The Heme Pocket Afforded by Gly\textsuperscript{117} Is Crucial for Proper Heme Ligation and Activity of CooA*

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CooA, a CO-sensing homodimeric transcription activator from \textit{Rhodospirillum rubrum}, undergoes a conformational change in response to CO binding to its heme prosthetic group that allows it to bind specific DNA sequences. In a recent structural study (Lanzilotta, W. N., Schuller, D. J., Thorsteinsson, M. V., Kerby, R. L., Roberts, G. P., and Poulos, T. L. (2000) \textit{Nat. Struct. Biol.} 7, 876–880), it was suggested that CO binding to CooA results in a modest repositioning of the C-helices that serve as the dimer interface. Gly\textsuperscript{117} is one of the residues on the C-helix within 7 Å of the heme iron on the Pro\textsuperscript{2} side of the heme in CooA. Analysis of a series of Gly\textsuperscript{117} variants revealed altered CO-sensing function and heme ligation states dependent on the size of the substituted amino acid at this position; bulky substitutions perturbed CooA both spectrally and functionally. A combination of spectroscopic and mutagenic studies showed that a representative Gly\textsuperscript{117} variant, G117I CooA, was specifically perturbed in its Pro\textsuperscript{2} ligation in both Fe(III) and Fe(II) forms, but comparison with other CooA variants indicated that perturbation of Pro\textsuperscript{2} ligation is not the basis for the lack of CO response in G117I CooA. These results have led to the hypothesis that (i) the heme and the C-helix region move toward each other following CO binding and the interaction of the heme with the C-helix is crucial for CooA activation, and (ii) this event occurs only when a properly sized heme pocket is afforded.

Proteins that sense small gaseous molecules, such as NO, O\textsubscript{2}, and CO, play important roles in biological systems ranging from prokaryotes to mammals. These include soluble guanylyl cyclase, which binds NO and exerts a variety of physiological responses, including neurotransmission and smooth muscle vasodilation (1–3), and FixL, which binds O\textsubscript{2} and regulates transcriptional regulators (14). These proteins exert their function by binding a cognate DNA sequence after responding to their specific effectors (cAMP for CRP; reducing conditions for FNR; CO for CooA). The only structure known for CRP is the cAMP-bound form (15), and a variety of analyses have suggested that a substantial conformational change occurs upon effector binding (16–18). No structure has been obtained for FNR, although it appears to be activated by a very different mechanism of a monomer-dimer transition rather than a conformational change within a dimer, as in the case of CRP and CooA (19). The structural comparison of Fe(II) CooA in the absence of effector (CO) with CRP bound to its effector (cAMP) suggested a number of differences between them, among which was a modest repositioning of the long C-helices that serve as the dimer interface in both CRP and CooA (8). This has lead to

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The atomic coordinates and structure factors (code 1ft9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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† The abbreviations used are: EPR, electron paramagnetic resonance; CRP, cAMP receptor protein; FNR, fumarate and nitrate reductase activator protein; WT, wild-type; MOPS, 4-morpholinepropanesulfonic acid; P-450cam, cytochrome P-450cam; DTT, dithiothreitol; CN\textsuperscript{−}, cyanide anion.
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the hypothesis that in CooA, effector binding causes a change in the relative positions of the C-helices, which transmits a signal to the DNA-binding domains. Presumably this repositioning either destabilizes the inactive conformation, stabilizes the active conformation, or both.

It is unclear how CO binding might affect the repositioning of the C-helix, although it seems plausible that CO displacement of Pro allows the heme, still tethered to His, to interact with the C-helices. To test this hypothesis, we examined the surface of the C-helices in the structure of Fe(II) CooA and noted that there are relatively few residues that are in a position to interact with the heme (8): Gly, Leu, Ile, and Leu. In this paper, we report the importance of the heme pocket affected by substitution at the Gly position and the analysis provides a number of insights into the response of wild-type (WT) CooA to CO.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—WT CooA and CooA variants were constructed in an E. coli overexpression system and a β-galactosidase reporter system as described previously (7).

In Vivo β-Galactosidase Assay—In vivo β-galactosidase activity in an E. coli reporter system was monitored as described previously (7) and quantitated using the standard protocol (20).

