Activation Mechanism of the CO Sensor CooA

MUTATIONAL AND RESONANCE RAMAN SPECTROSCOPIC STUDIES

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CooA is a CO-dependent heme protein transcription factor of the bacterium Rhodospirillum rubrum. CO binding to its heme causes CooA to bind DNA and activate expression of genes for CO metabolism. To understand the nature of CO activation, several CooA mutational variants have been studied by resonance Raman spectroscopy, in vitro activity measurements, and DNA binding assays. Analysis of the Fe–C and C–O stretching Raman spectroscopy bands permits the conclusion that when CO displaces the Pro2 heme ligand, the protein forms a hydrophobic pocket in which the C-helix residues Gly117, Leu118, and Ile113 are close to the bound CO. The displaced Pro2 terminus is expelled from this pocket, unless the pH is raised above the pKₐ, in which case the terminus remains in H-bond contact. The pKₐ for this transition is 8.6, two units below that of aqueous proline, reflecting the hydrophobic nature of the pocket. The proximal Fe–His bond in Fe(II)CooA is as strong as it is in myoglobin but is weakened by CO binding, an effect attributable to loss of an H-bond from the proximal His77 ligand to the adjacent Asn42 side chain. A structural model is proposed for the position of the CO-bound heme in the active form of CooA, which has implications for the mechanism of CO activation.

CooA is a member of the emerging class of heme sensor proteins that modulate biological activity in response to fluctuations in the concentration of the gaseous molecules CO, NO, or O₂ (1, 2). Binding of these diatomic ligands to the heme induces the biological response. The mechanisms whereby the protein responds to the binding event are of great current interest.

CooA is isolated from the bacterium Rhodospirillum rubrum, which grows anaerobically on CO as sole energy source (3). CooA regulates the expression of genes, termed coo, for CO oxidation, whose products are associated with CO metabolism. The protein is a homodimer (222 amino acids per monomer) containing two 6-type hemes that reversibly bind CO. The CO binding enables the protein to bind a specific DNA sequence and induce transcription of the coo genes. CooA contains DNA-binding and signal domains analogous to those found in the catabolite gene activator protein CAP (or CRP) (1, 2) (Fig. 1). The heme is bound within the signal domain, where it is ligated by a histidine (His77) side chain (although this ligand is replaced by the Cys75 side chain when the Fe(II) is oxidized to Fe(III) (4)). The sixth Fe(II) ligand is unprecedented; it is the N-terminal proline residue (Pro2) from the opposite chain, which is displaced by CO binding (5).

The structure of CO-bound CooA is unknown, but on the basis of the CAP structure (6) it is reasonable to assume that the DNA-binding domains are brought into proper position for DNA binding by movement of the C-helix, which runs the length of the protein and passes close to the heme (1, 2, 7). It has been shown that a repositioning of the C helices at the dimer interface is a major communication pathway between the hemes and the DNA-binding domains (8), but the basis for this repositioning upon CO binding remains unclear. To elucidate the nature of the CO-bound form of CooA and the mechanism of its CO activation, we have prepared a series of CooA variants by site-directed mutagenesis and examined in vitro activity, specific DNA binding, and the Fe–CO and C–O stretching resonance Raman (RR) bands. The residues chosen for mutagenesis are in the region of the heme groups, as defined by the Fe(II)CooA structure. Fig. 2 gives a close up view of this region.

The data permit us to identify the approximate position of the heme-COO in CooA and propose a specific model for how that positioning affects CooA activation and leads to the C-helix repositioning. An important aspect of the active form involves the breakage of a critical interaction that stabilizes the inactive structure: an H-bond from the His77 proximal ligand to the side chain of Asn42.

These structural inferences also provide a basis for understanding the cooperative binding and complex kinetics of CooA. Finally, we propose a structural model for the heme movement that is consistent with the data and will serve as a working hypothesis for future work.

EXPERIMENTAL PROCEDURES

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), and in vitro activities were quantified.

1 The abbreviations used are: CAP, catabolite gene activator protein; RR, resonance Raman; Mb, myoglobin; WT, wild-type; Mops, 4-morpholinepropanesulfonic acid.

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tated using the standard protocol (10). 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).

CooA Purification—The purification of WT CooA and most CooA variants was performed with our standard method as described previously (12). Some CooA variants such as C75S, L116K, and M124R CooA were purified with modified methods, which are also described elsewhere (12–14). The purity of WT CooA and CooA variants was estimated to be >95% based on SDS-PAGE (in the case of L116K CooA, ~90%). The heme content of CooA preparations was estimated using the extinction coefficient of Fe[III] WT CooA (3) or by a modified reduced pyridine-hemochromogen method (3), and protein concentration was measured using the BCA assay (Pierce).

In Vitro DNA Binding Assays—In vitro DNA binding assays of WT CooA and CooA variants were performed using the method described elsewhere (11). As a fluorescence probe, a 26-bp target DNA containing the concentration of 6.4 nM. Salmon sperm DNA was used as the nonspecific DNA competitor. Dissociation constants ($K_D$) were calculated by fitting of the binding data to a nonlinear equation with correction to the lower of the two values we observe, but their CooA–CO structure is definitively.

Structurally Distinct Populations of CO-bound CooA—We have previously reported RR spectra of CooA–CO showing a Fe–CO stretching band ($\nu_{FeC}$) at 487 cm$^{-1}$ and an unusually high CO stretching band ($\nu_{CO}$) at 1982 cm$^{-1}$ (16). In contrast, Uchida et al. (17) reported $\nu_{CO}$ = 1969 cm$^{-1}$ for their CooA–CO preparation although they reported the same $\nu_{FeC}$ value as ours, 487 cm$^{-1}$. To resolve this discrepancy we reexamined the $\nu_{CO}$ and $\nu_{FeC}$ regions of the RR spectrum at high amplification (Fig. 3), using $^{13}$CO shifts to identify the bands definitively. $\nu_{FeC}$ was measured at 487 cm$^{-1}$ as before, and we also reproduced the 1982 cm$^{-1}$ $\nu_{CO}$ band. However the newly measured $\nu_{CO}$ has a pronounced shoulder at 1962 cm$^{-1}$. Upon $^{13}$CO substitution, this 1962 cm$^{-1}$ band shifted appropriately, indicating a second $\nu_{CO}$ band.

The 487 cm$^{-1}$ $\nu_{FeC}$ band is a single band. Deconvolution from the 465 cm$^{-1}$ band of excess dithionite (Fig. 3) yields a bandwidth, 14.5 cm$^{-1}$, which is less than that of the CO adduct of myoglobin (data not shown), 18 cm$^{-1}$, ruling out multiple $\nu_{FeC}$ contributions in CooA–CO. Therefore, we conclude that the molecules having $\nu_{CO}$ = 1982 and 1962 cm$^{-1}$ both have the same $\nu_{FeC}$ = 487 cm$^{-1}$.

The $\nu_{CO}$ reported by Uchida et al. (17), 1969 cm$^{-1}$, is closer to the lower of the two values we observe, but their CooA–CO preparation also had a fraction with $\nu_{CO}$ = 1979 cm$^{-1}$; this is the frequency reported in picosecond FTIR experiments (18) for CooA–CO molecules undergoing photolysis. Thus, CooA–CO has two populations of molecules, having distinct $\nu_{CO}$ values (1962 and 1982 cm$^{-1}$ in our hands), but the same $\nu_{FeC}$ value, 487 cm$^{-1}$. The relative size of the populations appears to be preparation-dependent. In our preparation the 1962-cm$^{-1}$ fraction is small; the species is labeled CooA–CO$. We suggest that the species represented by 1982 cm$^{-1}$ is the active form and that CooA–CO$ is a form that has not rearranged to the active, DNA binding structure. There may be an equilibrium between active and inactive forms, or else a fraction of the molecules are stuck in the inactive configuration because of an energy barrier of some type.

Conformational Change of CooA upon CO Binding Weakens the Fe–His$^{77}$ Bond by Breaking the His$^{77}$–Asp$^{42}$ H-bond—The anomalous $\nu_{CO}$ of the major form of CooA–CO, 1982 cm$^{-1}$, is produced by a weakened Fe–His$^{77}$ bond. This can be seen in a
plot of ρFeC against ρCO (Fig. 4). Because of backbonding, ρFeC and ρCO are negatively correlated in heme proteins (19, 20), as illustrated for a set of Mb variants in Fig. 4 (solid line). Changes in the polarity of the binding pocket augment or diminish backbonding and shift the points along the line (19, 20). In addition, the correlation is displaced upward when the Fe-proximal ligand bond is weakened, because of diminished σ bonding competition between the proximal ligand and the CO (19, 21). The upper dashed line in Fig. 4 represents data for Fe(III) porphyrin CO adducts without any trans ligand (21).

When the CooA–CO values are plotted on the graph, the minority form, CooA–CO, is seen to fall near Mb variants with hydrophobic pockets, in which the distal histidine is replaced by hydrophobic residues (cluster of points marked −H64 in Fig. 4). However, the distinctly higher CO of the dominant form, CooA–CO, places it above the Mb line by 7 cm⁻¹, implying a weakened bond from the Fe to the proximal ligand, His₇⁷ in CooA (see Fig. 2).

