this region. It is our working hypothesis that the E60G substitution in CooA simply optimizes the region further for interaction with the sigma

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5 P. J. Kiley, personal communication.
subunit of RNAP of *E. coli*. The basis for the increased activity apparently caused by the elimination of the Glu residue (E60A also had modestly higher activity *in vivo*) is unclear, but perhaps bulky groups provide some steric hindrance at this position. The results of these targeted mutations together with the variant found in our screen suggests that the AR3 of CooA is functionally similar to those of CRP and FNR, in the sense that substitutions at apparently similar positions have similar effects in CooA, CRP, and FNR. The striking effects of substitutions in the analogous region of CooA strengthen the hypothesis that this region is of general importance to the CRP superfamily.

AR2 and AR3 Are Not Widely Separated Patches on CooA—Two of our gain-of-function substitutions found in the initial screen, E38K and E41K, map to a region on the surface of CooA roughly halfway between the putative AR2 and AR3 patches discussed above (Fig. 1). E38A and E41A CooA variants were also constructed and supported activities even higher than those of the original Lys substitutions (Table I), suggesting that it is the removal of the acidic side chains rather than the introduction of the basic side chains that is responsible for the detected activities. Isolated E38A CooA bound target DNA with a similar affinity as did WT CooA in our *in vitro* assay (Fig. 3B and Table I). Consistent with the notion that these variants are altered in their interaction with RNAP, E38A CooA supported a higher level of transcription product *in vitro* than did WT CooA (Fig. 4, lower panel).

Because the structure of CooA is rather different from that of CRP in this region, due to the presence of the nearby heme, it is impossible to make a strong case for an analogous region in that protein. Variants that might be similar have been found in FNR, where A61T and F112L were found in a screen for loss-of-function variants (18), and K60R and K60M were found in a screen for suppressors of an AR1 loss-of-function mutation (33). Lys60 of FNR aligns with Glu38 of CooA, and Ala61 and Phe112 of FNR are both spatially adjacent to Lys60 of FNR in a predicted FNR structure based on the structure of CRP and sequence alignment. These residues in FNR have been considered a part of AR3 based on proximity to the 4/5 loop region (18). In any event, the location of Glu38 and Glu41 in effector-free CooA (Fig. 1) strongly suggests that the AR regions on this family of proteins might not be the discrete patches of AR2 and AR3 as shown in the figure but actually reflect a more or less continuous surface of the effector-binding domain capable of interacting with RNAP.

The Putative AR1 Region of CooA—As outlined in the introduction, the presence of a functionally important AR1 region in CooA was suggested by an earlier study that demonstrated the necessity of RNAP α-CTD for CO- and CooA-induced transcriptional activation both *in vivo* and *in vitro* (13). In CRP, AR1 consists of a set of consecutive amino acids in the DNA-binding domain (15). None of the gain-of-function variants found in our screen of randomly mutagenized CooA variants mapped to this region, because we specifically mutagenized only the effector-binding domain. Although some residues in the effector-binding domain of FNR appear to be involved in its AR1 (18, 32), substitutions affecting analogous residues in CooA were not found due to our limited screen.

Important Implications of These Results—Irrespective of possible structural similarities of this CooA region with those of CRP and FNR, the position of these CooA variants at Glu38 and Glu41 invites speculation on a possible property of effector binding: repositioning of AR regions for optimal contact with RNAP. Although a comparison of the structure of effector-bound CRP with effector-free CooA shows a significant repositioning of the AR3 region (8) (Fig. 1), this might be the result of new surfaces of the DNA-binding domain being available for interaction with the effector-binding domain after the reorientation caused by effector binding. However, the Glu38-Glu41 region has no obvious connection to the DNA-binding domain but does potentially sense effector binding in the following way: Asn42, adjacent to Glu41, appears to make close contact with His77, the heme ligand that is retained upon CO binding (28, 30). There is strong reason to believe that CO binding perturbs the position of the heme and, therefore, His77, Asn42, and Glu41 residues as well. It is therefore possible that CO binding not only triggers a conformational change that supports DNA binding but also directly affects specific residues involved in RNAP interactions. Although the data in this paper do not address this hypothesis, we have found effector-independent CooA variants that display high affinity DNA binding, but not comparably high levels of transcription activation *in vivo*, suggesting that the causative mutations (typically near the dimer interface) support the conformational changes necessary for DNA binding but not all the changes for optimal interaction with RNAP.

Another important implication concerns the observations that all three AR regions of CooA appear to be important for function. Until recently, the view was that WT CRP lacks a functional AR3, though one can be revealed by a single substitution, and conversely the AR2 region of FNR is thought not to be important. At least in the case of CRP, more complete mutational analysis has revealed both activating and inhibitory determinants within AR3 (16). Previous results (13), combined with the present study with CooA, are consistent with a view that all members of the family probably have analogues of all three AR regions in close proximity to the appropriate surfaces of RNAP. The functional differences merely reflect the fact that, for a specific activator, some regions make more thermodynamically important contacts than do others, and the important regions have been defined as “functional ARs.” In our approach, CooA may be a particularly sensitive test case for these interactions, because we are examining the interactions between *R. rubrum* CooA and the heterologous RNA polymerase of *E. coli*, with which CooA has not evolved for optimal interactions.

We should note that the results in this work indicate an importance of the identified residues for proper interaction between CooA and RNA polymerase but do not imply that these residues actually make that contact. A reasonable alternative hypothesis is that some of these residues are important for the proper positioning of nearby residues that actually interact with RNA polymerase.

Conclusions—The identification of all three AR regions in CooA, together with the information from its crystal structure, expands and clarifies the paradigm that has arisen from the analysis of CRP and FNR. This conservation suggests an early origin for the AR1-, AR2-, and AR3-activating regions in this family of transcriptional activators. The location of “AR-like” regions between AR2 and AR3 suggests that a continuous surface of interaction between the activator and RNAP is possible. The proximity of this region to the CO-binding heme is intriguing and suggests a different pathway by which an effector can lead to the acquisition of transcriptional competence in this protein family: Effector binding might directly and specifically modify AR regions, in addition to the alteration in the positioning of the DNA-binding domains.

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CooA-RNA Polymerase Interactions

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Mapping CooA-RNA Polymerase Interactions: IDENTIFICATION OF ACTIVATING REGIONS 2 AND 3 IN CooA, THE CO-SENSING TRANSCRIPTIONAL ACTIVATOR

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