Purification of WT CooA and CooA Variants—The purification of WT CooA and CooA variants to ~95% purity was performed as described previously (7). The heme content of CooA preparations was quantitated by the reduced pyridine-hemochromogen method (21).

Preparation of Hydroxyapatite Batch-treated CooA Samples—For the initial screening of the heme coordination states of different CooA variants, a 50-mL culture of the cells was harvested, resuspended in 5 ml of 25 mM MOPS buffer, 0.2 mM NaCl, 10% glycerol, pH 7.4, 10 mM potassium phosphate, pH 7.4, 14 mM potassium chloride, and 0.1 M NaCl. After the supernatant was then mixed with 0.3 g of solid hydroxyapatite resin. After unknown materials were removed from the resin, a high salt buffer containing 25 mM MOPS, pH 7.4, 10 mM potassium phosphate, pH 7.4, 1.2 mM KCl, and 5% glycerol was added, and the resin was washed twice. CooA was then eluted with high salt buffer containing 25 mM MOPS, pH 7.4, 160 mM potassium phosphate, pH 7.4, 50 mM KCl, and 5% glycerol. The eluent was precipitated with ammonium sulfate with a final saturation of 50% and stored at -20 °C until use. CooA prepared with this procedure resulted in an enrichment of CooA to ~10% of total protein.

Spectroscopic Measurements—Electronic absorption spectra of CooA variants were measured with a Shimadzu UV-2401 spectrophotometer. Anaerobic reduction of CooA samples was performed as described previously (7). EPR spectra were recorded on a Varian E-4 spectrometer equipped with an Oxford Cryostat (31). For the measurement at the microwave frequency of 9.25 GHz at 4 K and 200 microwatts (µW) for high spin signals and 22 K and 20 µW for low spin signals. EPR spectra at pH 7.4 were obtained in 25 mM MOPS buffer, pH 7.4, containing 0.1 mM NaCl. For the preparation of Fe(III) G117I CooA at pH 6.6 and 9.4 for EPR, samples were buffered by mixing 150 µl of the purified protein (dissolved in 25 mM MOPS, pH 7.4, and 0.1 mM NaCl) with 150 µl of 200 mM buffer (MOPS, pH 6.5, glycine/NaOH, pH 9.5) containing 0.1 mM NaCl to provide a final buffer concentration of ~100 mM. EPR spectra of Fe(III) GI171 CooA and WT CooA at pH 3.4 were buffered by mixing 150 µl of the purified protein (dissolved in 25 mM MOPS, pH 7.4, and 0.1 mM NaCl) with 150 µl of 400 mM glycine/NaCl, pH 3.1, and 0.1 mM NaCl to provide a final buffer concentration of ~200 mM. For the EPR analysis of Fe(III) G1171 CooA at pH 10.2, isolated Fe(III) G13P4 CooA was buffered by using 200 mM glycine/NaOH, pH 10.5, and 0.1 mM NaCl. The pH of all samples for EPR spectra was confirmed after mixing using an Orion model 611 pH meter equipped with a Ross semi-micro temperature compensation electrode. The heme concentrations of WT, G117I, and G117V CooA were determined using the standard protocol (20).

pH Titrations of Fe(III) WT CooA—The effect of pH on the UV-visible spectrum of WT CooA was monitored by adding 10 µl of 160 mM WT CooA to 990 µl of different buffer solutions. The following buffer solutions were used: pH 7.4, 100 mM MOPS, 0.1 mM NaCl, pH 6.9, 100 mM MOPS, 0.1 mM NaCl, pH 6.1, 100 mM potassium phosphate, 0.1 mM NaCl, pH 5.5, 5.3, 5.0, 4.5, 4.0, 100 mM acetate, 0.1 mM NaCl; pH 3.4, 100 mM glycine/NaCl, 0.1 mM NaCl.

In Vitro Fluorescence Polarization Assay—In vivo DNA binding assays were performed using the fluorescence polarization assay described previously (10). Target DNA containing pCooF (purchased from Genosys) was labeled with the fluorescent dye, Texas Red, on one end of the duplex and used at the concentration of 6.4 nM. Dissociation constants (KD) were calculated by fitting the binding data to a non-linear fitting equation described by Lundblad et al. (24).

