The Role of the Hydrophobic Distal Heme Pocket of CooA in Ligand Sensing and Response*

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CooA from Rhodospirillum rubrum is a heme-containing transcriptional activator that becomes activated only upon binding CO. The basis for this specificity has been probed in a CooA variant, termed ΔP3R4 CooA, lacking two residues adjacent to the Pro\(^{2}\) heme ligand, which weakens that ligand. ΔP3R4 CooA can bind imidazole and CN\(^{-}\), as well as CO, and form a 6-coordinate low spin adduct with each. However, in contrast to the case with CO, imidazole and CN\(^{-}\) do not stimulate the DNA binding activity of ΔP3R4 CooA. This result indicates that the CO-specific activation of CooA is not merely the result of creation of a 6-coordinate CooA adduct but that there must be another element to this response. One feature of CooA activation is modest repositioning of the C-helices upon CO binding, so we altered a portion of the C-helix (residues Ile\(^{113}\) and Leu\(^{116}\)) located near the heme-bound CO in wild type CooA, and we investigated the effect on CO-specific activation. Surprisingly, the sizes of Ile\(^{113}\) and/or Leu\(^{116}\) positions are not critical for CooA activation by CO, disproving a precise interaction between these residues and the CO-bound heme as a basis for the CO activation mechanism and CO ligand specificity. In contrast, hydrophobic residues at these positions contribute to the activation. Some CooA variants altered at these positions in the background of ΔP3R4 were also found to show low but reproducible activation in response to imidazole binding to the heme. A model for the role of hydrophobicity in CooA activation and specificity is suggested.

Rhodospirillum rubrum is a photosynthetic bacterium that can grow with CO as a sole energy source (1). The response of this organism to CO is regulated by the CooA protein, which binds CO and then activates the transcription of a series of genes encoding the CO oxidation system of R. rubrum (2). CooA contains a b-type heme prosthetic group as do many other gas-sensing proteins such as soluble guanylyl cyclase, FixL, DOS, and HemAT (3–6). The heme of CooA is 6-coordinate and low spin in all oxidation and ligation states (7), indicating that the incoming CO must displace one of the internal protein ligands. The structure of Fe(II) CooA (without CO and there-low spin in all oxidation and ligation states (7), indicating that the effector-bound form of CooA can bind imidazole and CN\(^{-}\), as well as CO, and form a 6-coordinate low spin adduct with each. However, in contrast to the case with CO, imidazole and CN\(^{-}\) do not stimulate the DNA binding activity of ΔP3R4 CooA. This result indicates that the CO-specific activation of CooA is not merely the result of creation of a 6-coordinate CooA adduct but that there must be another element to this response. One feature of CooA activation is modest repositioning of the C-helices upon CO binding, so we altered a portion of the C-helix (residues Ile\(^{113}\) and Leu\(^{116}\)) located near the heme-bound CO in wild type CooA, and we investigated the effect on CO-specific activation. Surprisingly, the sizes of Ile\(^{113}\) and/or Leu\(^{116}\) positions are not critical for CooA activation by CO, disproving a precise interaction between these residues and the CO-bound heme as a basis for the CO activation mechanism and CO ligand specificity. In contrast, hydrophobic residues at these positions contribute to the activation. Some CooA variants altered at these positions in the background of ΔP3R4 were also found to show low but reproducible activation in response to imidazole binding to the heme. A model for the role of hydrophobicity in CooA activation and specificity is suggested.

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The abbreviations used are: CRP, cAMP receptor protein; WT, wild-type; MOPS, 3-(N-morpholino)propanesulfonic acid; PDB, Protein Data Bank; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
binding oxidizes CooA. It was therefore a reasonable hypothesis that the specificity for CO simply reflected its liganding strength. If correct, this would predict that any ligand that could displace Pro2 yet allow a 6-coordinate heme would be similarly active. On the other hand, this result would not be seen if CO bound to the heme affected the achievement of the active form in ways other than simply creating a 6-coordinate species with Pro2 displacement.

In this report, we have examined the issue of the role of CO in CooA activation and the related issue of CO specificity. We reasoned that any direct role of the heme-bound CO would almost certainly be affected by the protein residues nearest to that CO, and we have examined the importance of these through a combination of mutational, functional, and spectroscopic analyses.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and in Vivo Activity Assays—The construction of strains overexpressing wild-type (WT) CooA and CooA variants in an Escherichia coli background having a cooA-dependent β-galactosidase reporter system in the chromosome was described previously (17). In vivo activities of WT CooA and CooA variants were measured using the above system and quantitated using the standard protocol (18). All the CooA variants were constructed in a pEXT20-based expression plasmid that provides tight control of cooA expression (10).

