Roles of Heme Axial Ligands in the Regulation of CO Binding to CooA

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CooA is a CO-dependent transcription factor of the bacterium *Rhodospirillum rubrum* that contains a six-coordinate heme. It has as its heme axial ligands Pro² and Cys⁷⁵ in the ferric state and Pro² and His⁷⁷ in the ferrous state. To probe the regulation of CO binding and the ligand switching mechanism in CooA, we have prepared site-directed mutants in which the residues contributing the axial ligands are substituted. The properties of these mutants were investigated by resonance Raman and CO titration methods. Wild-type CooA binds CO with a modest dissociation constant ($K_d$) of 11 μM, which is similar to that in wild-type CooA. Thus, Pro² is the ligand that is indeed replaced by exogenous CO, and the changes in the CO-sensing mechanism of CooA necessarily involves the displacement of one of these two ligands. The displaced ligand in the ferric state is Cys⁷⁵ in the thiolate form, and Pro² is proposed indirectly to control the His⁷⁷ acting as a proton reservoir.

A new class of heme proteins is emerging that senses small gaseous molecules such as NO, O₃, and CO (1–3). Binding of these diatomic ligands to the heme triggers the biological response. Soluble guanylyl cyclase binds NO and exerts a variety of physiological responses including neurotransmission and smooth muscle vasodilation (4–6). FixL, Dos, and HemAT bind O₃ and regulate gene expression in nitrogen-fixing rhizobia (7), phosphodiesterase activity in *Escherichia coli* (8), and the swimming behavior of *Bacillus subtilis* (9), respectively. Npas2, a very recent member in this class, binds CO and regulates the transcription related to the circadian clock in the brain (10, 11). The heme moieties in these sensor proteins are directly involved in binding their respective effector molecules and thereby regulating the activity of the proteins. Thus, the mechanisms whereby these proteins respond to the binding of gaseous molecules are currently of great interest.

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The abbreviations used are: WT, wild type; mW, milliwatt.

CooA is isolated from the photosynthetic bacterium *Rhodospirillum rubrum*, which can utilize CO as a sole energy source (12). CooA regulates the expression of genes, termed *coo* for CO oxidation, in which products are associated with CO metabolism (13). CooA is a homodimer (221 amino acids/monomer) containing two b-type hemes that reversibly bind CO. The signal of CO binding to the heme is transmitted to the DNA binding domain, enabling the protein to bind its target DNA sequence and induce transcription of the *coo* genes. The DNA binding and signaling domains are analogous to those found in the catabolite gene activator protein CAP (or CRP) (14, 15). The heme in CooA is bound within the signaling domain where it is ligated by a histidine (His⁷⁷) side chain in the ferrous state (14). This ligand is replaced by the Cys⁷⁵ side chain in the ferric state (16, 17). Although ligand switching indicates that the position of the heme cofactor is flexible relative to the surrounding protein matrix of CooA, the physiological role and exact mechanism of the ligand switch remains elusive.

The crystal structure of ferrous CooA revealed unexpectedly that the heme ligand *trans* to His⁷⁷ is the amino-terminal proline residue (Pro²) from the opposite chain in the homodimer (see Fig. 1) (14). Previous studies (16, 18), including electron paramagnetic resonance analysis and site-directed mutagenesis showed that one of the ligands in the ferric state is Cys⁷⁵ in the thiolate form, and Pro² is proposed indirectly to be the *trans* ligand (19, 20). Because the ferrous heme in CooA is six-coordinate and low spin with Pro²/His⁷⁷ ligands, the CO-sensing mechanism of CooA necessarily involves the displacement of one of these two ligands. The displaced ligand in the CO-bound ferrous CooA is suggested to be Pro² by nuclear magnetic resonance and time-resolved resonance Raman studies (21, 22). However, it has been argued elsewhere (23, 24) that CO displaces His⁷⁷ instead of Pro² and that the changes in His⁷⁷ ligation trigger DNA binding. Although the release of Pro² from the heme iron by incoming CO triggers the sequential events that result in the activation of CooA, a variety of Pro² variants was found to be CO-responsive, indicating that Pro² is not critical for activation of CooA in response to CO (19, 20). Thus, the role of the Pro² ligand in the CO binding event remains to be clarified.

In this paper, we present resonance Raman and CO titration analyses of wild-type (WT*) and a series of mutants of CooA in their ferric, ferrous, and ferrous-CO bound forms. Through the analysis of various mutants, it is established that the Pro² and His⁷⁷ residues regulate CO binding to CooA. Pro² is revealed to be the ligand that is indeed replaced by exogenous CO, and the His⁷⁷ ligand is required to direct the CO to the Pro² side of the heme. The roles of these ligands are discussed in the light of CO...
Recognition and redox-linked ligand-switching mechanisms in CooA.

**Materials and Methods**

Cloning and Purification of CooA—To achieve high expression, a gene encoding WT CooA was synthesized using a modification of the recursive PCR strategy (25–27). Based on the known amino acid sequence of *R. rubrum* CooA, six forward (A1–3, B1–3) and six reverse (A1–3, Br1–3) primers with lengths ranging from 69 to 117 bases were designed (see Fig. 2) and custom-synthesized. Because the designed sequence is long, subsequent chain extension by KOD Plus DNA polymerase (Toyobo) was performed for two sets of primers, A and B, in which each pair of forward and reverse primers overlaps by about 20 bases. The full-length PCR product of set A was selectively amplified in a second PCR with a forward primer AF, which creates tandem Xhol and NdeI sites and a reverse primer AR, which generates a HindIII site (Fig. 2). The full-length PCR product of set B was also amplified with a forward primer BF, which creates a HindIII site and a reverse primer BR, which generates a termination codon (TAA) and a BamHI site. The resultant products were inserted at the Xhol/HindIII and HindIII/BamHI sites, respectively, of the plasmid pBluescriptII KS(−) (Stratagene) and joined at the common HindIII site, and the ligation products were used to transform *E. coli* XL-1 Blue MRF′ (Stratagene). The DNA sequence of the recombinant plasmid product was confirmed with a Li-Cor Model 4200S2 DNA sequencer. The QuikChange system (Stratagene) was used to introduce mutations into the CooA coding sequence. The NdeI/BamHI insert containing the full-length CooA coding sequence was excised from the pBluescript derivatives and ligated into a pET3a-based expression vector (pBEX) (27). The ligation products were introduced into *E. coli* strain BL21 Gold(DE3) (Stratagene). Cell growth and protein purification were performed as described previously (16). Proteins were purified to homogeneity as judged by SDS-PAGE criteria. CooA concentrations were evaluated from the heme content, which was measured by a pyridine hemochromogen assay and hence were based on a monomer unit.

Resonance Raman Spectroscopy—Spectra were recorded using a double monochromator (Jasco R-800) with a slit width of 6 cm⁻¹, following excitation by a krypton ion laser (406.7 nm line, Coherent I-302). A photomultiplier detector was used (Hamamatsu Photonics, R595), and the frequencies were calibrated with indene. A spinning Raman cell was used (Hamamatsu Photonics, R595), and a photomultiplier detector was used (Hamamatsu Photonics, R595). A spinning Raman cell was used (Hamamatsu Photonics, R595). A spinning Raman cell was used (Hamamatsu Photonics, R595).

**Results**

P2H and H77A—In Fig. 3, resonance Raman spectra of ferric WT, H77A, and P2H CooAs are shown. Resonance Raman spectroscopy is a powerful tool for revealing the oxidation states, spin states, and coordination numbers of heme iron (28, 29). In the higher frequency region for WT and H77A CooA, the ν₂, ν₃ₐ₈, ν₃, and ν₄ lines are observed at 1583, 1549, 1501, and 1372 cm⁻¹, respectively. These frequencies are typical of six-coordinate ferric low spin hemes and similar to those reported previously for the WT CooA (23). The profiles of WT and H77A are essentially the same, supporting the notion that His⁷⁷ is not coordinating the iron in ferric CooA. In contrast, the spectra of P2H is altered with the ν₃ₐ₈ line shifted to 1490 cm⁻¹ with a broad line around 1580 cm⁻¹. The ν₄ frequency indicates the presence of a ferric five-coordinate heme, indicating that Pro² is an axial ligand in the WT protein. A shoulder on the higher frequency side of the ν₃ₐ₈ line indicates that there is a minor six-coordinate low spin component present. The broad profile around 1580 cm⁻¹ also supports the presence of multiple components.

In Fig. 4, resonance Raman spectra of ferric WT, H77A, and P2H CooAs are shown. In the higher frequency region for WT CooA, the ν₂, ν₃ₐ₈, ν₃, and ν₄ lines are observed at 1581, 1555, 1493, and 1362 cm⁻¹, respectively. These frequencies are typical of six-coordinate ferric low spin hemes and similar to those reported previously for the WT CooA (23). His⁷⁷ and Pro² are revealed to be the axial ligands of the ferric CooA (Fig. 1) (14). As expected, a resonance Raman line for a ferric five-coordinate high spin component is apparent at 1470 cm⁻¹ in the spectra for both H77A and P2H. However, other features are quite similar to those of WT CooA indicating that six-coordinate low spin species still dominate in these ligand mutants. In P2H, His² may coordinate the ferric heme, originating the low spin component. Such an explanation is not possible for H77A where His⁷⁷ is replaced by non-coordinating alanine. This suggests strongly that an alternative ligand coordinates the ferrous heme in this mutant. This ligand could be the thiol moiety of Cys⁷⁵ as will be discussed later.

CO Titrations—There is disagreement concerning the identity of the residue that is displaced by exogenous CO. Early studies suggested that CO displaces the His⁷⁷ ligand (23, 24), whereas more recent studies indicate that the Pro² is the ligand that is replaced (21, 22). To resolve this controversy, we measured the CO binding properties of the axial ligand mutants of CooA (Fig. 5). The Q-band region of the absorption spectra of heme proteins is sensitive to the nature of the ligand. The spectrum of ferric WT CooA shows sharp absorption bands around 560 and 530 nm, which are replaced by broad bands around 570 and 540 nm upon CO binding. These spectral changes are clearly associated with one set of isosbestic points, indicating that CO binding is a one-step equilibrium process (Fig. 5, top panel). Thus, the titration curve was analyzed in terms of a simple equilibrium between the ferrous and CO-bound forms of the protein. The absorbance changes at the Q-band maximum of the ferrous protein were normalized and plotted against the CO/CooA ratio (Fig. 5, bottom panel). A theoretical curve drawn, assuming that binding involves 1 equivalent of CO, provides a satisfactory fit to the titration curve, and an apparent Kₐ value of 11 μM was calculated for CO binding to WT CooA (Table 1). The P2H mutant has higher affinity for CO, its Kₐ value was estimated to be 53 nM. The titration curve saturates at a CO/CooA ratio of unity, indicating unequivocally that 1 mol of CO binds to each heme in the P2H mutant. Thus, the Pro² residue regulates the CO binding affinity of CooA lowering the affinity by more than 2 orders of magnitude.

In H77A, exogenous CO was observed to bind in two steps. The first CO molecule clearly binds with a 1:1 stoichiometry to the H77A mutant with a Kₐ value of 85 nM (Fig. 5, bottom panel).
panel), which is similar to that for P2H. This is reasonable, because the His$^{77}$ ligand is replaced by a non-coordinating alanine in this mutant, and a vacant site is expected to be created at the fifth coordination site of iron. The second CO molecule binds with much lower affinity, the $K_d^2$ value being estimated to be 85 $\mu$M. The presence of two binding steps indicates that His$^{77}$ is essential for directing exogenous CO to the appropriate side of the heme. This is likely to be important for inducing conformational changes in CooA that lead to binding to the target DNA. The $K_d$ values for the axial ligand mutants of CooA are summarized in Table I. Two-step binding is prevalent among the His$^{77}$ mutants, and the $K_d$ values are affected by the nature of the replacement at position 77. Bi- 

phasic CO binding in the His$^{77}$ mutants indicates that Pro$^{2}$ must be being replaced by CO in the second binding step. The second CO binding event was not observed in the P2H mutant even at saturating concentrations of CO. This indicates unequivocally that Pro$^{2}$ is more easily displaced by exogenous CO than His$^{77}$.

Fe-CO Stretch—To evaluate the properties of bound CO, the $\nu$(Fe-CO) stretching Raman line was measured. The $\nu$(Fe-CO) stretching frequency is sensitive to the polarity of the residues around the bound CO and is therefore an excellent probe of the distal environment (30–34). As seen in Fig. 6, a Raman line is observed at 490 cm$^{-1}$ in CO-bound WT CooA. This line has been assigned to the $\nu$(Fe-CO) stretching mode in experiments using isotopically labeled CO (35, 36). In the Pro$^{2}$ mutant, a
similar line is observed at 491 cm\(^{-1}\), indicating that the environment of the bound CO is nearly the same as that in WT CooA. On the other hand, in the H77A mutant the corresponding \(\nu(\text{Fe-CO})\) stretch is observed at 497 cm\(^{-1}\), which is 7 cm\(^{-1}\) higher than that in WT CooA and should be assigned to the vibration from the Pro\(^2\)-Fe-CO moiety. Many heme proteins have a histidine ligand \(\text{trans}\) to CO in the ferrous CO-bound state, and the higher \(\nu(\text{Fe-CO})\) stretch is a signature of a positively charged CO environment (31–33). The higher frequency in H77A suggests that the bound CO is in a more positively polar environment than that in WT and P2H proteins, although the \(\nu(\text{Fe-CO})\) frequency in the proline-coordinated heme-CO complex has not been determined to date.

In some of the His\(^{77}\) mutants, an additional Raman line is observed at about 525 cm\(^{-1}\) in the frequency region of the \(\nu(\text{Fe-CO})\) stretch (Fig. 6). The relative intensity of this extra line depends on the residue at position 77. Positions 77 side chains having a labile proton (H77D and H77E) exhibit a stronger line. This is especially noticeable in the H77D mutant. The appearance of two lines indicates two distinct structures for the Fe–CO bond, and this in turn may be related to the two binding steps observed in the CO titration experiments. It should be noted that the \(K_{d2}\) value for H77D is the smallest among the H77 mutants (Table I), and this implies that the second CO binds easily to the ferrous heme in this mutant. H77T, which exhibits a minor \(\nu(\text{Fe-CO})\) stretching band around 525 cm\(^{-1}\), has a relatively small \(K_{d2}\) value (Fig. 6). Thus, the labile proton is not a prerequisite for the appearance of the second \(\nu(\text{Fe-CO})\) component.

Ferrous and Ferric Heme in His\(^{77}\) Mutants—Because some of the His\(^{77}\) mutants showed two \(\nu(\text{Fe-CO})\) components, the incoming CO molecule must recognize structural differences in the mutants in the ferrous state. As seen in Fig. 7, the ferrous high spin component of \(v_3\) is observed at 1471 cm\(^{-1}\) in every His\(^{77}\) mutant. In addition, a line seems to be buried around 1600 cm\(^{-1}\) in the His\(^{77}\) mutants, and this line can be assigned to the \(v_{57}\) mode of the ferrous high spin component. The relative intensity of the \(v_3\) line, however, varies among the mutants, and those having labile protons (H77E and H77D) give rise to a stronger line at 1471 cm\(^{-1}\). Again, this proton is not a prerequisite for the strong 1471 cm\(^{-1}\) line, because H77T shows a
similar profile to those of H77E and H77D. To clarify whether the replacements may affect the Cys coordination in the ferric CooA, we studied the coordination structure by resonance Raman spectroscopy (Fig. 8). In the ferric state, spectral differences among the His77 mutants are not observed. Thus, it is clear that coordination of the ferric heme by the Cys ligand, is unaffected by the nearby His77 replacements.

DISCUSSION

Roles of Pro2—Through CO titration measurements and resonance Raman spectroscopy, we were able to reveal that the heme axial ligand in CooA that is displaced by exogenous CO is Pro2. Because the ν(Fe-CO) stretching frequency in the WT and P2H proteins is quite similar (Fig. 6), the dissociated Pro2 ligand may be remote from the ferrous heme. Indeed, truncation of the amino-terminal residues has little effect on the activation of CooA, and the amino-terminal residues are known to be highly flexible (19, 20). As seen in the resonance Raman spectra of ferric (Fig. 3) and ferrous (Fig. 4) P2H, a six-coordinate component dominates in this mutant even though one of the axial ligands has been replaced. Although the identity of the ligand trans to His77 is unclear at present, the imidazole nitrogen or the terminal amino group of His2 in P2H could possibly be the sixth ligand. In this case, however, the coordination would be weak enough to lead the appearance of a five-coordinate component.

The CO binding affinity of heme proteins is usually determined from kinetic studies. When the kinetics are monophasic, the ratio of $k_{on}$ and $k_{off}$ is equal to the dissociation constant $K_d$. In the case of CooA, however, the CO binding kinetics is multiphasic (35, 37), and the estimation of the $K_d$ value has been hampered. In this study, we could estimate the CO affinity of WT CooA for the first time using an equilibrium titration method (Table I), and the $K_d$ value is similar to those for other proteins that sense gaseous ligands (8, 38). Most of the gas sensors contain a six-coordinate heme, and this character seems to be unique in this class of proteins. Six coordination is inherently unsuitable for the binding of exogenous gaseous ligand, because the sixth ligand would be expected to present a barrier to binding. As seen in P2H, the substitution of the sixth Pro2 ligand greatly enhances the CO affinity, and the $K_d$ value approaches that of myoglobin (39), which contains a five-coordinate heme. The presence of a five-coordinate component in the P2H mutant is apparent from the resonance Raman spectra (Fig. 4). Thus, the presence of Pro2 as a sixth coordinating ligand is a way to fine tune the sensing of CO in the media. It should be noted that neuroglobin shows a relatively small $K_d$ value, although this globin protein contains a six-coordinate heme with His/His axial ligands (27, 40). This may imply that neuroglobin is equipped with a special device that facilitates the binding of gaseous molecules.

Two Fe-CO Lines—In the His77 mutants, the CO binds in a two-step manner, and the $K_{1,2}$ value is similar to that observed for P2H (Table I). The estimated $K_{1,2}$ values are reasonable, because one of the axial ligands is replaced in these mutants, and the first CO molecule “sees” the vacant coordination site (Fig. 9). However, the second CO molecule attacks the trans sixth site after the binding of the first CO has taken place with 1:1 stoichiometry (Fig. 5). Thus, the coordination of His77 is essential both to direct the CO molecules to the correct sixth coordination site (otherwise occupied by Pro2) and to block the binding of a second CO species. This observation is in accord with the previous report (21) that CO binds to the “wrong” side of the heme in the H77Y mutant.

Ferrous His77 mutants contain both six- and five-coordinate ferrous hemes (Fig. 7), and the ligand trans to Pro2 in the six-coordinate species is suggested to be the thiol moiety of Cys75 as will be discussed later. It may be argued that the first CO replaces the Pro2 even in the six-coordinate His77 mutants. If Pro2 is replaced by the first CO, we should expect the appearance of a ν(Fe-CO) stretching line around 520 cm$^{-1}$ from the Cys75-Fe-CO moiety in the His77 mutants, because weak ligands such as thiol should give rise to a trans ν(Fe-CO) stretch around this frequency. Although such a line is observed at 525 cm$^{-1}$ in these mutants (Fig. 6), this line is related more to the five-coordinate component in the ferrous protein (Fig. 7). Thus, H77D, H77E, and H77T show a relatively strong five-coordinate component at 1471 cm$^{-1}$, and these mutants also...
show relatively strong 525 cm$^{-1}$ lines. Thus, the 525 cm$^{-1}$ line should not be attributed to the vibration from the Cys$^{75}$-Fe-CO moiety.

The CO-bound WT CooA was very photolabile, and the laser power had to be reduced to 1 mW to measure the $\nu$(Fe-CO) stretch Raman line (Fig. 6). However, the CO adducts of the His$^{77}$ mutants were relatively stable and could be probed with a higher laser power (3 mW). This greater stability may be related to the higher affinity of these mutants for CO as reflected in the lower $K_{d2}$ values (Table I). The second CO molecule, however, binds with much lower affinity as revealed by the larger $K_{d2}$ values relative to WT CooA. Thus, the $\nu$(Fe-CO) stretch at 497 cm$^{-1}$, which is commonly observed in the His$^{77}$ mutants, should be assigned to the first CO molecule bound to the ferrous heme. Among the His$^{77}$ mutants, H77D showed the lowest $K_{d2}$ value, and this value is lower than the $K_d$ for WT CooA (Table I). This suggests that the second CO molecule ought to be observed in the H77D mutant. H77E and H77T showed $K_{d2}$ values comparable with the $K_d$ for WT CooA, and a weak but definite Raman line is observed around 525 cm$^{-1}$ in these mutants. Thus, the 525 cm$^{-1}$ component should be assigned to the second CO molecule that is bound to the ferrous heme trans to the first CO molecule.

The CO binding scheme for the His$^{77}$ mutants is illustrated in Fig. 9. The first CO molecule coordinates the ferrous heme with high affinity and 1:1 stoichiometry as revealed by the CO titration experiments (Fig. 5). The vacant fifth site in these mutants favors CO binding, giving rise to the 497 cm$^{-1}$ $\nu$(Fe-CO) stretch. The second CO molecule replaces the Pro$^2$ ligand trans to the first CO, and the resultant CO-Fe-CO moiety gives rise to the 525 cm$^{-1}$ line.

**Ligand Switch**—As seen in Fig. 7, the ferrous His$^{77}$ mutants contain six- and five-coordinate hemes, and the amount of the latter component seems to be related to the availability of a proton that is capable of hydrogen bonding in the presence of a hydrogen bond donor group. In the ferric state, Cys$^{75}$ coordination is unaffected by the residue at position 77 (Fig. 8). The ferric heme favors anionic thiolate coordination, which neutralizes the positive charge of the iron. In the ferric state, the iron is already neutralized by the pyrrole nitrogens of the porphyrin ring, and no balancing charge is required. Hence the neutral thiol form is the favored ligand (Fig. 9). In the crystal structure of ferrous CooA, the only residue capable of coordination in the vicinity of the ferrous heme is Cys$^{75}$ (14). When a proton is available, the neutral thiol group of Cys$^{75}$ may make a hydrogen bond with the side chain of residue 77, directing the thiol group off the ferrous heme (Fig. 9). In this context, His$^{77}$ is a proton reservoir controlling the protonation state of the Cys$^{75}$ side chain. In the ferric state, the imidazole moiety of His$^{77}$ takes up a proton and facilitates the formation of thiolate, whereas in the ferrous state, the proton is passed to the thiolate, and the resultant neutral imidazole favors binding to the ferrous heme.

In summary, we have been able to determine the CO binding affinity of CooA, and the affinity was found to be similar to other heme-based gas sensors. Pro$^2$ is assigned unequivocally to the ligand that is replaced by exogenous CO. Pro$^2$ and His$^{77}$ ligands are essential for regulating CO binding to CooA and hence for sensing the levels of CO in the medium. His$^{77}$ plays a key role in the ligand switch in CooA depending on the iron-redox state in that the imidazole group is a proton reservoir affecting the protonation state of the Cys$^{75}$ side chain.

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