RESULTS

Ligation Structure and in Vivo Activity of Gly Variants Are Closely Related with Residue Size—The crystal structure of Fe(II) CooA revealed that relatively few residues are present in the vicinity of the heme on the side of Pro, one of the axial ligands in Fe(II) CooA (8). These residues include Gly, and we tested the hypothesis that the portion of the pocket provided by this residue might be important for heme interaction with the C-helix upon CO binding (Fig. 1). The set of Gly variants studied here included G117A, G117S, G117V, G117I, and G117H. As shown in Table I, the activities of these Gly variants in response to CO are severely perturbed in our in vivo assay. As the size of substitution at the position 117 increased (see “mole vol” column in Table I), CO-sensing function was reduced. This CO-sensing function is indicated in Table I by the ratio of the activity of each CooA variant in the presence of CO to that in the absence of CO; this ratio is an approximate measure of the -fold activation caused by effector binding. There is also a less striking correlation between residue size and effector-independent activity: Larger residues at position 117 provided greater effector-independent activity in the Fe(III) and Fe(II) forms (Table I). It is important to note that the differences in activity in vivo of the variants are not the result of poor accumulation of heme-containing CooA, because we have shown that ~5% active CooA (compared with WT CooA) is sufficient for maximal in vivo activity (25) and all the Gly117 variants accumulate ≥25% of WT CooA (Table I).

The Soret wavelength maxima of Gly variants are also listed in Table I. Although substitutions at position 117 with relatively smaller side chains such as Ala and Ser showed wavelength maxima that were indistinguishable from that of WT CooA, G117V CooA and G117I CooA revealed a bleached Soret peak at 387 nm in the Fe(III) form, characteristic of a five-coordinate high spin heme. The Fe(II) forms of G117V CooA and G117I CooA are also severely perturbed in terms of Soret maxima, and the detailed spectral properties of purified G117I CooA will be discussed below. The extent of the perturbation of both UV-visible spectra and the CO-sensing function
in Gly^{117} variants was well correlated with the size of the substitutions (Table I), with the exception of G117H, which will be discussed below. This result indicates that the portion of the heme pocket created by Gly^{117} is important for the ligation structure and CO-sensing function of CooA.

*Cys^{75}, but Not Pro^{2}, Is a Ligand in Fe(III) G117I CooA*—As shown in Fig. 2B, the UV-visible spectrum of purified Fe(III) G117I CooA is typical for a thiolate-ligated five-coordinate high spin heme with a Soret peak at 407 nm and ligand-to-metal charge-transfer peak appearing at ~640 nm. The EPR spectrum of Fe(III) G117I CooA corroborated the results of the UV-visible spectrum, displaying only high spin signal with a charge-transfer peak appearing at 640 nm. The rhombicity (expressed as E/D (26)) of G117I CooA is comparable to that of a model heme-thiolate complex (Table II). On the other hand, the rhombicity of G117I CooA is significantly higher than those of nitrogen-based ligand high spin signal such as FixL (30) and soluble guanylyl cyclase (31) (Table II).  

As controls, two other variants were constructed: G117I H77A and G117I ΔP3R4. H77A CooA lacks the normal His ligand of the Fe(II) form of WT CooA, and ΔP3R4 CooA did not show any spectral difference with and without 5 mM DTT. The visible ranges of the spectra were multiplied by five for better display.

### Table I

| CooA          | Soret maximum | Soret peak ratio | β-galactosidase activity | Molec Vol | Accumulation |
|---------------|---------------|------------------|--------------------------|-----------|--------------|
|               | Fe(III)       | Fe(II)           | Fe(II)-CO                | Ox^{-}    | Red^{2} -CO  | Red^{2} + CO | Ratio       | Mole Vol | Accumulation |
| pKK223-3      | 422           | 424.5            | 422                      | 0.5       | 0.5          | 1            | 0           |          |              |
| WT            | 423.5         | 424.5            | 422                      | 2         | 3            | 100          | 33.3        | 60.1     | 100          |
| G117A         | 387           | 425              | 387                      | 9         | 13           | 16           | 1.2         | 140.0    | 30           |
| G117I         | 387           | 425              | 387                      | 1         | 3            | 16           | 28          |          |              |
| ΔP3R4         | 387           | 425              | 387                      | 2         | 2            | 70           | 5           |          |              |
| G117A ΔP3R4   | 387           | 425              | 387                      | 2         | 7            | 121          | 10          |          |              |
| G117I H77A    | 387           | 417              | 419                      | 0.82      | 1            | 69           | 10          | 11       | 8            |
| G117I H77A    | 387           | 417              | 419                      | 0.82      | 1            | 69           | 10          | 11       | 8            |