Creation and Screening of cooA Mutations—Site-directed mutagenesis involved PCR amplification of cooA with primers designed to incorporate the desired nucleotide changes, as described elsewhere (19). The method used for codon randomization was essentially identical to the method used for site-directed mutagenesis, except that the primers contained randomized codons for the desired positions. Screening of CooA variants involved the analysis of their ability to cause β-galactosidase accumulation in colonies on agar plates incubated under different growth conditions as described previously (10). Based on colony color, CooA variants were classified as active, weakly active, and inactive. Selected variants were examined quantitatively by the in vivo β-galactosidase assay, after which the cooA genes were sequenced to determine the causative residue changes. Imidazole (25 mM final concentration) was used for the screening of imidazole-activated CooA variants.

Purification of WT CooA and CooA Variants—The purification of WT CooA and the CooA variants (>95% homogeneity) was performed as described previously (17). The heme content of CooA preparations was estimated using the extinction coefficient of WT CooA (7) or by a modified reduced pyridine-hemochromogen method (7), and protein concentration was measured using the BCA assay (Pierce).

Preparation of Hydroxylapatite Batch-treated CooA Samples—Preparation of hydroxylapatite batch-treated CooA samples was carried out using the procedure described previously (20). By this method, heme-containing CooA was enriched to ~10% of total protein in case of WT CooA. These preparations were used for the preliminary measurement of UV-visible spectra and in vitro DNA binding activities of some CooA variants.

Measurement of Heme-containing CooA Accumulation—20 ml of the cells of WT CooA or CooA variants grown in 1× MOPS-buffered media in the presence of CO were harvested by centrifugation. Cell pellets were dissolved in 50 μl of H2O, vortexed, treated with 50 μl of 2 N HCl, vigorously vortexed, and then treated with 1000 μl of a 7:2 acetone/ methanol solution. This solution was vigorously vortexed and centrifuged, and the spectra of the supernatant were immediately measured. The peak intensity at 383 nm, normalized for cell mass, was used for determination of the heme-containing CooA accumulation.

In Vitro DNA Binding Assays—In vitro DNA binding assays of WT CooA and CooA variants were performed using the fluorescence polarization technique with a Beacon 2000 fluorescence polarization detector (Panvera Corp., Madison, WI) as described previously (10). For the measurement of DNA binding of CO-, imidazole- and CN−-bound Δ384 CooA at pH 9.5, the following assay buffer (high pH anisotropy buffer) was used: 40 mM glycine-NaOH, pH 9.5, 6 mM CaCl2, 50 mM KCl, 5% (v/v) glycerol, and 5 mM dithiothreitol. As a fluorescence probe, a 26-bp target DNA containing Pcoo was labeled with Texas Red on one end of the duplex and used at the concentration of 6.4 μM. Salmon sperm DNA at 6.4 μM was included in the reaction mixture to eliminate possible nonspecific DNA binding. Dissociation constants (Kd) were calculated by fitting of the binding data to an equation that incorporated a fluorescence quenching factor upon DNA-protein interaction as described elsewhere (21).

UV-visible Absorption Spectroscopy—UV-visible absorption spectroscopy of COA samples was performed at room temperature in quartz cuvettes using a Shimadzu UV-2401PC spectrophotometer. UV-visible spectra of COA samples were routinely obtained using 25 mM MOPS buffer, pH 7.4, with 0.1 mM NADH, unless stated otherwise. The UV-visible spectra of CO-, imidazole-, and CN−-bound Δ384 CooA were obtained with high pH anisotropy buffer for proper comparison with DNA binding activities of these forms.

RESULTS AND DISCUSSION

Weakening the Pro2 Ligand Allows CooA to Bind Other Exogenous Ligands Such as Imidazole and CN−—As noted in the Introduction, WT CooA is only activated in response to CO. It
has been demonstrated that other potential exogenous ligands cannot create the 6-coordinate CooA adduct; NO is the only small molecule, other than CO, that can form a stable adduct to the heme of CooA (16), but NO binding results in a 5-coordinate form (8). (ii) The C-helices in the vicinity of the heme are the obvious candidates for this sensing function for the following reasons. (i) The C-helices of CooA are the closest residues to the heme iron (20). The following results show that ΔP3R4 CooA is perturbed in the Fe(II) form as well and that imidazole and CN− can bind to this form. In the 6-coordinate adducts produced, the small molecules have presumably displaced the perturbed Pro2 ligand, although this has not been demonstrated. Fig. 2A shows the UV-visible spectra of various forms of ΔP3R4 CooA in high pH anisotropy buffer. A comparison of the spectra of Fe(III) and Fe(II) ΔP3R4 CooA (Fig. 2A) with those of WT CooA (data not shown) measured in the presence of 0.2 mM KCl suggested the existence of a population of proteins in both forms of the ΔP3R4 CooA with an open heme coordination site. The addition of imidazole (final 0.2 mM) to Fe(II) ΔP3R4 CooA resulted in increase in signal intensity with concomitant red shifts of Soret, α, and β bands to 426, 560, and 530 nm (Fig. 2A), respectively, indicating that imidazole-bound 6-coordinate CooA heme was formed. Similarly, the addition of KCN (final 0.2 mM) into Fe(II) ΔP3R4 CooA resulted in shifts of Soret, α, and β bands to 435, 567.5, and 538.5 nm (Fig. 2A), characteristic of a 6-coordinate low spin CN−-adduct of the heme.

This imidazole- and CN−-binding property is also observed in other CooA variants with perturbed Pro2 ligation. These include ΔP3-I7 CooA (data not shown), which lacks five residues from Pro3 to Ile7, and G117I CooA, where Ile117 sterically perturbs Pro2 ligation (20). In each case, it appears that the Pro2 perturbation creates a population with an open heme coordination, to which these exogenous ligands bind. We assume that imidazole and CN− first bind on the Pro2 side to the low level of 5-coordinate species in Fe(II) form and shift the equilibrium to the 6-coordinate adduct with the small molecule adduct. This open heme coordination position would almost certainly be the Pro2 side, because Pro2 is the endogenous ligand that is perturbed in these CooA variants. This implies that those exogenous ligands are binding to the same side of the heme as does CO to WT CooA. This situation allowed us to test the ability of “properly bound” exogenous ligands other than CO to activate CooA.

**Imidazole and CN− Binding Cannot Activate ΔP3R4 CooA**—We then tested whether Fe(II)-imidazole or Fe(II)-CN− ΔP3R4 CooA showed DNA binding activity under the same conditions as were used to measure the spectra. As shown in Fig. 2B, Fe(II) ΔP3R4 CooA responds to CO with a significant increase in signal by fluorescence anisotropy but shows no response to imidazole or CN−. When CO was then added to head space of Fe(II)-imidazole or Fe(II)-CN− ΔP3R4 CooA, DNA binding activity was restored in each case. The appearance of partial activity in the sample with both CN− and CO (Fig. 2B) reflects the ability of high levels of CN− to compete for heme binding with the modest levels of CO used, as revealed by UV-visible spectrum (data not shown). Surprisingly, CO addition to Fe(II)-imidazole ΔP3R4 CooA actually resulted in DNA binding activity that is reproducibly higher than that of the Fe(II)-CO form. Under this condition, CO completely displaced imidazole from the heme, as revealed by UV-visible spectrum (data not shown), so that the stimulation by imidazole was not due to its being a ligand. The exact mechanism of this interesting secondary effect of imidazole is under further investigation. In the absence of added effectors, Fe(III) and Fe(II) ΔP3R4 CooA failed to show any DNA binding activity at this CooA concentration (Fig. 2B).

These results indicate that imidazole or CN− binding to Fe(II) ΔP3R4 CooA cannot trigger the conformational change that leads to DNA binding, and therefore that CO provides a level of specificity for the CooA activation process in addition to its ability to form a 6-coordinate CooA adduct. If the nature of the small molecule ligand is sensed, then the surfaces of the C-helices in the vicinity of the heme are the obvious candidates for this sensing function for the following reasons. (i) The C-helices of CooA are the closest residues to the heme iron other than Pro2, at least in the known Fe(II) structure (8). (ii) Resonance Raman analysis indicated that Ile113, Leu116, and Gly117 are the residues close to the bound CO. (iii) As discussed in the Introduction, there are several lines of evidence that C-helix repositioning occurs upon CO binding and that
Approximately 6,000 colonies, with small changes of "Red" activity (denominator) potentially result in large differences in this ratio.

Hydrophobic Residues at Positions 113 and 116 Are Important for a Normal CooA Function—Because all C-helix residues near the distal side of the heme are hydrophobic (Fig. 1C), it was our working hypothesis that specificity in activation by CO would be the result of steric interaction between the bound CO and these residues (8). Based on the known structure of Fe(II) CooA, Ile"113 and Leu"116 are within 8 Å of the heme iron on Pro2 side (8), and we have already shown a functional importance for Gly117, although the basis for this remains unknown (20). We therefore created CooA variants with substitutions of small (Ala) and large (Phe) residues at positions 113 and 116. Table I shows in vivo activities of these CooA variants with a CooA-dependent β-galactosidase reporter system, showing that these variants were somewhat altered in their CO responsiveness of CooA. It should be noted that there is excess amount of CooA in the cells under the conditions of these assays, so that activities below 80% actually represent a meaningful loss of CooA functionality. The basis for the low but significant CO-independent activity of I113F CooA is unknown.

To change more dramatically the distal heme pocket volume, we created two CooA variants with double substitutions, I113A/L116A and I113F/L116F CooA variants. Although I113A/L116A CooA was severely perturbed in its CO-sensing function, I113F/L116F CooA was relatively normal (Table I). The synergistic effect of the combination of the I113A and L116A substitutions led to the hypothesis that these two residues might form a functional pair in some way, such that modification of a single residue might have only a modest effect. In order to understand the functional requirements of that pair, we simultaneously randomized the codons for both residues 113 and 116 and screened for those CooA variants with significant activity in the presence of CO, as well as seeking less functional variants for comparison. Such a randomization and screening procedure allows a clearer understanding of the requirements at both positions for CO responsiveness, because a large number of possibilities are tested. Approximately 6,000 colonies, with cooA randomized at these two codons, were screened in the presence of CO. CooA variants with high activity (blue colonies; −10%), intermediate activity (pale blue, −30%), and negligible activity (white colonies; −60%) colonies were seen. Selected variants were then examined more quantitatively for β-galactosidase activity in the presence and absence of CO and their cooA genes sequenced (Table II). The first conclusion is that, although a variety of residues can support normal CooA function at positions 113 and 116, neither charged nor hydrophilic residues are acceptable for good activity in the presence of CO. Such residues are absent among the normally active variants, yet are common among those variants with little or no activity (Table II). The requirements for a functional CooA appear to be rather more restrictive at position 116 than at position 113, as a narrower range of residues is found at that position among normally active variants. This suggests that residue 116 has a more important role than does residue 113 for the in vivo CO response of CooA.

Because Leu at position 116 was so common in the double random mutagenesis, we wanted to probe further the acceptability of other residues at that position. We then randomized only the codon for residue 116, screened in the presence of CO for variants with a range of activities, identified causative changes, and measured quantitative β-galactosidase activity in the presence and absence of CO. As suggested by the 113/116 double randomization results, hydrophobic residues at position 116 such as Val and Phe gave high activity in the presence of CO, whereas hydrophilic residues such as His, Asn, Arg, and Gly allowed very low activity (Table III).

Because of the possible synergy between the residues at positions 113 and 116, we analyzed the variants from the double-codon randomization for informative patterns. There was no apparent correlation between CO-dependent activity and the amino acid volume of either residue or of the sum of their volumes (data not shown). However, there was a clear correlation between hydrophobicity at position 116 and in vivo activity, with a suggestive pattern for the combination of the 113/116 positions (Fig. 3). At position 116 (Fig. 3A), there are three exceptions to the pattern as follows: L116C, L116H, and L116R CooA variants. L116C CooA can be explained by the fact that the hydrophobicity of Cys can assume a range of hydrophobic natures, depending on context, and that value used in other reports would move it to a more consistent position in the figure (22). L116R CooA accumulates heme-containing protein at negligible levels, so its inactivity probably reflects an absence of that species, as discussed below. L116H CooA is intriguing because His could be a ligand in the Fe(II) form, as has strongly been suggested for Lys in L116K CooA (23). We previously showed that Lys ligation in this variant led to the unusually high activity of Fe(II) form and decrease of its activity upon CO binding (23). It is a reasonable hypothesis that His"116 ligation could be the origin for this phenotype in L116H CooA.

At position 113, although the correlation between hydrophobicity and CooA function is much less obvious (Fig. 3B), non-hydrophobic residues were found at this position in CooA variants lacking in CooA function. Asp, Ser, and Asn were found to be detrimental: I113D CooA lacked CooA function, and Ser and Asn at 113 position were responsible for perturbed activities of I113S/L116I and I113N/L116C CooA variants, respectively, because Ile and Cys are acceptable at position 116 (Tables II and III). Whereas the analysis in Fig. 3, A or B, reveals an important role of hydrophobicity at individual residue 116 or 113 in the CO response of CooA in vivo, the data shown in Fig. 3C suggest the importance of overall hydrophobicity (sum of hydrophobicity at residues 113 and 116). For example, this analysis suggests why I113A/L116A CooA displays poor CooA function, although the individual I113A and L116A substitutions would seem to be acceptable (Fig. 3C). The functional dependence on overall hydrophobicity might be the core of the previously hypothesized synergistic relationship between positions 113 and 116.

| CooA          | % β-Galactosidase activity<sup>a</sup> | % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO and showed variability <10%. | % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO and showed variability <10%. | % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO and showed variability <10%. | % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO and showed variability <10%. | % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO and showed variability <10%. |
|---------------|----------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| pEXT-20       | 0.7                                    | 0.7                                                                                              | 1.0                                                                                              | 1.0                                                                                              | 1.0                                                                                              | 1.0                                                                                              |
| WT            | 1.9                                    | 100                                                                                              | 52.7                                                                                              | 52.7                                                                                              | 52.7                                                                                              | 52.7                                                                                              |
| I113A/L116A   | 0.7                                    | 106                                                                                              | 117.8                                                                                             | 117.8                                                                                             | 117.8                                                                                             | 117.8                                                                                             |
| I113F/L116F   | 8.8                                    | 137                                                                                              | 15.8                                                                                              | 15.8                                                                                              | 15.8                                                                                              | 15.8                                                                                              |
| L116F         | 1.2                                    | 71                                                                                               | 59.2                                                                                              | 59.2                                                                                              | 59.2                                                                                              | 59.2                                                                                              |
| L113F/L116F   | 1.7                                    | 91                                                                                               | 53.5                                                                                              | 53.5                                                                                              | 53.5                                                                                              | 53.5                                                                                              |

<sup>a</sup> Cells grown anaerobically were used for the activity.

<sup>b</sup> The ratio will be informative only when considered with "Red + CO" activity since small changes of "Red" activity (denominator) potentially result in large differences in this ratio.

The ratio will be informative only when considered with "Red + CO" activity since small changes of "Red" activity (denominator) potentially result in large differences in this ratio.
Hydrophobic Nature at Position 113 and 116 Is Important for Proper Accumulation of Heme-containing CooA—Based on resonance Raman analysis of CooA variants, Ile\textsuperscript{113} and Leu\textsuperscript{116}, together with Gly\textsuperscript{117}, are primary distal heme pocket residues of the Fe(II)-CO form of CooA.\textsuperscript{2} Heme pocket residues are known to affect greatly the heme stability in myoglobin (24, 25). We therefore examined the \textit{in vivo} accumulation of heme-containing CooA in some CooA variants in order to test whether the introduction of hydrophilic or charged residues at position 113 and/or 116 affected that property. The accumulation of heme-containing CooA was measured by the method described under “Experimental Procedures” on the same culture samples as were used for the \textit{in vivo} activity measurement (1\times MOPS-buffered media, in the presence of CO). As shown in Fig. 4, all of the CooA variants tested accumulated heme less well than did WT CooA, and hydrophilic CooA variants such as I113D, L116R, and L116N failed to accumulate detectable levels of heme under this condition. I113K CooA was also highly perturbed in accumulation of heme-containing protein. This result suggests that hydrophobic residues at these positions are important for heme stability when CO is present.

\[\text{CO Activation of CooA} 2337\]

\[\text{TABLE II}\]

Properties of CooA variants altered in the vicinity of heme through double-randomized mutagenesis at positions 113 and 116

| CooA | Sequence | % β-galactosidase activity<sup>a</sup> | Ratio' (Red + CO/Red) |
|------|----------|-----------------|---------------------|
| pEXT-20 | | | |
| WT (Blue\textsuperscript{4}-1) | Ile\textsuperscript{a} Leu\textsuperscript{b} | 0.7 | 1.0 |
| Blue-2 | Cys | 0.7 | 1.0 |
| Blue-3 | Phe | 1.0 | 1.0 |
| Blue-4 | Pro | 0.7 | 1.0 |
| Blue-5 | Met | 0.7 | 1.0 |
| Blue-6 | Trp | 1.0 | 1.0 |
| Blue-7 | Leu | 1.0 | 1.0 |
| Blue-8 | Tyr | 1.0 | 1.0 |
| Blue-9 | Cys | 1.0 | 1.0 |
| Blue-10 | Ala | 1.0 | 1.0 |
| Blue-11 | Phe | 0.7 | 1.0 |
| Blue-12 | Val | 0.7 | 1.0 |
| Blue-13 | Ile | 0.7 | 1.0 |
| Blue-14 | Cys | 0.7 | 1.0 |
| Blue-15 | Leu | 0.7 | 1.0 |
| Blue-16 | His | 0.7 | 1.0 |
| Blue-17 | Gly | 0.7 | 1.0 |
| Pale blue-1 | Ser | 0.7 | 1.0 |
| Pale blue-2 | Ser | 0.7 | 1.0 |
| Pale blue-3 | Asn | 0.7 | 1.0 |
| Pale blue-4 | Ala | 0.7 | 1.0 |
| Pale blue-5 | Leu | 0.7 | 1.0 |
| Pale blue-6 | Leu | 0.7 | 1.0 |
| Pale blue-7 | Asp | 0.7 | 1.0 |
| Pale blue-8 | Ile | 0.7 | 1.0 |
| Pale blue-9 | Ala | 0.7 | 1.0 |
| White-1 | Asp | 0.7 | 1.0 |
| White-2 | Ile | 0.7 | 1.0 |

<sup>a</sup> % activity indicates the mean value of double measurements of activity relative to that of WT CooA in the presence of CO and showed variability<sup>b</sup>\textsuperscript{<10%}.<sup>c</sup> Cells grown anaerobiocally were used for the activity.<sup>d</sup> The ratio will be informative only when considered with Red + CO activity since small changes of Red activity (denominator) potentially result in large differences in this ratio.<sup>e</sup> Blue, pale blue, and white indicate the color of a colony in a screening plate, respectively. Blue color indicates active CooA variant; white color indicates inactive CooA variant or one that fails to accumulate heme.<sup>f</sup> Wild type amino acid sequence.

\[\text{TABLE III}\]

Properties of CooA variants altered in the vicinity of heme through double-randomized mutagenesis at position 116

| CooA | % β-galactosidase activity<sup>a</sup> | Ratio' (Red + CO/Red) |
|------|-----------------|---------------------|
| pEXT-20 | | |
| WT | | |
| L116V | | |
| L116C | | |
| L116F | | |
| L116T | | |
| L116A | | |
| L116Q | | |
| L116R | | |

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However, despite the importance of hydrophobicity at 113 and 116 residues, the size range of acceptable residues at these positions appears to disprove the original hypothesis that CO specificity results from a precise interaction between the CO and the C-helix residues.
under this condition (1/11003, Experimental Procedures, and sum of the residues, 113 residue (B) at 116 residue (C) altered at residues 113 and/or 116 were plotted against hydrophobicity important for CooA function. The in vivo activities of CooA variants altered at residues 113 and/or 116 were plotted against hydrophobicity at 116 residue (A), 113 residue (B), and sum of the residues (C). Whereas A and B contain CooA variants singly altered at residues 116 and 113, respectively, C contains all the CooA variants altered at residues 113 and/or 116. Amino acid index of hydrophobicity was from Karplus (26).

DNA binding, proper interaction with RNA polymerase, and the accumulation of heme-containing CooA. Because of their internal location in CooA, we doubt that 113 and/or 116 substitutions alter its interaction with RNA polymerase. Therefore, the above examination implies that hydrophobicity at positions 113 and/or 116 has a role in both proper conformational response to CO and normal heme retention.

A Representative Hydrophilic CooA Variant, I113D, Is Perturbed in DNA Binding Activity—In order to probe more directly the effects of a hydrophobic residue in this region on the conformational response of CooA to CO, we purified a CooA variant with a representative hydrophilic substitution, I113D CooA, and directly measuring DNA binding activity of the protein. We chose I113D CooA variant because of its good heme stability during the preliminary manipulations and its adequate CooA accumulation in a heme-containing form under aerobic growth conditions in rich medium (LB). (It is unknown why the same I113D CooA variant accumulates poorly heme-containing CooA in 1× MOPS-buffered media anaerobically in the presence of CO.) Originally, we planned to purify a CooA variant with hydrophilic residues at position 116 as well, but we failed because such variants were unstable. Instability of heme-containing CooA variant at this position has been already reported (23). Fig. 5A shows the titration of a Texas Red-labeled DNA probe with the purified I113D CooA. Although Fe(III) and Fe(II) forms of 113D CooA did not show any DNA binding activity up to 4,000 nM CooA, the Fe(II)-CO form showed highly perturbed DNA binding activity corresponding to a $K_d$ value of 1,320 nM, in contrast to the 23 nM $K_d$ value of Fe(II)-CO WT CooA (Fig. 5A). Upon CO binding I113D CooA is therefore highly defective in performing the conformational change necessary for DNA binding. However, UV-visible spectra of I113D CooA were normal in all three forms (Fig. 5B), indicating that the perturbed DNA binding of Fe(II)-CO I113D CooA is not a result of a dramatic change of heme ligation states. These data show that the presence of a charged residue in this region greatly affects the ability of CooA to undergo the proper conformational change in response to CO.

Proper Heme Positioning May Be Critical for CooA Response to CO—In the analysis of I113D CooA above, there was no evidence that perturbation of the vicinity of the bound CO was the basis for the altered activity, and we wondered if the problem might instead be one of heme positioning. Namely, it is known that the heme of CooA must move with respect to the protein during oxidation and reduction (17), and it is certainly possible that there is further heme movement upon CO binding. A role of residues 113 and 116 might therefore be in proper heme positioning. We therefore asked if an indirect perturbation of the heme position might mollify the problem caused by the I113D substitution. We have already found that the I95W substitution can increase the CO-responsive activity of a variety of CooA variants, consistent with the notion that this residue on the His77 side of the heme might sterically move the heme to a position consistent with that in CO-bound WT CooA. Ile95 lies on the B-helix (8), and bulkier substitutions might push the heme toward the C-helices, thereby reducing the heme pocket size, and reorienting the heme close to Leu116 and away from Ile113 in the known Fe(II) structure. For this reason, we introduced the I95W substitution into a strain that already had the I113D substitution. As shown in Fig. 5A, the $K_d$ value of Fe(II)-CO I113D/I95W CooA was determined as 98 nM, which is 13-fold lower than that of Fe(II)-CO WT CooA (Fig. 5A). Upon CO binding I113D CooA is therefore highly defective in performing the conformational change necessary for DNA binding. However, UV-visible spectra of I113D CooA were normal in all three forms (Fig. 5B), indicating that the perturbed DNA binding of Fe(II)-CO I113D CooA is not a result of a dramatic change of heme ligation states. These data show that the presence of a charged residue in this region greatly affects the ability of CooA to undergo the proper conformational change in response to CO.

3 H. Youn, unpublished data.
of the results identified imidazole-dependent CooA variants. Clones displaying imidazole-dependent \( \beta \)-galactosidase activity were sequenced. Table IV shows the quantitative \textit{in vivo} activities of selected imidazole-responsive CooA variants with control, WT CooA, \( \Delta P3R4 \) CooA in “anaerobic,” “anaerobic + imidazole,” and “anaerobic + CO” conditions. The four imidazole-activated CooA variants showed 6–18% full activity under the anaerobic + imidazole condition, which is dramatically above the activity seen with WT or \( \Delta P3R4 \) CooA (Table IV). As explained earlier in this report, these levels of activity would be relatively modest in an assay where CooA was limiting, but the response is still striking. It is also interesting that \( \Delta P3R4 \) L116T CooA, which showed the highest \textit{in vivo} activities in the presence of imidazole, is only slightly more active in the presence of CO. The negligible effector-free activity of \( \Delta P3R4 \) L116T CooA also indicates that the ligand-free form is not close to the active conformation, and imidazole induces a substantial conformational change in the CooA variant. The UV-visible spectra of the hydroxylapatite-batch preparations of the imidazole-activated CooA variants (listed in Table IV) in Fe(II) forms were all changed upon addition of 25 mM imidazole (data not shown), implicating that imidazole was a ligand in these conditions. Preliminary analysis of \textit{in vitro} DNA binding by \( \Delta P3R4 \) L113F/L116F CooA in the presence of saturating imidazole showed DNA affinity below that of WT CooA with CO but well above background. These results demonstrate that imidazole binding in this variant does not lead to fully active CooA. However, this screening has hardly optimized the potential of CooA to respond to imidazole, but the fact that modification of positions 113 and 116 give readily detectable activity demonstrates a clear role of these residues in effector specificity once the physical barrier (Pro2 ligation) was removed.
Summary and Working Hypothesis—CooA and CRP function as dimer. In each protein, the two C-helices provide a dimerization interface. The comparison of the structures of inactive Fe(II) CooA with active cAMP-bound CRP revealed the repositioning of the C-helices with respect to each other, and it became our working hypothesis that CO binding activates CooA by inducing this repositioning. The importance of the repositioning in CooA activation has been confirmed by the finding that CooA variants where alteration of positions 121–126 generated CooA variants with significant effector-independent activity (27).

The goal of this study was to determine the basis for the CO specificity of WT CooA and, by implication, the mechanism by which CO binding causes C-helix repositioning. The simple hypothesis that CO specificity was due to the selective ability of CO to displace on Pro^2 was disproved by the demonstration that other effectors could bind to ΔP3R4 CooA, yet not to CooA activation. Structural and spectral evidence had indicated that positions 113 and 116 were close to the heme-bound CO in WT CooA (8), and the results reported here show the significance of these residues in response to effectors. The nature of these residues is clearly important for activation in response to CO binding as well as for proper heme retention, with the specific residue at position 116 being particularly critical. As described below, we believe that these effects can be explained with a model whereby a hydrophobic pocket is critical for both heme retention and response to CO. We note that the C-helices in the CooA dimer assume a coiled-coil helical structure where Leu^{116} is in the d position and Ile^{121} is in the a position. The residue requirements for an optimal leucine zipper are also substantially consistent with the acceptable residues that we detect at positions 113 and 116. Nevertheless, these CooA variants with very good leucine zipper residues (including WT CooA) at these positions do not result in effector-independent activity, indicating that the leucine zipper effect itself is not sufficient to afford the transition energy to active conformation.

Although these residues play an important role in activation, their role in CO specificity is less clear. We expected that specificity might be provided by steric contacts between the heme-bound CO and these residues, but the variety of acceptable residues at these positions for CO activation disproves this hypothesis. Nevertheless, an important role of these residues in effector specificity is certainly shown by the results with imidazole. The weakening of the Pro^2 ligand (by the ΔP3R4 alteration) permits binding of imidazole, but it is not sufficient for activation. In contrast, perturbation of residues 113 and 116 in the ΔP3R4 background allows significant activation in response to imidazole binding, although the molecular rules that govern this response have not been elucidated. Although it is not clear if the process of activation by imidazole in these variants is mechanistically similar to that of WT CooA by CO, it nevertheless establishes a potential role for these residues in sensing the heme-bound effector. It might well be that CO specificity is provided by the exclusion of other small molecules by an unknown mechanism and that a variety of residues have this property. Further analysis of the requirements of these residues at 113 and 116 to various small molecules should clarify this important issue.

Our present working hypothesis for the results in this paper is the following. CO binding displaces Pro^2, which is protonated and therefore expelled from the hydrophobic heme pocket. Therefore, CO binding will certainly expose the hydrophobic C-helix residues near the CO-bound heme in Fe(II)-CO CooA. The hydrophobic interaction between the CO-bound heme and residues 113 and 116 allows it to reposition within that hydrophobic cavity, which directly or indirectly affects C-helix repositioning. In this view, hydrophobicity at positions 113 and 116, and exclusion of water from the heme cavity of Fe(II)-CO CooA, would be critical for CO responsiveness of CooA. Proper positioning of the CO-bound heme by the residues at positions 113 and 116 is also important, as suggested by the ability of the I95W substitution to restore activity to certain variants, presumably through a different heme-positioning mechanism.

The results reported here show that C-helix residues 113 and 116 are important for heme retention, effector response, and effector specificity in CooA. Further analysis will better define the molecular basis of those effects.

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REFERENCES
1. Kerby, R. L., Ludden, P. W., and Roberts, G. P. (1995) J. Bacteriol. 177, 2241–2244
2. He, Y., Shelver, D., Kerby, R. L., and Roberts, G. P. (1996) J. Biol. Chem. 271, 120–123
3. Marletta, M. A. (1994) Cell 78, 927–930
4. Gilles-Gonzales, M. A., Ditta, G. S., and Helinski, D. R. (1991) Nature 350, 170–172
5. Delgado-Nixon, V. M., Gonzalez, G., and Gilles-Gonzales, M. A. (2000) Biochemistry 39, 2685–2691
6. Hsu, S., Larsen, R. W., Boudko, D., Riley, C. W., Karatan, E., Zimmer, M., Oriol, G. W., and Alam, M. (2000) Nature 403, 540–544
7. Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11216–11220
8. Lanziolita, W. N., Schaller, D. J., Thorsteinsson, M. V., Kerby, R. L., Roberts, G. P., and Poulo, T. J. (2000) Nat. Struct. Biol. 7, 876–880
9. Yamamoto, K., Ishikawa, H., Takahashi, S., Ishimori, K., Morishima, I., Nakajima, H., and Aono, S. (2001) J. Biol. Chem. 276, 11473–11476
10. Thorsteinsson, M. V., Kerby, R. L., Conrad, M., Youn, H., Staples, C. R., Lanziolita, W. N., Poulo, T. J., Serate, J., and Roberts, G. P. (2000) J. Biol. Chem. 275, 38332–38338
11. Shelver, D., Kerby, R. L., He, Y., and Roberts, G. P. (1995) J. Bacteriol. 177, 2157–2163
12. Passner, J. M., Schultz, S. C., and Steitz, T. A. (2000) J. Mol. Biol. 304, 847–859
13. Cheng, X., Kovac, L., and Lee, J. C. (1995) Biochemistry 34, 10816–10820
14. Cheng, X., and Lee, J. C. (1998) J. Biol. Chem. 273, 705–712
15. Kiley, P. J., and Reznikoff, W. S. (1991) J. Bacteriol. 173, 16–22
16. Reynolds, M. P., Parks, R. B., Hurstyn, E. T., Lanzilotta, W. N., Serate, J., and Roberts, G. P. (2000) Biochemistry 39, 388–396
17. Shelver, D., Thorsteinsson, M. V., Kerby, R. L., Chung, S. Y., Roberts, G. P., Reynolds, M. P., Parks, R. B., and Hurstyn, J. N. (1999) Biochemistry 38, 2669–2678
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1995) Short Protocols in Molecular Biology, 3rd Ed., pp. 13–30, John Wiley & Sons, Inc., New York
19. Chiang, L. W., Kovari, I., and Howe, M. M. (1993) PCR Methods Applications 2, 210–217
20. Youn, H., Kerby, R. L., Thorsteinsson, M. V., Conrad, M., Staples, C. R., Serate, J., Beack, J., and Roberts, G. P. (2001) J. Biol. Chem. 276, 41603–41610
21. Lundeblad, J. R., Lauranne, M., and Goodman, R. H. (1996) Mol. Endocrinol. 10, 607–612
22. Nagano, N., Ota, M., and Nishikawa, K. (1999) FEBS Lett. 458, 69–71
23. Youn, H., Kerby, R. L., Thorsteinsson, M. V., Clark, R. W., Hurstyn, J. N., and Roberts, G. P. (2002) J. Biol. Chem. 277, 33616–33623
24. Alayash, A. I., Ryan, B. A. B., Rich, R. F., Olson, J. S., and Cashon, R. E. (1999) J. Biol. Chem. 274, 2029–2037
25. Liang, E. C., Dou, Y., Scott, E. E., Olson, J. S., and Phillips, G. N., Jr. (2001) J. Biol. Chem. 276, 9093–9100
26. Karplus, P. A. (1997) Protein Sci. 6, 1302–1307
27. Kerby, R. L., Youn, H., Thorsteinsson, M. V., and Roberts, G. P. (2003) J. Mol. Biol., in press
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