### Table I

| CooA          | β-Galactosidase activity | CooA–CO Kᵣ for DNA binding | CooA–CO Raman band |
|---------------|--------------------------|-----------------------------|-------------------|
|               | Fe(III)³ | CooA–CO¹ | νₑₑ₅ (ΔWT)² | ν₉₉₃ (ΔWT)² |
| Control       | 0.5   | 1      |               |               |
| WT            | 2     | 100    |               |               |
| WT, pH 7      | ND    |        | 487 (0)       | 1982 (0)      |
| WT, pH 8      | 21    |        | 487 (0)       | 1982 (0)      |
| WT, pH 9.5    | 40    |        | 487 (0)       | 1979 (−3)     |
| WT, pH 10     | 85    |        | 487 (0)       | 1979 (−3)     |
| WT, pH 10.5   | 500   |        | 487 (0)       | 1979 (−3)     |
| WT, pH 11     | ND    |        | 487 (0)       | 1980 (−2)     |
| P₂Y           | 1     | 59     | 487 (0)       | 1982 (0)      |
| ΔP₅R₄         | 2     | 114    | 487 (0)       | 1982 (0)      |
| N₄₂A          | 1     | 10     | 487 (0)       | 1979 (−3)     |
| N₄₂D          | 1     | 83     | 487 (0)       | 1979 (−3)     |
| N₄₂K          | 1     | 59     | 487 (0)       | 1979 (−3)     |
| B₅₉₅          | 4     | 124    | 487 (0)       | 1979 (−3)     |
| E₉₉₉L         | 2     | 140    | 487 (0)       | 1979 (−3)     |
| W₁₁₆₀L        | 3     | 89     | 487 (0)       | 1979 (−3)     |
| I₁₃₅A         | 1     | 106    | 487 (0)       | 1979 (−3)     |
| I₁₃₅Y         | 3     | 111    | 487 (0)       | 1979 (−3)     |
| I₁₃₅H         | 6     | 81     | 487 (0)       | 1979 (−3)     |
| I₉₉₉D         | 1     | 4      | 487 (0)       | 1979 (−3)     |
| L₁₁₆₂F        | 1     | 78     | 487 (0)       | 1979 (−3)     |
| L₁₃₆₉K        | 99    | 73     | 487 (0)       | 1979 (−3)     |
| G₁₇₇I         | 4     | 4      | 487 (0)       | 1979 (−3)     |
| G₁₇₇S         | 3     | 11     | 487 (0)       | 1979 (−3)     |
| G₁₇₇H         | 4     | 4      | 487 (0)       | 1979 (−3)     |
| M₁₂₄₄R        | 13    | 116    | 487 (0)       | 1979 (−3)     |

¹ Anaerobically grown cells were used for the activity.
² Anaerobically grown cells in the presence of CO were used for the activity.
³ Shift in the Raman band center, relative to WT CooA–CO.
⁴ % activity, relative to WT CooA–CO; mean value of multiple measurements, <10% variability.
that form the binding pocket are part of a helix.

The inference of a weakened Fe–His$_{77}$ bond in CooA–CO is strongly supported by the picosecond RR study by Uchida et al. (23), which identified the Fe–His stretching band at 211 cm$^{-1}$ in the immediate photoproduct of CooA–CO. This band, a direct measure of the Fe–His bond strength, is enhanced in five coordinate Fe[III] hemes, and is found at 220 cm$^{-1}$ in deoxyMb (24). The distinctly lower value found by Uchida et al. (23) shows that the Fe–His$_{77}$ bond is weaker than in Mb for the immediate photoproduct, consistent with a weaker bond in the CO adduct itself. The minority form, CooA–CO’, which lies on the Mb backbonding line (Fig. 4), does not have a weakened Fe–His$_{77}$ bond and is expected to have an Mb-like photoproduc"
With regard to the minor CooA–CO form, we note that the low frequency shoulder on the N42A CO band is attenuated relative to the wild-type 1962 cm\(^{-1}\) shoulder. Curve resolution gives a higher frequency, 1966 cm\(^{-1}\) for the N42A shoulder, suggesting a weakened H-bond in the N42A CooA–CO structure.

Although Asn42 interacts with His77 in the Fe\(^{III}\) form and not the CO-bound form, the nature of the residue is of some importance to activation. N42A, N42D, and N42K CooA all displayed poorer DNA affinity in the presence of CO (and poorer β-galactosidase activity) than did WT CooA (Table I), suggesting some additional role for this residue in the active form. The basis for that effect is unknown. Note that the activity assays that measure DNA affinity are somewhat easier to interpret than the \textit{in vivo} assays reporting β-galactosidase activity, because the former only measures DNA binding, whereas the latter measures interaction with RNA polymerase and has other complications (8).

These results indicate that an element of the activation mechanism is the perturbation of the His–Fe bond. The data

**Fig. 5.** RR spectrum of G117I CooA variant in the Fe\(^{II}\) form, obtained with 442-nm excitation to enhance the five-coordinate high spin fraction, whose Soret band is at ~440 nm (7). The 1355-cm\(^{-1}\) \(v_s\) band position is diagnostic for five-coordinate high spin heme (23), and a strong Fe–His stretching band is seen at 220 cm\(^{-1}\). The spectrum closely resembles that of deoxyMb (21).

**Fig. 6.** \(v_{FeC}\) and \(v_{CO}\) RR bands, showing negligible shifts upon mutation of Asn\(^{42}\) and Cys\(^{75}\) residues, which abut the His\(^{77}\) ligand in the Fe\(^{II}\) structure (see also Fig. 4). Asterisks mark positions of the 465-cm\(^{-1}\) di-thionite band.
and arguments below strongly suggest that the His–Fe bond is perturbed by displacement of the heme upon CO binding, which is critical for CooA activation.

Hydrophobic C-helix Residues, but Not Pro2, Form the CO Pocket of CooA That Is Necessary for Activation—The distal heme pocket has an important role in ligand discrimination in many heme proteins (27). CooA activation is specific to CO, and an understanding of the CO binding pocket should be valuable in understanding both specificity and the activation in response to CO. We have already shown that hydrophobic residues at positions 113 and 116 are critical for normal CO response to CO. We have already shown that hydrophobic residues at positions 113 and 116 are critical for normal CO response to CO.

Pro2 is the ligand of Fe[II]CooA displaced by CO, so we tested the hypothesis that the displaced Pro2 helped form the CO pocket. As shown in Table I, neither replacement of the Pro2 terminus by tyrosine (P2Y CooA) nor deletion of the two penultimate residues (∆P3R4 CooA) showed noticeable effect on the νFeC or νCO band. This implies that Pro2 in WT CooA does not form the CO pocket and is consistent with the previous observation that Pro2 is not essential for activation of CooA by CO (11). We note, however, that the DNA binding affinities of these two Pro2 variants are 5–10-fold poorer than WT CooA, suggesting a minor role for the displaced Pro2 in the active form of CooA.

Properties of the Pro2 variants are further considered below. Because Pro2 is not near the bound CO, then the structure of Fe[II]CooA suggests that the most probable candidates for CO pocket residues are on the C-helix, with Ile113, Leu116, and Gly117 being particularly attractive (1, 28). The RR analysis of CooA variants altered at these positions establishes significant influences on νFeC and νCO consistent with a positioning of these residues close to the bound CO (see Fig. 7 and Table I). When these residues are replaced by H-bond donors, histidine and lysine νFeC shifts up, and νCO shifts down from the position in WT CooA, as expected for a positive polar interaction.3

The effect is particularly marked for the Gly117 position. G117H CooA displayed an 11-cm⁻¹ upshift of νFeC and an 18-cm⁻¹ downshift of νCO. These shifts are opposite in sign and comparable in magnitude to those observed when the distal histidine of Mb is replaced by hydrophobic residues (19, 20). G117S CooA also showed a sizeable, though more modest effect on the RR spectrum, shifting νFeC up by 7 cm⁻¹ and νCO down 12 cm⁻¹. These results indicate that the newly introduced His117 or Ser117 (albeit to a smaller extent) lie sufficiently close to the CO to exert a positive potential, via H-bond donation. However, the introduced His117 in G117H CooA is not well ordered and exerts a heterogeneous effect on the CO as evidenced by broad νCO and νFeC bands (Fig. 7). Consistent with the polaron interpretation, introduction of steric hindrance at 117 position (G117I CooA) had very little effect on νFeC and νCO (Table I), because the CO environment remains non-polar. The importance of Gly117 to CooA activation has already been described (7) and is reflected in the dramatically reduced activity of Gly117 variants seen in Table I. Because Gly117 is critical for activity, and its substitution perturbs the RR spectra, it would be tempting to conclude that its role is to contact the bound CO. Although this hypothesis cannot be discounted, it is also possible that Gly117 is an important determinant of the C-helix structure per se and simply happens to be near the bound CO.

At position 113, the effect of mutation on RR spectra is smaller, as νFeC shifts up only 3 cm⁻¹ and νCO down 11 cm⁻¹ in I113H CooA (see Fig. 7 and Table I). Interestingly, essentially the same effect was seen in I113A and I113D CooA. A possible interpretation is that in these cases water molecules may be brought into contact with the CO, filling the cavity that results from substituting the smaller Ala residue, or attracted to the negatively charged Asp side chain. These results suggest that Ile113 is close to the bound CO but not as close as Gly117. In the case of I113Y CooA, the RR spectrum gives a slight indication of negative polarity, shifting νFeC down 2 cm⁻¹ and νCO up 3 cm⁻¹. This effect could result from proximity to the π electron cloud of the Tyr ring to the CO. Substitutions at position 113 typically have modest effects on activity, as reported previously (28) and shown in Table I. The exception is I113D, and we have already shown that hydrophilic residues at positions 113 and 116 severely perturb activation (28). It is our working hypothesis that the effect is because the hydrophobic pocket is important for eliciting the proper conformational change in the C-helices upon displacement of the Pro2 ligand by CO.

At position 116, Lys and Phe substitutions were tested. Other possible H-bond donors were not examined, because they

\[\text{Fig. 7. Segments of the 406.7-nm excited RR spectra of WT CooA-CO, and of several distal residue CooA variants, showing the Fe-CO and CO stretching bands and their shifts from the WT positions. The asterisk in the G117I spectrum marks a feature that is because of excess dithionite. This CooA variant required more dithionite for reduction than the other proteins.}\]
have been shown to accumulate heme-containing CooA very poorly, presumably because of disruption of the hydrophobic pocket (28). L116K CooA variant was chosen, because it showed very high CO-dependent DNA binding activity (comparable with that of wild-type CooA in the presence of CO) and diminished CO-dependent DNA binding activities (see Ref. 14 and Table I). As shown in Fig. 7, the introduction of Lys at position 116 exerted an almost identical effect on the RR spectrum as did G117H, shifting νFeC by 13 cm⁻¹ up and νCO by 18 cm⁻¹ down (Table I). This result is consistent with the hypothesis that the L116K substitution, like G117H, introduces positive polarity into what is otherwise a non-polar environment around the CO. This CooA variant also showed broad νCO and νFeC bands. CooA-CO activity is impaired by the L116K substitution but is still considerable, indicating a modest perturbation of the active structure. The high activity of L116K CooA in the absence of CO has already been analyzed, and the result strongly implies that the substituted Lys residue serves as a ligand in both the Fe(III) and Fe(II) forms. We have suggested that this ligation leads to a similar helix repositioning as that seen in WT CooA when bound to CO (14). The absence of significant νFeC or νCO shifts in L116F is again consistent with maintenance of a hydrophobic environment for the bound CO.

Importantly, in the G117H and L116K CooA variants, the νFeC/νCO point shifts up and to the left in the backbonding plot (Fig. 4, only L116K is shown, for clarity) but remains displaced from the Mb line by the same amount as WT CooA-CO. This behavior indicates that the Fe–His⁷⁷ band is unaffected by the distal residue replacement, although backbonding is enhanced by the distal polarity. CO binding weakens the Fe–His⁷⁷ bond in these variants, just as in WT CooA-CO.

It should be noted that these observations apply only to the major population of CooA-CO molecules as described at the beginning of the “Results.” It is impossible to tell how the substitutions affect the minor CooA–CO’ fraction because of its weak signal and the breadth of the perturbed bands. However, a non-polar binding pocket is also indicated for the minor WT CooA–CO by its position in the νFeC/νCO correlation (Fig. 4), close to those of Mb variants with non-polar pockets.

In summary, the RR data establish that Gly¹¹⁷, Leu¹¹⁶, and Ile¹¹³ are all in or near the CO binding pocket. However, the data also indicate that substitutions of the Ile¹¹³ and Leu¹¹⁶ side chains are much more permissive with respect to CO activation of CooA than are those at Gly¹¹⁷. A possible interpretation is that the CO directly contacts the Gly¹¹⁷ and that any steric hindrance destabilizes the DNA binding conformation of the protein. However, it is also possible that Gly¹¹⁷ induces a critical bend in the C-helix, which is disrupted by substitutions.

We also examined other candidate residues for their effect on RR spectra. These included C-helix variants M124R and W110L and B-helix variants E99L and I95W (the B-helix is the short helix on the “bottom” of CooA as shown in Fig. 1). M124R (13) was chosen because of its significant impact on CO-sensing function of CooA. The others were chosen because of their potential proximity to the heme iron. As shown in Table I, all these variants except M124R had little effect on RR spectra, suggesting that these residues are not near the bound CO and consistent with the pocket being formed primarily by Gly¹¹⁷, Leu¹¹⁶, and Ile¹¹³.

The M124R replacement has little effect on the CooA-CO activity (Table I) but produces a double peak νCO band; one component is at the WT CooA position, 1982 cm⁻¹, whereas the other is −17 cm⁻¹ lower. The νFeC band is lowered slightly and is broadened (see Table I and Fig. 7). Although Met¹²⁴ is further from the heme iron than are Gly¹¹⁷, Leu¹¹⁶, and Ile¹¹³, it is possible that the long Arg side chain reaches the vicinity of the bound CO in a fraction of the CooA–CO molecules, producing the downshifted νCO component. Modeling of the introduced arginine in Fe(II)CooA (13) suggested two favored conformations of the side chain, the guanidine cation interacting either with the seven-propionate of the heme or with the backbone carbonyl of Ser⁷⁶. It is possible that in the M124R-CO structure one of the Arg conformers interacts instead with the CO.

The Strongly Basic Pro² Ligand Is Expelled from the Hydrophobic CO Pocket in the CO-bound Form of CooA at Physiological pH—Extensive mutational studies have shown that Pro² is not critical for the response of CooA to CO (11). Nevertheless, the Pro² ligand is a central aspect of cooperativity in CO binding by CooA.² Because the global conformational change of CooA upon CO binding is initiated by the displacement of the Pro² ligand by CO, it would be informative to trace the fate of the displaced Pro². We have shown above that Pro² does not appear to be near the bound CO at normal pH, but when the solution pH was increased above 7, the νFeC and νCO of WT CooA–CO were affected significantly (Fig. 8). The νFeC band at 487 cm⁻¹ was replaced by one at 497 cm⁻¹ whereas the νCO band at 1982 cm⁻¹ was replaced by one at 1964 cm⁻¹. Although the latter frequency is close to that of CooA–CO’, the νFeC frequency is distinctly higher than in CooA–CO’ Thus the change at high pH is not simply an increase in the CooA–CO’ fraction, but instead a new species is formed in basic solution.

The high pH νFeC and νCO values are similar to those displayed by the L116K and G117H variants (see Fig. 7 and Table I). The νFeC and νCO point remains well above the MbCO backbonding correlation (Fig. 4), indicating little change in the Fe–His⁷⁷ bond. We infer that the effect of raising the pH is to bring a H-bond donor into proximity with the bound CO. As in L116K and G117H, the high pH νCO and νFeC bands are broadened, suggesting heterogeneity in the H-bond donor position.

What might this H-bond donor be? Key evidence comes from the Δ3PR4 CooA variant, whose νFeC and νCO bands are unperturbed when the pH is raised, up to 11 (Fig. 8). We conclude that the H-bond donor is the N-terminal Pro², interacting with the bound CO via its secondary amine NH group. When the two penultimate residues, Pro³ and Arg⁴, are removed, the N terminus is constrained from interacting with the CO.

The Pro² interaction in WT CooA requires pH elevation, presumably because the secondary amine becomes protonated when it is displaced from the Fe(II) at pH 7. Because the CO binding pocket is hydrophobic, the protonated N terminus would be expelled from the binding site, because it is positively charged, just as the distal histidine is expelled from the MbCO binding pocket upon protonation at low pH (29, 30). At high pH, the displaced Pro², being neutral, can remain in contact with the CO and enhance backbonding, just as the distal histidine does in Mb.

What is the pKₐ of the displaced Pro²? In aqueous solution the pKₐ of the Pro amine is 10.5, but it should be lowered in CooA–CO because of the energy cost of expelling Pro² from the hydrophobic pocket. In Mb, the pKₐ of the distal His is 4.3 (29, 30), nearly three units lower than the aqueous His pKₐ. We examined the pH dependence of WT CooA–CO by decomposing the νFeC band into 487- and 497-cm⁻¹ components and plotting their intensities against the solution pH (Fig. 9). The resulting titration curves yield a pKₐ of 8.6, two units lower than the aqueous pKₐ of proline. If the assignment of the pKₐ to Pro² is correct, then the energy cost for expelling the distal
H-bond donor is about two-thirds as high in CooA as it is in Mb. We note that the absorption spectrum is not sensitive to the $p_Ka$ 8.6 process. The Soret band position, which is 422 nm at pH 7, only shifts significantly when the pH is raised to values greater than 10; at pH 12 the band maximum is 419 nm. We found that the RR spectral changes could not be fully reversed for solutions whose pH was raised to 10 or higher, indicating some irreversible change in the protein structure. However the Soret band shift could be reversed, even from pH 12, showing that there is a different physical mechanism for perturbation of the absorption spectrum.

These results indicate that Pro\textsuperscript{2} does not lie near the bound CO under physiological conditions, and the role of Pro\textsuperscript{2} in the active form of CooA is uncertain. The DNA affinity of the CO-bound forms of P2Y and ΔP3R4 CooA is 5- to 10-fold poorer than that of WT CooA, implying some small perturbation of the active structure when the N terminus is altered. However the primary roles of Pro\textsuperscript{2} appear to be to maintain the inactive form of CooA in the absence of CO and to provide a mechanism for the cooperative binding of CO when it is present.\textsuperscript{2}

The ligating nitrogen atom of Pro\textsuperscript{2} is part of a strongly basic secondary amine and is expected to be a strong donor to the Fe\textsuperscript{II}. Evidence for strong donation is seen in the RR spectrum of Fe\textsuperscript{II}CooA, which reveals a depressed value, 1532 cm\textsuperscript{-1}, for the porphyrin ring stretching vibration (25) (Fig. 10, 568-nm excitation was used to enhance the 1532 cm\textsuperscript{-1} via resonance with the heme Q bands (25)). This mode is known to be sensitive to the strength of the axial ligand field in low spin Fe\textsuperscript{II} porphyrin complexes. For example, the band shifts from 1547 to 1533 cm\textsuperscript{-1} when methionine is replaced by lysine in Fe\textsuperscript{II} cytochrome c (31) and from 1539 to 1527 cm\textsuperscript{-1} upon deprotonation of the imidazole complexes of microperoxidase or of bisimidazole Fe\textsuperscript{II} protoporphyrin (32, 33). The sensitivity to imidazole deprotonation was the basis of our earlier suggestion (16) that His\textsuperscript{77} might be deprotonated or strongly H-bonded in Fe\textsuperscript{II}CooA. However, His\textsuperscript{77} is now revealed by the crystal structure to have a normal H-bond with Asn\textsuperscript{42}, and the position of the porphyrin ring stretching vibration is unaffected when Asn\textsuperscript{42} is replaced by the non-H-bonding Ala (Fig. 10).

We conclude that the $\nu_{11}$ depression is unrelated to His\textsuperscript{77} but instead reflects the strong ligand field of Pro\textsuperscript{2}. This interpretation is supported by the observation (Fig. 10) that $\nu_{11}$ shifts up, by 4 cm\textsuperscript{-1}, in the P2Y and ΔP3R4 variants. When Pro\textsuperscript{2} is replaced by Tyr, the secondary amine is replaced by a primary amine at the terminus, producing a somewhat weaker ligand field. In the ΔP3R4 variant, the penultimate pair of residues is deleted, shortening the N terminus. The Fe\textsuperscript{II} protein remains.

![Fig. 8. pH dependence of the $\nu_{\text{FeC}}$ and $\nu_{\text{CO}}$ bands for the CO adducts of WT CooA and of the ΔP3R4 CooA variant. The shift in $\nu_{\text{FeC}}$ for WT CooA is also seen in the position of the $\sim$1860-cm\textsuperscript{-1} band assigned to a combination band with $\nu_{11}$ (1371 cm\textsuperscript{-1}, the strongest Soret-excited RR band). Another band, at 2049 cm\textsuperscript{-1}, is because of a combination of $\nu_{1}$ with $\nu_{\text{FeC}}$ (677 cm\textsuperscript{-1}, another strong fundamental), which is unaffected by pH (and whose intensity indicates the weakness of resonance enhancement for the $\nu_{\text{CO}}$ band). The asterisks mark the excess dithionite band.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

![Fig. 9. pH titration curves of WT CooA-CO. For the analysis, $\nu_{\text{FeC}}$ band envelopes of RR spectra were deconvoluted into 487- and 497-cm\textsuperscript{-1} components and then the fractional intensity of each band was plotted against the solution pH. The solid curves represent best fits to single proton release and uptake processes having a $p_Ka$ of 8.6.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
mainly six-coordinate, but a small fraction of the heme is five-coordinate, as judged by a shoulder on the ν4 porphyrin band at the expected five-coordinate position, 1355 cm⁻¹ (25). Pro2 might remain the ligand in ΔP3R4, but the shortening of the polypeptide chain would strain the Pro2-Fe bond, again weakening the field. This interpretation is consistent with the very rapid binding of CO to the ΔP3R4 variant.

**DISCUSSION**

The objective of this study was to gain insight into the nature of the CO-bound form of CooA and its implications for the conformational change that allows CooA-CO to bind its DNA target. RR signals arising from the heme and its ligands were examined for selected variants, whose activity in vivo and in a DNA binding assay were determined. It is not expected that spectroscopic and activity perturbations should be correlated. On the contrary, it is an important feature of this study that quite different aspects of the CooA molecule are sensed by the spectroscopic and activity probes. Full activity assures that the protein has the correct conformation to bind its DNA target; alterations in many parts of the molecule can destabilize the correct conformation. The RR signals are sensitive to the structure of the heme and its ligands. It is this information that we seek to integrate into a model of how the binding of CO at the heme initiates the conformation change from inactive to active protein.

Two principal findings are central to our model. 1) The C-helix residues Ile113, Gly117, and Leu116 become part of the CO binding pocket in CooA-CO. This is revealed by the spectral perturbations observed when their side chains are replaced with H-bond donors. This result implies repositioning of the hemes relative to the C-helices, because all three residues are at substantial distances from the heme iron in the FeII[CooA crystal structure (1). Measured Fe-Cα distances are 9.4 (10.2) Å for Leu116, 13.0 (7.0) Å for Gly117, and 11.2 (9.3) Å for Ile113. The first number refers to the residue on the same subunit as the heme, whereas the second number refers to the residue on the opposite subunit. Although not required crystallographically this part of the structure is nearly 2-fold symmetric; the Fe-Cα distances from the second heme are within 0.1 Å of those from the first. It is notable that only in the case of Leu116 does the closer residue reside on the same subunit as the heme. For both Ile113 and Gly117, the residue from the opposite subunit is much closer than is the residue from the same subunit. Thus a simultaneous rearrangement of both hemes and both C-helices is suggested to bring all three residues close enough to allow H-bond donor replacements to interact with the bound CO. 2) The His77-Asn42 H-bond, which is evident in the FeII[CooA crystal structure, appears to be broken upon CO binding. The strongest evidence is that the νCO and νFeC RR bands are unaffected when Asn42 is replaced by the non-H-bonding residue Ala (or by any other residue), despite the known sensitivity of these bands to the H-bond status of the proximal histidine ligand in heme-CO adducts (19, 20). Moreover, the position of the νCO and νFeC on the backbonding correlation reveal that the Fe–His bond is weaker in CooA-CO than in MbCO, consistent with His77 having no H-bond acceptor. Yet the Fe–His bond is equally strong in Mb and in FeIII[CooA as revealed by equal Fe–His stretching frequencies (revealed for FeII[CooA in the five-coordinate heme of the G117I variant), indicating the same extent of proximal His H-bonding. Thus CO binding evidently leads to loss of the His77-Asn42 H-bond in CooA.

These are the findings that flow from the present study. Together with the FeII[CooA crystal structure, they lead to a model for the initial motions leading to activation of CooA. The proposed motions are shown as dashed arrows in Fig. 2.

Upon binding CO, the heme is suggested to slide upward, bringing the FeCO unit closer to the side chain of Leu116 on the same subunit. As discussed above, the L116K variant is active in the absence of CO, suggesting that a Lys at this position induces the same heme displacement by displacing Pro2 as the distal ligand. We modeled a Lys side chain in the orientation of the Leu116 side chain and measured a 4.0-Å separation from the iron to the amine nitrogen. Thus a ~2.0-Å displacement of the heme group would be required to form a bond. This displacement provides an explanation for loss of the His77-Asn42 H-bond, because His77 remains bound to the heme and is displaced with it. It is likely that this heme displacement is also involved in the His77/Cys79 ligand switch that is known to occur...
upon oxidation to Fe[III]CooA (4), because the shifted heme position would accommodate Cys175 ligation. Similar heme movement and ligand switching has been described for cytochrome cd$_1$ (35). Although WT Fe[III]CooA is not active, there are a number of mutations that induce activity in the Fe[III] form of the protein (13).

It is further suggested that when the heme is displaced upward, it is approached more closely by the C-helix from the second subunit, whose Gly117 and Ile113 residues help form the hydrophobic binding pocket for the CO. A modest motion of the second C-helix would permit H-bond donors at these positions to perturb the CO, whereas a much larger rearrangement of the first C-helix would be required to produce this effect. Thus the overall motion that initiates the conformation change to the active CooA-CO structure is viewed as a clockwise rotation of both hemes and both C-helices, in the plane of Fig. 2.

Some CooA molecules do not undergo this motion. The minority CooA–CO$^+$ fraction represented by the 1962 cm$^{-1}$ CO shoulder appears to have an intact His77-Asn62 H-bond (the vFeC=C point falls on the Mb backboning line), indicating that the heme is undisplaced from its position in the Fe[II]CooA structure. Presumably these molecules are not active in binding DNA. We do not know whether they are in a dynamic equilibrium with the active molecules or else are prevented from undergoing activation by some kind of energy barrier.

What is the driving force for the proposed heme and C-helix displacements? We propose that hydrophobic interactions are responsible (28), perhaps abetted by the electronic reorganization of the heme that results from a π acceptor ligand (CO) replacing a δ donor ligand (the N terminus). Examination of the Fe[II]CooA crystal structure shows the heme to be partially exposed to solvent, whereas it would become more buried by sliding into the cavity (Fig. 11). Hydrophobic interactions would be enhanced by the suggested displacement of the opposite C-helix toward the heme. The hydrophobic character of the CO binding pocket is evidenced by the −two-unit pK$_a$ reduction of the displaced Pro$_2^+$, relative to the expected aqueous pK$_a$.

CONCLUSIONS

RR spectroscopy, combined with activity measurements, on variants of CooA has provided evidence about the nature of the CO adduct. Three C-helix residues, Ile$_{112}$, Leu$_{116}$, and Gly$_{117}$, are shown to be close to the bound CO, forming a hydrophobic pocket. The displaced Pro$_2^+$ ligand is expelled from this pocket, unless the amine is unprotonated; the pK$_a$ for this process is 8.6. The RR evidence points strongly to breaking of the proximal His$_{77}$-Asn$_{62}$ H-bond when CO binds. On the basis of the Fe[II]CooA crystal structure, these observations lead to the proposal that when CO binds, the heme is displaced into an adjacent cavity and is approached by the C-helix of the opposite subunit. These motions are suggested to trigger the protein conformation change required for DNA binding.

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