A % activity indicates the mean value of multiple measurements of activity relative to that of WT CooA in the presence of CO, and showed variability ~10%.

% accumulation indicates the heme accumulation of variant CooA in cell-free extracts variant relative to that of WT CooA, based on the spectra of CO-bound form.

* Aerobically grown cells were used for the activity.

* Anaerobically grown cells were used for the activity.

* Molecular volume (35).

* Purified sample was used for measurement of Soret maximum.

* For comparison, A_{Fe(II)}, for WT CooA was obtained from the UV-visible spectrum at pH 3.4 and A_{Fe(III)} and A_{Fe(II)-CO} for WT CooA were obtained from those at pH 7.4. The same amount of WT CooA was used for the UV-visible spectra at both pH 3.4 and 7.4.

* WT indicates the same Soret maximum and peak shape as WT CooA.

* Hydroxyapatite batch-enriched CooA sample was used for Soret maximum.

* Indicates reduced intensity in Soret band compared to WT CooA.

* ND, not detectable.
However, unlike G117I CooA, the UV-visible spectrum of Fe(III) ∆P3R4 CooA at pH 7.4 exhibited a mixed-spin state with a five-coordinate high spin heme as a major population, indicated by a 387-nm Soret peak and small amount of six-coordinate low spin heme, indicated by a shoulder at 423 nm (Fig. 2D). EPR spectra of Fe(III) ∆P3R4 CooA showed two kinds of high spin signals, in which the major one roughly corresponded to that of Fe(III) G117I CooA, whereas the minor one was similar to that of other high spin thiolate-ligated hemes such as P-450cam, endothelial nitric oxide synthase, and chloroperoxidase (Table II). Unlike Fe(III) G117I CooA, the spin state of Fe(III) ∆P3R4 CooA was found to be pH-dependent based on UV-visible (data not shown) and EPR spectra (Fig. 3). Finally, the open coordination site of Fe(III) ∆P3R4 CooA was accessible to exogenous imidazole and CN⁻ as judged by the changes in the UV-visible spectrum (data not shown). Thus, although Pro² ligation appears to be perturbed in the Fe(III) forms of both G117I and ∆P3R4 CooA, the different effects of pH and exogenous ligands on the spectra indicate that the perturbations are not identical.

Pro² Ligation Is pH-dependent in Fe(III) WT CooA—We had previously found that WT CooA appeared to start precipitating as the pH was lowered to ~6, so our ability to lower the pH of G117I CooA to 3.4 initially surprised us. We therefore wondered if WT CooA could also be analyzed at this pH and found that a rapid transition to a lower pH allowed WT CooA to remain in solution. We therefore asked how low pH perturbed the ligation state of WT CooA. As shown in Fig. 4A, as the pH was decreased from 7.4, the fraction of five-coordinate high spin form of Fe(III) WT CooA increased as indicated by the Soret band increase at 387 nm in the UV-visible spectrum. Above pH 7.4, no significant spectral changes were observed, which is consistent with a previous study (7). Fitting the absorbance at 387 nm as a function of pH using the Henderson-Hasselbalch equation showed a single protonation process with a pKᵣ of ~5.5 (Fig. 4B). This is most easily rationalized by supposing that one of the ligands being protonated and displaced at acidic pH. To identify the retained ligand at lower pH, we measured the EPR spectrum of Fe(III) WT CooA at pH 3.4. As shown in Fig. 3 and Table II, the EPR spectrum of Fe(III) WT CooA at pH 3.4 displayed a high spin rhombic symmetry with g values of gz = 2.29, gx = 4.99, and g₀ = 1.92 similar to that of Fe(III) G117I CooA, suggesting that the retained ligand of Fe(III) WT CooA at this pH is Cys⁷⁵, not Pro². Our apparent ability to protonate Pro² at low pH and cause its deligation suggests that it is the neutral form of Pro that serves as the normal ligand, in at least the Fe(III) form. The EPR spectrum of Fe(III) WT CooA also exhibited a minor high spin signal having axial symmetry with g values of gz = gₓ = 2.6 and g₀ = 2 (Table II), which might reflect free heme. 

His²⁻ Is a Retained Ligand and Pro² Is a Perturbed Ligand in Fe(II) G117I CooA—The known CooA Fe(II) structure (8) suggested that bulky substitutions at position 117 might produce steric hindrance between Pro² and the substituted residue, so we anticipated that the Fe(II) form of G117I CooA would be highly perturbed. As expected, the UV-visible spectrum of Fe(II) G117I CooA is significantly different from that of Fe(II) WT CooA, characterized by a reduction of the peak intensity in Soret, α, and β bands with a concomitant appearance of a shoulder at ~440 nm (Fig. 2B), which suggests a five-coordinate high spin Fe(II) heme. To test this hypothesis, we investigated the effect of pH on spectral features and the ability of exogenous ligands such as imidazole and CN⁻ to bind the heme iron. As shown in Fig. 5B, upon raising the pH to 9.5 the peak intensity in Soret, α, and β bands was increased at the expense of the ~440-nm shoulder. Also, addition of imidazole (Fig. 5A)
Henderson-Hasselbach equation.

CooA in pH 7.4 (\(pH_{77A}\)) and pH 9.5 (\(pH_{9004}\)), respectively. Dotted lines indicate the changes in the Soret peak corresponding to a decrease in pH.

Spectral changes in Fe(II) G117I CooA as a result of \(\Delta\)P3R4 with those of G117I H77A CooA (Fig. 2B). The summary listed in Table I shows changes in the UV-visible spectra. These results are consistent with the existence of an open coordination site in Fe(II) G117I CooA, and that Pro2 is the perturbed ligand, in Fe(II) G117I CooA.

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or CN\(^-\) (Fig. 5B) to Fe(II) G117I CooA produced significant changes in the UV-visible spectra. These results are consistent with the existence of an open coordination site in Fe(II) G117I CooA, strongly suggesting that the Fe(II) form of G117I CooA contains a significant amount of five-coordinate heme and that Ile\(^{117}\) does not preclude exogenous ligands in this redox state, in contrast to the Fe(III) state.

The two ligands in Fe(II) WT CooA are Pro\(^2\) and His\(^{77}\) (8). To identify the retained and perturbed ligand of Fe(II) G117I CooA, we compared the UV-visible spectrum of Fe(II) G117I CooA with those of G117I \(\Delta\)P3R4 (data not shown) and G117I H77A CooA (Fig. 2D). The summary listed in Table I shows that the UV-visible spectrum of Fe(II) G117I CooA corresponds exactly to that of G117I \(\Delta\)P3R4 CooA but is markedly different from that of G117I H77A CooA. This result suggests that His\(^{77}\) is the retained ligand, and that Pro\(^2\) is the perturbed ligand, in Fe(II) G117I CooA.

Bulk Residues at Position 117 Do Not Appear to Interact with Bound CO—In contrast to the significant perturbation of ligation states in Fe(III) and Fe(II) G117I CooA, the UV-visible spectrum of Fe(II)-CO G117I CooA was highly similar to that of WT CooA (Fig. 2B), with a similar \(A_{Fe\text{-CO}}/A_{Fe\text{III}}\) ratio (Table I). This similarity is surprising, given the extreme functional perturbation seen \(in\) \(vivo\) (Table I). To test the hypothesis that bulky residues at position 117 interact with the bound CO but fail to perturb the UV-visible spectrum, we performed a preliminary resonance Raman spectroscopic analysis of purified G117H CooA. We reasoned that G117H has a similar bulk, as does G117I, and causes similar effects on \(in\) \(vivo\) activity (Table I), but histidine is more likely to reveal an interaction between the position 117 residue and the CO in resonance Raman spectroscopy. The analysis showed the same \(v_{Fe\text{-CO}}\) stretching band (487 cm\(^{-1}\)) as did WT CooA,\(^2\) suggesting that the bound CO in G117H CooA is in a similar environment as it is in WT CooA and is therefore apparently not binding to the opposite side of the heme as has been proposed to be the basis for the inactivity of H77Y CooA (13). The absence of an effect by His\(^{117}\) itself on this frequency indicates that this introduced residue does not interact effectively with the bound CO. We did not pursue resonance Raman analysis of G117I CooA because its frequency shift of the \(v_{Fe\text{-CO}}\) stretching band would be much lower than that of G117H CooA. This result implies that the lack of CO-sensing function of G117H CooA (and possibly G117I CooA) is not caused by either hindering the CO-binding process or changing the environment of bound-CO.

In Vitro DNA Binding Assay Shows that the Fe(II)-CO Form of G117I Is Functionally Perturbed in DNA Binding, Whereas the Fe(III) Form Binds DNA—Because the \(in\) \(vivo\) activity is measured under the conditions of excess amounts of CooA (10), it is not a realistic measure of CooA specific activity. Furthermore, activity in the \(in\) \(vivo\) assay system requires proper interaction of CooA with RNA polymerase in addition to DNA binding. To examine the DNA-binding property of G117I CooA only under a condition where CooA is limiting, we carried out an \(in\) \(vitro\) DNA binding assay of purified G117I CooA using the

### Table II

| Proteins                     | \(\delta_x\) | \(\delta_y\) | \(\delta_z\) | \(E/D\) | \(\delta_x\) | \(\delta_y\) | \(\delta_z\) | \(E/D\) | Reference |
|-----------------------------|--------------|--------------|--------------|--------|--------------|--------------|--------------|--------|-----------|
| WT CooA (pH 7.4)            | ND\(^a\)     | ND           | ND           |        | ND           | ND           | ND           |        |           |
| WT CooA (pH 3.4)            | 7.29         | 4.99         | 1.92         | 0.048  | 2.46         | 2.25         | 1.89         | 0.093  |           |
| G117I CooA (pH 7.4)         | 6.0          | 6.0          | 2.0          | 0      | ND           | ND           | ND           |        | This work |
| \(\Delta\)P3R4 CooA (pH 7.4)| 7.15         | 5.02         | 1.93         | 0.044  | ND           | ND           | ND           |        | This work |
| \(\Delta\)P3R4 CooA (pH 10.2)| 8.07         | 3.61\(^c\)   | 1.71\(^d\)   | 0.093  | 2.45         | 2.26         | 1.90         | 0.050  |           |
| Fe\(PPX(DME)(SC_6H_4Cl)\)\(^e\)| 7.2         | 4.8          | 1.9          | 0.069  | ND           | ND           | ND           |        | 27        |
| eNOS                        | 7.67         | 4.34         | 1.87         | 0.081  | ND           | ND           | ND           |        | 26        |
| P-450                        | 7.55         | 3.97         | 1.75         | 0.069  | ND           | ND           | ND           |        | 29        |
| Chloroperoxidase             | 7.5          | 4.3          | 1.8          | 0.069  | ND           | ND           | ND           |        | 30        |
| FxL                          | 6.16         | 5.75         | 1.99         | 0.009  | ND           | ND           | ND           |        | 31        |

\(^a\) E/D is used as a measure of rhombicity.

\(^b\) ND, not detectable.

\(^c\) Calculated value.

\(^d\) NT, not determined.

\(^e\) PPIX(DME) = protoporphyrin IX dimethyl ester; \(SC_6H_4Cl\) = \(p\)-chlorobenzenethiol.

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\(^2\) R. D. Williams and T. G. Spiro, unpublished data.
fluorescence polarization method. As shown in Fig. 6A, the Fe(II)-CO form of purified G117I CooA showed only negligible DNA binding above 1 μM CooA. The failure of G117I CooA to bind DNA in the presence of CO is not due to a poor affinity for CO; consistent with its open heme coordination state, its $K_d$ for CO is actually lower than that of WT (data not shown). This result indicates that the functional perturbation of Fe(II)-CO G117I CooA is due to a poor ability to bind target DNA. Surprisingly, Fe(III) G117I CooA showed a concentration-dependent increase of anisotropy value at somewhat high protein concentrations, corresponding to a $K_d$ of 1.8 μM. This property of Fe(III) G117I CooA is different from that of Fe(III) WT CooA (Fig. 6B), implying that some structural change that is caused by the Ile substitution at the Gly117 position partially mimics the DNA-binding conformation of the CO-bound form of WT CooA. Although the Fe(III) form of G117I CooA is much more active than the Fe(II) and Fe(II)-CO forms in terms of in vitro DNA binding, the in vivo activity of Fe(III) G117I CooA is very low, similar to those of Fe(II) and Fe(II)-CO G117I CooA. We were surprised by this low activity, because other variants with this level of affinity for DNA typically display substantial activity in vivo (data not shown). This result therefore suggests that, although the structure of Fe(III) G117I CooA supports DNA binding, it does not allow the proper interactions with RNA polymerase that are necessary for transcription activation (32). It is our hypothesis that CO binding to WT CooA causes conformational changes that properly position the DNA-binding domains and also the regions of CooA that interact with RNA polymerase; we are actively pursuing this latter issue.

We were concerned that the substitutions at position 117 might affect DNA binding indirectly by precluding CooA dimerization, because Gly117 lies on the C-helices that form the dimer interface. However, gel filtration analysis of Fe(III) G117I CooA showed that it migrated as a single peak at the position of dimeric WT CooA (data not shown).

Functional Perturbation of Gly117 Variants Does Not Originate from the Alteration of Pro2 Ligation—Because some of the Gly117 variants appeared to prevent Pro2 from ligation of the heme in the Fe(III) and Fe(II) forms, we investigated the in vitro DNA binding properties of ΔP3R4 CooA. In contrast to the case of G117I CooA, ΔP3R4 CooA showed effective DNA binding in the Fe(II)-CO form, corresponding to a $K_d$ of 158 nM (Fig. 6C). Under the same experimental conditions, Fe(II)-CO WT CooA displayed a $K_d$ of 16.2 nM (Fig. 6B), a value roughly similar to that reported in a previous study (10). These results indicate that the direct perturbation of Pro2 ligation in ΔP3R4 CooA does not eliminate CO-responsive DNA binding but does decrease the affinity of the CO-bound form for DNA, implying that the CO-bound form of ΔP3R4 CooA is not optimal. Although the deletion of Pro2 and Arg2 residues must perturb Pro2 ligation, it is possible that Pro2 is not eliminated as a ligand, and we therefore investigated the activity of a deletion variant, which lacks five N terminus residues (from Pro3 to Ile7) and is highly unlikely to have Pro2 in the vicinity of the heme. As shown in Table I, ΔP3-I7 CooA still retained some ability to respond to CO in vivo, in contrast to the lack of response in G117I CooA. This phenotype is quite similar to those of variants completely missing Pro2 such as P2Y CooA (10) or CooA ΔN5 (Pro2-Pro5 deleted (11)). This result substantiates the notion that the perturbation of Pro2 ligation seen in some Gly117 variants is not the basis of the lack of function in these variants. The severe functional perturbation of G117A CooA and G117S CooA (Table I) despite apparent normal ligation in the Fe(III) and Fe(II) forms also supports this.

To rule out the steric interaction between the substituted amino acids at position 117 and Pro2 as the basis for the lack of CO-sensing function of Gly117 variants, we also constructed G117A, G117H, and G117I CooA variants in the background of ΔP3R4. Like single Gly117 variants, these Gly117 ΔP3R4 double variants also showed loss of CO-sensing function (Table I). These results suggest that the severe functional perturbation of Gly117 variants is not caused by the possible interaction of substituted residue at the Gly117 position with Pro2.

**DISCUSSION**

A series of Gly117 variants shows a close correlation between the CO-sensing function and ligation states of CooA and the
size of the substituted amino acid at this position, although even an Ala substitution was found to be sufficient for some perturbation of CO-sensing function. Because Gly ranks next to Pro as a helix breaker (33), and even Ala perturbs CO-responsiveness, we cannot completely rule out helix perturbation as a functional aspect of the Gly117 variants. However, we note that there is no helix bending at Gly117 in the solved Fe(II) CooA structure (8). