Heme Displacement Mechanism of CooA Activation

MUTATIONAL AND RAMAN SPECTROSCOPIC EVIDENCE*

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The heme-containing protein CooA of Rhodospirillum rubrum regulates the expression of genes involved in CO oxidation. CooA binds its target DNA sequence in response to CO binding to its heme. Activity measurements and resonance Raman (RR) spectra are reported for CooA variants that bind DNA even in the absence of CO, those in which the wild-type residues at the 121–126 positions, TSCMRT, are replaced by the residues AYLLRL or RYLLRL, and also for wild-type residues at the 121–126 positions, TSCMRT, are also for variants that bind DNA poorly in the presence of CO, such as L120S and L120F. The Fe-C and C-O stretching resonance Raman (RR) frequencies of all CooAs examined deviate from the expected back-bonding correlation in a manner indicating weakening of the Fe-His-77 proximal ligand bond, and the extent of weakening correlates positively with DNA binding activity. The (A/R)YLLRL variants have detectable populations of a 5-coordinate heme resulting from partial dissociation of the endogenous distal ligand, Pro-2. Selective excitation of this population reveals downshifted Fe-His-77-stretching RR bands, confirming the proximal bond weakening. These results support our previous hypothesis that the conformational change required for DNA binding is initiated by displacement of the heme into an adjacent hydrophobic cavity once CO displaces the Pro-2 ligand. Examination of the crystal structure reveals a physical basis for these results, and a mechanism is proposed to link heme displacement to conformational change.

An increasing volume of research has revealed the ubiquity of heme sensor proteins (1, 2), which regulate a range of biological activities in response to changing levels of the gaseous molecules CO, NO, or O₂. In these proteins nature has taken advantage of the ability of heme to bind these nonpolar molecules and has coupled the elementary act of ligand binding to a change of protein activity. How this is accomplished has become a central issue in chemical biology.

One of the best opportunities to probe this question is presented by the well studied molecule CooA (3, 4) from Rhodospirillum rubrum, which grows on CO as the sole energy source under anaerobic conditions. When bound by CO, CooA binds its target DNA sequence and activates transcription of genes coding for proteins that oxidize CO to CO₂ and reduce protons to H₂. CooA is a homodimer, and each 221-residue monomer contains an N-terminal heme binding domain and a C-terminal DNA binding domain. The x-ray crystal structure has been determined for the CO-free inactive state of Fe(II) CooA (5) but not for the CO-bound active state. However, CooA is homologous to another well studied transcription factor, cAMP receptor protein (CRP)§ (6–8), whose crystal structure has been determined in the cAMP-bound active state. Comparison of the two structures (Fig. 1) reveals similar folds for both the DNA and effector binding domains but very different domain orientations. In the symmetric structure of active CRP, the DNA binding domains are positioned near to the effector binding domains in such a way as to expose the F helices to properly interact with target DNA. In contrast, the structure of inactive CooA is asymmetric, presumably because of crystal forces (5). The B chain has an extended structure due to a fusion of the C helix at the dimer interface with the D helix of the DNA binding domain. The A chain has a bend between the C and D helices as in active CRP, but the domain orientation is completely different. In both monomers of inactive CooA, the F helices are buried from solvent and should be inactive for DNA binding. In both CRP and CooA, the F helices are associated in a coiled-coil, and each is in contact with both effector domains. The heme groups of inactive Fe(II) CooA are ligated on one side by His-77 and on the other by the Pro-2 N terminus of the opposite chain. The Pro-2 ligands are displaced when CO binds (9). These interconnections between the two chains no doubt account for the cooperativity observed in CO binding (10).

The question of how CO binding to the CooA hemes can induce reorientation of the DNA binding domains has been addressed via mutagenesis and spectroscopic studies (10–15). From resonance Raman (RR) evidence on altered proteins, we have proposed (10) that the displacement of Pro-2 by CO induces displacement of the hemes into adjacent hydrophobic cavities, accompanied by readjustment of the C helices. The

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4 The abbreviations used are: CRP, cAMP receptor protein; RR, resonance Raman; WT, wild-type; MOPS, 4-morpholinepropanesulfonic acid; Mb, myoglobin; 6-c, 6-coordinate.
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importance of C-helix repositioning has also been shown by a substantial amount of functional analysis of variants altered in this region (16). We now elaborate our model with further evidence including RR studies of variants that are constitutively active (active in the absence of CO) as well as other variants with low activity even in the presence of CO. In addition we suggest a mechanism for the initiation of C-helix bending required for reorientation of the DNA domains through protein tension generated by the heme displacement. Finally we present evidence that CO-bound wild-type (WT) CooA is not entirely in the “on” conformation but is in an intermediate conformation or else in an equilibrating mixture of on and off populations. The latter alternative is supported by previous kinetic measurements (17), indicating that CO-bound CooA is a mixture of forms that are “closed” and “open” with respect to CO dissociation and has recently been discussed more fully in Roberts et al. (3).

MATERIALS AND METHODS

Strains, Plasmids, and in Vivo Assays—The construction of strains overexpressing WT CooA and CooA variants in an *Escherichia coli* background having a β-galactosidase reporter system in the chromosome was described previously (8). All the site-directed and region-randomized *cooA* mutations were constructed in a pEXT20-based expression plasmid, which provides tight control of *cooA* expression (11). The selection for and properties of WT CooA and CooA variants were investigated; (a) those in which changes to the C-helix, and (b) those with changes at position Leu-120, an invariant residue in CooA homologues (21), (c) those in which changes to the C-helix position 121–126 CooA variants (27) and ΔP3R4 CooA (14, 20) have been described.

CooA Purification—The purification of WT CooA and the variants was performed with our standard method as described previously (12) or by modified protocols (13, 14). The purity of WT CooA and CooA variants was estimated to be >95% based on SDS-PAGE. The heme content of CooA preparations was estimated using the extinction coefficient of WT Fe(II)-CO CooA (220 mM−1 cm−1 (31)), and protein concentration was measured using the BCA assay (Pierce).

*In vitro* DNA binding assays of WT CooA and CooA variants were performed using the conditions described elsewhere.5 As a fluorescence probe, a 26-base pair target DNA containing PcooF was labeled with Texas Red on one end of the duplex and used at the concentration of 6.4 nM. Salmon sperm DNA was used at a 1000-fold excess as the nonspecific DNA competitor. Dissociation constants (*Kd*) were calculated by fitting the binding data to a nonlinear equation with correction of the fluorescence quenching as described elsewhere (15).

Sample Preparation—Purified CooA was diluted in buffer (25 mM MOPS/0.1 mM NaCl, pH 7.4) to a heme concentration of ~20 μM. For AYLLRL and RYLLRL variants, whose solubility and degree of Pro-2 ligation is salt-dependent, a 25 mM MOPS, 0.5 mM NaCl, 50 mM CaCl₂, pH 7.4, buffer was used. CooA samples were purged with N₂ for 20 min then with CO for ~5 min followed by reduction of the Fe(III) to the Fe(II) form with sodium dithionite (final concentration, ~20–60 mM). Reduction and CO binding were monitored by changes in the absorption spectra.

The Fe(II) CooA samples were prepared by first purging with N₂ for 20 min followed by the reduction with sodium dithionite. To increase the 5-coordinate (5-c) fraction, a buffer with higher salt concentration (25 mM MOPS, 0.5 mM NaCl, 50 mM CaCl₂, pH 7.4) was used.

DNA-bound Fe(II)-CO CooA was prepared according to the published procedures (19, 20) with the following modifications. The double-stranded DNA sample (provided by Prof. Thomas Poulos, University of California, Irvine), 5'-GCATAACTGCT-ACCTGGCCGACAGCATGG-3' and 3'-GTATTTGACA-GTAGACCGCTGTCTGTGTACCCG-5', was heated at 80°C for 5 min in a water bath and then cooled at room temperature for ~2 h. Then the annealed DNA (final concentration 15 μM) was mixed with a sample of either WT or RYLLRL CooA (final concentration 15 μM) in 40 mM Tris, 50 mM KCl, 6 mM CaCl₂, pH 8.0, buffer and incubated at room temperature for ~30 min. The Fe(II)-CO sample was then prepared as described above.

RR Spectroscopy—RR spectra were obtained with excitation wavelengths of 406.7 and 568.1 nm from a Kr⁺ laser (Spectra Physics, 2080-RS) and 441.6 nm from a He-Cd (Liconix) laser in a 270° backscattering sample geometry. Photodissociation of the bound CO was minimized by using low laser power (~1 milliwatt at the sample) and by spinning the sample. The scattered light was collected and focused onto a triple spectrograph (Chromex) equipped with a CCD detector (Roper Scientific) operating at 77 K. Spectra were calibrated with dimethyl formamide, ethyl acetate, and dimethyl sulfoxide-d₆.

RESULTS

In Vitro DNA Binding Analysis Identifies Variants Affected in the Heme Vicinity with a Range of Activities—To explore the connection between ligand binding and protein conformation change, a set of CooA variants was chosen with substitutions predicted to be near the heme. Three classes of variants were investigated; (a) those with changes at position Phe-74, which forms part of the interior heme pocket on the proximal side (His-77, Fig. 1) and is conserved (Phe or Tyr) in CooA homologues (21), (b) those with changes at position Leu-120, an invariant residue in CooA homologues (21), which forms part of the heme pocket on the distal side and simultaneously serves as an “a” position coiled-coil residue in the C-helix, and (c) those in which changes to the C-helix position 121–126 segment (Fig. 1) have significantly stabilized the DNA-binding conformation of the protein.5

These variants were produced by the mutagenesis scheme described below and selected on the basis of *in vivo* screens in which CooA activity is linked to the expression of β-galactosidase.5 The *in vivo* activity depends upon the variant accumulation levels, specific DNA binding affinity, and ability to interact with RNA polymerase. Consequently the biochemical properties of the identified variants were examined further through *in vitro* DNA binding.

Randomization of the codon for residue 74 followed by screening for variants with CO-dependent activity in *vivo* yielded two distinct classes with activity above background.

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5 R. L. Kerby, N. D. Lanz, H. Youn, and G. P. Roberts, submitted for publication.
Highly active variants possessed either Phe or Tyr residues, whereas variants with lower activity contained Leu, Gly, or His residues. At position 120, similar mutagenesis and screening showed that only Leu afforded CO-dependent activity similar to WT CooA; L120F and L120S, which were chosen for further study, were poorly activated by CO. The selection and analysis of the CO-independent, constitutively active variants possessing changes in the C-helix 121–126 region is elaborated elsewhere. These changes produce CooA that is active even without added CO.

In vitro DNA binding affinity (Table 1) was measured with a fluorescence polarization assay, which monitors binding of CooA to a Texas Red-labeled target DNA (20). The 121–126 C-helix variants precipitate from solution at high protein concentrations but were stabilized at higher salt concentrations. This property and their exceptional DNA affinity made analysis of CO-inactive variants possessing changes in the C-helix 121–126 region impossible at lower salt levels (20). In terms of DNA binding, F74Y and F74L CooAs showed CO-dependent affinities similar to that of WT CooA (Table 1), suggesting that the poor in vivo activity of F74L CooA represents a defect in its interaction with RNA polymerase. In contrast, changing Leu-120 to Phe or Ser diminished activity in vivo and in vitro, with a >10-fold decrease in DNA affinity relative to CO-bound WT CooA (Table 1). The activities of the 121–126 C-helix variants were unusual in two respects: (a) these variants possessed DNA binding affinities in vitro in the absence of CO that were similar to those of CO-bound WT CooA and (b) in the presence of CO their DNA binding affinity exceeded that of WT CooA. Two rather similar variants in this region were studied, with the following two sequences at positions 121–126: AYLLRL and RYLLRL. In the following text, we will refer to them by these names or by (A/R)YLLRL for the pair.

**TABLE 1**

| CooA          | Fe(II) $K_d$ (nM) | Fe(II)-CO $K_d$ (nM) |
|--------------|------------------|----------------------|
| WT           | >5,000           | 212 (13)             |
| F74Y         | >5,000           | 152 (1)              |
| F74L         | >5,000           | 383 (136)            |
| L120F        | >5,000           | ~2900 (estimated)    |
| L120S        | >5,000           | ~3200 (estimated)    |

*Average of independent assays with 95% confidence interval, shown in parentheses. All assays were performed in high salt fluorescence polarization buffer that included 250 mM KCl and 20 mM CaCl$_2$. 

When there are changes in back-bonding due electrostatic influences in the vicinity of the bound CO, then $v$CO and $v$FeC are anticorrelated; as one increases, the other decreases as a result of changes in back-bonding (22). This behavior is illustrated in Fig. 3 (solid line) for a series of myoglobin (Mb) variants having substitutions among residues distal to the bound CO. However, increasing or decreasing the donor strength of the proximal ligand, trans to the CO, produces negative or positive deviations from the back-bonding correlation (22).

The $v$CO/$v$FeC point for WT CooA falls significantly above the Mb line, implying, as noted earlier (10), a significant weakening of the bond to the proximal imidazole ring relative to Mb. This was attributed to displacement of the heme as part of the activation mechanism of CooA induced by CO binding. We now observe that the data for the variants in the present study fall on a horizontal line that intersects the Mb line at about the position occupied by Mb variants in which the distal histidine is replaced by apolar residues. This is exactly the behavior expected if the CO pocket is apolar in CooA (as inferred earlier), but the Fe-His bond is weakened to different extents depending on the variant. The higher $v$CO reflects diminished...
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back-donation because of diminished forward donation by the His ligand; however, the expected inverse effect on $\nu$FeC is compensated by diminished competition between the Fe-His and Fe-C bonds (22). Density functional theory (DFT) calculations by Franzen (23) find $\nu$CO lowering but little change in $\nu$FeC when the bond to a proximal imidazole is strengthened by donating a H-bond, as does the proximal ligand in Mb (see the lower inset in Fig. 3). We infer that the CooA Fe-His is weakened to various extents, reflecting variable displacement of the heme group.

Significantly, $\nu$CO is increased when CO-bound WT CooA binds its target DNA sequence, implying a further weakening of the Fe-His bond (Fig. 2). This same higher frequency is seen for CO adducts of the constitutively active (A/R)YLLRL variants, in the absence of DNA, and no further shift is seen when DNA is bound. In the presence of CO, DNA binding affinity is about three times higher for (A/R)YLLRL than for WT CooA (Table 1). We interpret these results to mean that the position 121–126 substitutions have fully stabilized the on conformation of CooA-CO, whereas CO-bound WT CooA is in an equilibrium between on and off forms or else it is in an intermediate form, which is turned on by DNA binding. Although an equilibrium between two forms should produce a superposition of two spectral responses, the spectral resolution is insufficient to distinguish a superposition from an intermediate response; the difference in the central position of the $\nu$CO band is less than its width (Fig. 2). An equilibrium mixture of on and off populations would be consistent with kinetic evidence for "open" and "closed" forms of CooA coexisting in the CO-bound WT protein (17).

On the other hand the L120S and L120F variants, which display low CO-dependent activity, have significantly stronger Fe-His bonds, as judged by their $\nu$CO/$\nu$FeC points being closer to the Mb line than is the WT protein. Thus, the low activity of these mutants can be attributed to destabilization of the on conformation, with an attendant diminution in the average heme displacement. The L120S variant displays a small but noticeable (3 cm$^{-1}$) downshift in $\nu$FeC relative to the L120F variant and a corresponding upshift (4 cm$^{-1}$) in $\nu$CO. These opposite shifts are a signal of decreased back-donation, associated with negative polarity among distal groups near the bound CO. This effect implies that the Leu-120 side chain is close to the CO and that replacement with Ser introduces negative polarity via the OH lone pair, similar to the H64V/V68T variant of Mb. Yamashita et al. (15) reached a similar conclusion by examining the L120N variant, although in that case there was a 6-cm$^{-1}$ upshift in $\nu$FeC, presumably reflecting positive polarity from the asparagine NH$_2$ group (15).

Aono and co-workers (19) reported that adding DNA to CooA induced an additional $\nu$FeC RR band at 519 cm$^{-1}$ and a narrowing of the main $\nu$FeC band at 487 cm$^{-1}$. We do not observe these effects (Fig. 2) and note that their experiments were carried out without added divalent cations, which have subsequently been shown to be essential for DNA binding (24). Also, the CooA preparation studied by Aono and co-workers (19) exhibited the $\nu$CO band at 1969 cm$^{-1}$, much lower than the 1982 cm$^{-1}$ we observe but close to a subsidiary $\nu$CO band seen in our spectra at higher amplification and assigned earlier to a fraction of inactive molecules with undisplaced heme (10).

The preparation of Aono and co-workers did contain a fraction with $\nu$CO = 1979 cm$^{-1}$, the position found in picosecond Fourier transform IR experiments for CO-bound CooA molecules undergoing photolysis (25). This can be taken to represent the active fraction of molecules.
Constitutively Active Variants without CO Show Weakened Fe-His Bonds—To assess the Fe-His bond strength in the absence of bound CO, we determined the frequency of the Fe-His stretching vibration. This mode can only be detected in RR spectra of 5-c hemes, with no sixth ligand. It is not enhanced for 6-c hemes (26). WT CooA has a sixth ligand, Pro-2, but there are variants with detectable populations of 5-c heme because of perturbations to the Fe-Pro-2 bond. One such variant is G117I, in which the bulky Ile side chain sterically hinders the Pro-2 ligand (27). By tuning the Raman laser to 442 nm, the wavelength of the 5-c heme Soret absorption (seen as a shoulder on the dominant 420-nm band in Fig. 4), one can selectively enhance the Fe-Pro-2 bond RR spectrum, which reveals a strong 218 cm\(^{-1}\) frequency of deoxyMb (28). Likewise, the variant \(\Delta P^3 R^4\), in which the two penultimate residues are deleted from the N-terminus, has a small 5-c fraction because the Fe-Pro-2 bond is weakened (10). Its 442-nm RR spectrum also reveals a 218 cm\(^{-1}\) frequency of Fe-His band (Fig 4). Thus, variants with 5-c populations resulting from distal perturbations of the Fe-Pro-2 bond have Fe-His bonds that are comparable with that of deoxyMb.

The (A/R)YLLRL variants also have significant 5-c populations, as revealed by prominent 440-nm absorption band shoulders (Fig. 4). Their 440-nm RR spectra reveal Fe-His bands at significantly downshifted frequencies, 214 cm\(^{-1}\). Inasmuch as these variants bind DNA in the absence of CO, we infer that the downshifted frequency is an indication of the protein being substantially in the on conformation. The downshift is an indication of a weakened Fe-His bond in this conformation. This inference is consistent with the detection by Uchida et al. (29) of a downshifted Fe-His band in the picosecond RR spectrum of the prompt photoproduct of WT CooA-CO. On this time scale the 5-c heme generated by photolysis has not yet relaxed to the structure it would have in the off conformation of WT CooA. The reported frequency, 216 cm\(^{-1}\) (30), was slightly higher than that of the (A/R)YLLRL variants, consistent with the view that the latter are shifted further toward the on conformation than is the WT CooA CO adduct.

The weakened Fe-His bond is, thus, attributed to heme displacement in the on conformation. The very existence of a 5-c fraction in the (A/R)YLLRL variants can also be attributed to heme displacement, which would strain both the Fe-His-77 and the Fe-Pro-2 bond, occasioning disruption of the latter.

Fe-His Bond Weakening and H-bonding—The Fe-imidazole bond strength in heme adducts is known to be modulated by H-bond donation from the N-H proton on the bound imidazole to nearby H-bond acceptors (22). The H-bond increases the negative charge on the imidazole ligand, increasing its interaction with the iron. DeoxyMb has an intermediate rFe-His frequency, 220 cm\(^{-1}\), consistent with an H-bond of moderate strength to the neutral OH group of a Ser residue and to a backbone carbonyl (see the lower inset in Fig. 3). An H-bond of similarly moderate strength is implied by the comparable frequency, 218 cm\(^{-1}\), observed for the 5-c population of the G117I variant of CooA (Fig. 4).

The crystal structure of inactive Fe(II) CooA reveals that the most obvious candidate for accepting an H-bond from His-77 is the amide side-chain carbonyl of Asn-42 (5) (Fig. 5); the N...O distance is short, 2.7 Å, consistent with a significant H-bond. To test the role of this H-bond, we replaced Asn-42 in the G117I variant with alanine and aspartate residues. Alanine eliminates the possibility of H-bonding, whereas aspartate might make the H-bond stronger by analogy with the peroxidases (28, 31). To our surprise, the Fe-His band remained at 218 cm\(^{-1}\) (Fig. 4). We speculate that both replacements induced a water molecule to enter the proximal region of the heme and H-bond with His-77, replacing the Asn-42 H-bond with one of about the same strength. We considered testing this hypothesis with D,0 exchange, but there is an exchange on the imidazole, increasing its effective mass slightly and causing a small shift whether an H-bond to H2O existed. Alternatively, the H-bond acceptor might be the Cys-75 residue, which is known to become the proximal ligand in the oxidized form (32, 33); a Cys...His interaction has been proposed in the heme sensor protein neuronal PAS protein 2 (34). However, such an interaction in CooA would require a significant conformation change between crystal and solution.

An alternative explanation for Fe-His bond weakening implied by the 214 cm\(^{-1}\) frequency is simple mechanical tension. Displacement of the heme would strain the Fe-His bond, because His-77 is anchored on a β-strand in the effector binding domain (Fig. 5). Hemoglobin provides a precedent, since T-state displacement of the F-helix, which holds the proximal histidine, induces Fe-His weakening in the α chains in hemoglobin tetramers (28). The energetics have been evaluated computationally by Marti et al. (35), who found an anomalously weak Fe-His bond in the deoxyhemoglobin α subunit resulting from protein forces, although H-bonding also plays a role. In general, effects of tension and H-bonding will be entangled.

FIGURE 4. RR spectra excited at 441.6 nm (left) in resonance with the 440-nm Soret absorption shoulder (right) from the 5-coordinate populations of the indicated CooA variants in their Fe(II) states. The Fe-His stretching band shifts from 218 cm\(^{-1}\) for the inactive variants (top) to 214 cm\(^{-1}\) for the constitutively active variants (bottom) in their Fe(II) states (a spectrum of Fe(II) WT CooA lacks these signals because it has no detectable 5-coordinate species).
since any motion of the histidine ligand in response to strain will also affect its H-bonding to nearby acceptors.

Negligible Heme Electronic Interaction with Phe-74 — The Phe-74 side chain is in close proximity to the heme (see Fig. 5), and the possibility existed of even closer contact in the CO adduct as a result of heme displacement. This might have induced an electronic interaction between the heme and the phenyl group. We tested for such an effect by measuring the position of the porphyrin \( v_{11} \) band for Phe-74 variants (Fig. 6). \( v_{11} \) is a non-totally symmetric mode of the heme and is selectively enhanced with 568-nm excitation, in resonance with the Q electronic transitions. The position of \( v_{11} \) is sensitive to electron donation effects at the heme iron. In WT CooA, its frequency is 1532 cm\(^{-1}\) but is shifted up to 1536 cm\(^{-1}\) in the \( \Delta P3R4 \) variant as a result of weakening of the strong donor interaction of the N-terminal proline with the heme (10). In CooA-CO the frequency shifts up further, to 1546 cm\(^{-1}\) (Fig. 6), as a result of electron withdrawal from the heme to the CO via back donation. When the CO adducts were compared for F74Y and F74L variants with WT CooA (Fig. 6), the frequency did not vary by more than 1 cm\(^{-1}\). The \( \nu_{FeC} \) and \( \nu_{CO} \) positions were likewise unaffected by the Phe-74 substitutions (data not shown), indicating no influence on the Fe-His bond weakening. We conclude that there is no evidence for a significant heme-phenyl electronic interaction.

**DISCUSSION**

*CooA Activation Is Initiated by Heme Displacement* — This study addresses the mechanism whereby the binding of CO to the heme group of CooA induces the protein to bind its target DNA sequence. The crystal structure of the inactive CO-free form reveals that the DNA binding domains of the two monomers are different from each other, but both are strikingly rearranged when compared with the structure of the active form of the homologous CRP protein (Fig. 1). It is less obvious, however, how representative the crystal structures are to the set of the protein structures in solution. Modeling of small angle x-ray scattering data led Akiyama et al. (36) to the conclusion that there is only a small change in the relative orientation of the effector and DNA binding domains when CO binds CooA in solution. However, this analysis did not take into account the possibility that CooA-CO is a mixture of conformations, as discussed below. In view of the flexibility of the interdomain hinge and the evidence from the CooA structure that crystal forces may affect the domain orientation, it seems quite possible that still other orientations are accessible in solution.

How is the transition from off to on induced by CO binding to the heme? In our earlier study we proposed that the primary impetus for the repositioning of the DNA binding domains was displacement of the heme toward an adjacent cavity that can be identified in the CooA crystal structure (Fig. 5) together with a complementary displacement of the C helices (10). The cavity is hydrophobic, and hydrophobic forces would favor the heme displacement toward it once the anchoring sixth ligand, the proline N terminus of the opposite chain (Fig. 1), is displaced by CO and expelled from the heme pocket (10), thus exposing the heme to solvent. The C-helix would then readjust to accommo-
**TABLE 2**

Residues lining the surfaces of CooA cavities adjacent to the hemes (Fig. 5)

| CooA       | Heme A          | Heme B          |
|------------|-----------------|-----------------|
| WT         | Val-54 (A)      | Val-54 (B)      |
|            | Leu-65 (A)      | Leu-65 (B)      |
|            | Phe-66 (A)      | Phe-66 (B)      |
|            | Phe-74 (A)      | Phe-74 (B)      |
|            | Ser-78 (A)      | Ser-78 (B)      |
|            | Cys-80 (A)      | Cys-80 (B)      |
|            | Ala-119 (A)     | Ala-119 (B)     |
|            | Ser-122 (A)     |                 |
|            | Cys-123 (A)     | Cys-123 (B)     |
|            | Leu-120 (B)     | Leu-120 (A)     |
|            | Met-124 (B)     | Met-124 (A)     |
| AYLLRL     | Val-54 (A)      | Val-54 (B)      |
|            | Leu-65 (A)      | Leu-65 (B)      |
|            | Phe-66 (A)      | Phe-66 (B)      |
|            | Phe-74 (A)      | Phe-74 (B)      |
|            | Ser-78 (A)      | Ser-78 (B)      |
|            | Cys-80 (A)      | Cys-80 (B)      |
|            | Ala-119 (A)     | Ala-119 (B)     |
|            | Ser-122 (A)     |                 |
|            | Cys-123 (A)     | Cys-123 (B)     |
|            | Leu-120 (B)     | Leu-120 (A)     |
|            | Leu-124 (B)     | Leu-124 (A)     |

In the (A/R)YLLRL variants both are replaced by leucine residues, thereby moderately increasing the hydrophobicity of the cavity. Also, the computed cavity size (assuming no change in backbone structure or side-chain orientation) increases in the (A/R)YLLRL variants, expanding from 74 to 104 Å for the cavity next to the B-chain heme and from 59 to 83 Å for the cavity next to the A-chain heme. The combination of increased cavity size and hydrophobicity is consistent with the enhanced heme displacement propensity of the (A/R)YLLRL variants, even in the absence of CO binding.

The computed cavity also suggests why heme displacement is restricted (small rFeC/rCO deviations, Fig. 3) in the L120S/L120F variants. The Leu-120 side chain forms a significant part of the hydrophobic cavity surfaces, consistent with the RR evidence for a polar influence on the bound CO in L120S (Fig. 2) and also in L120N (15). Introduction of a polar group in L120S reduces the hydrophobicity of the cavities and diminishes the driving force for heme displacement. On the other hand, phenylalanine substitution is expected to occlude the cavities, thereby impeding heme displacement sterically.

In addition to the cavity effects, it is likely that altered contacts within the C-helix coiled-coil contribute to stability of the off and on conformations. The two Cys-123 side chains are in contact at the critical “d” position of the heptad repeat; replacement of the Cys by Leu produces an optimal “leucine zipper” contact (16). On the other hand, the Leu-120 side chains are already in contact at the heptad “a” position, and substitution of Ser or Phe would interfere with this contact.

A caveat to this analysis is that the contacts and cavities computed from the CooA crystal structure cannot accurately represent the coil and heme contacts in the on state because of the modest repositioning of the C-helices, which accompanies the heme displacement. For example the residues Ile-113, Leu-116, and Gly-117 do not appear on the surface or side-chain orientation increase in the (A/R)YLLRL variants, expanding from 74 to 104 Å for the cavity next to the B-chain heme and from 59 to 83 Å for the cavity next to the A-chain heme.

The computed cavity also suggests why heme displacement is restricted (small rFeC/rCO deviations, Fig. 3) in the L120S/L120F variants. The Leu-120 side chain forms a significant part of the hydrophobic cavity surfaces, consistent with the RR evidence for a polar influence on the bound CO in L120S (Fig. 2) and also in L120N (15). Introduction of a polar group in L120S reduces the hydrophobicity of the cavities and diminishes the driving force for heme displacement. On the other hand, phenylalanine substitution is expected to occlude the cavities, thereby impeding heme displacement sterically.

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**Heme Displacement Mechanism of CooA Activation**

In the (A/R)YLLRL variants both are replaced by leucine residues, thereby moderately increasing the hydrophobicity of the cavity. Also, the computed cavity size (assuming no change in backbone structure or side-chain orientation) increases in the (A/R)YLLRL variants, expanding from 74 to 104 Å for the cavity next to the B-chain heme and from 59 to 83 Å for the cavity next to the A-chain heme. The combination of increased cavity size and hydrophobicity is consistent with the enhanced heme displacement propensity of the (A/R)YLLRL variants, even in the absence of CO binding.

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**Heme Displacement and C-helix Bending**—How is heme displacement connected to the C-helix bending that is required for reorientation of the DNA-binding domains? Again, the crystal structure points to a plausible connection (Fig. 5). The proximal ligand, His-77, is on a strand that is linked to the base of the β4/β5 hairpin loop. Heme displacement would apply tension to this hairpin, tension that is reflected in the observed weakening of the Fe-His-77 bond. The tip of the hairpin contacts the hinge region of the C-helix. Thus, there is a mechanical link between the heme and the point at which the C-helix bends.

We have no evidence on how this mechanical link may operate but note that Lanzilotta et al. (5) identified two switch residues in the hinge region, Phe-132 and Arg-138, that form alternative and complementary contacts in the B-chain of CooA, on the one hand, and in CRP, on the other. In the CooA B-chain, Phe-132 contacts the DNA binding domain, whereas Arg-138 forms a salt bridge with Glu-59 in the β4/β5 hairpin loop of the A-chain regulatory domain. However, in CRP the homologous
Heme Displacement Mechanism of CooA Activation

Phe-136 contacts the β4/β5 hairpin loop, whereas the homologous Arg-142 contacts a phosphate in the DNA backbone. In addition to the Arg-138—Glu-59 salt-bridge, CooA has a salt-bridge between Arg-118 and Asp-72 connecting the C-helix with the proximal β-strand (Fig. 5). A homologous pair exists in CRP, Arg-122 and Asp-68, but the salt-bridge is absent in the CRP structure, suggesting that activation breaks this salt-bridge as well. Asp-72 is only five residues from His-77, and it is reasonable that maintenance of the salt-bridge would be incompatible with heme displacement. By inducing heme displacement and β-strand tension, CO binding might break both salt-bridges.

The β-strand tension model can also explain why binding NO, unlike CO, does not activate DNA binding by CooA (37). Upon heme displacement the NO trans effect induces the Fe-His-77 to break, thereby relieving the tension on the β-strand. Intriguingly, Clark et al. (18) have discovered that NO does activate DNA binding in a CooA homolog from the thermophile Carboxydothermus hydrogenoformans and that the NO adduct is 6-coordinate, indicating that the Fe-His-77 bond is not broken. However, raising the temperature induced bond rupture and produced a 5-coordinate NO-heme (the DNA binding activity could not be determined at elevated temperature but would presumably be diminished). Apparently, Fe-His-77 bond breaking is prevented by the rigidity of the thermophile protein despite heme displacement but is induced at high temperature where the protein is more flexible. Clark et al. (18) found evidence in the absorption spectrum of R. rubrum CooA that lowering the temperature to 4 °C induced 6-coordination in a fraction of the NO adducts, supporting the idea that Fe-His-77 bond breaking is dependent on protein mobility. Kerby et al.5 have found that the (A/R)YLLRL variant also forms a 6-coordinate NO adduct. This finding suggests that, in addition to inducing heme displacement, these substitutions diminish protein flexibility in the proximal region of the heme, so that the Fe-His-77 bond does not break when NO binds.

CO Binding Produces Comparable On and Off Conformational Energies—The on and off conformations have similar energies in the CO adduct of CooA. Evidence for this is that the heme is fully displaced in the WT CO adduct only when DNA is added. The vFeC/vCO deviation from the Mb line (Fig. 3) is maximal for the (A/R)YLLRL variants with or without DNA and for the WT protein with DNA, but without DNA the WT deviation is at an intermediate value. We infer that the WT CO adduct has roughly comparable populations of on and off populations with displaced and undisplaced heme. DNA binding pulls the equilibrium toward the on conformation. Alternatively, the WT CO adduct may be in an intermediate conformation, with partially displaced heme, perhaps like the A-chain of the CooA crystal structure. This intermediate conformation would be pulled into the on conformation by DNA binding. Consistent with both of these postulates is the observation of Kubo et al. (30) that the Trp-110 UV-RR response is augmented still further when CooA binds DNA.

The hypothesis of coexistence of on and off populations is consistent with our previous finding (17) of comparable populations of open and closed molecules having different rates of CO dissociation. The open rate was similar to that of MbCO, whereas the closed rate was 10-fold slower. It seems likely that the open molecules are in the off conformation and have undisplaced heme, whereas the closed molecules are in the on conformation and have displaced heme. The movement of the heme into its hydrophobic cavity (Fig. 5) could account for the marked slowing in the CO dissociation rate.

The DNA binding constants (Table 1) support these inferences. Without CO, WT CooA does not bind DNA; it is in the off conformation. Once CO binds, the affinity is substantial (Kd = 206 nM), but it is significantly higher for the CO adducts of the (A/R)YLLRL variants (Kd ~ 50 nM), consistent with their being in a substantially on conformation. In the absence of CO, these variants bind DNA with approximately the same affinity as does CO-bound WT CooA. We surmise that heme displacement is significant but incomplete in the absence of CO because of the restraining effect of the Pro-2 ligand, which remains bound to the heme in the main population of the variant molecules (Fig. 4). The CO adducts of L120S and L120F have very low DNA affinities, indicating a shift of the energetics toward an off conformation, consistent with the small average heme displacements of these variants.

Conclusions—CooA responds to its effector molecule, CO, by realigning its DNA binding domains into the correct orientation for recognizing its target DNA sequence. In our model it does this by displacing the endogenous Pro-2 ligand and inducing displacement of the heme into an adjacent hydrophobic cavity. In turn, this displacement induces bending of the C-helix at the hinge connecting the regulatory and DNA domains by altering contacts between the hinge and the β4/β5 hairpin loop of the regulatory domain (of the opposite subunit). The β4/β5 hairpin loop is connected to the heme via the Fe-His-77 bond; heme displacement applies tension to this bond, which is transmitted to the β4/β5 hairpin loop.

The conformational change induced in WT CooA by CO binding is incomplete and is drawn to completion by DNA binding. Either the energetics are comparable for the on and off conformations or else the conformation of the CO adduct is intermediate between them. The former alternative is consistent with biphasic CO dissociation rates, which indicate comparable populations of closed and open forms. The energetics are biased toward the on conformation by replacement of the 121–126 residue C-helix segment, TSCMRT, with the sequence (A/R)YLLRL, because the Cys-123 side chain also forms part of the hydrophobic cavity surface and are replaced by leucine residues, which are more hydrophobic and enlarge the cavity (and perhaps because they improve the C-helix coiled-coil contacts). The (A/R)YLLRL variants are active even without CO. On the other hand, the L120S/L120F variants bias the energy toward the off conformation, because the Leu-120 side chain also forms part of the cavity surface and is replaced by a hydrophilic (Ser) or bulky (Phe) residue (and perhaps because these substitutions weaken the coiled-coiled contacts).

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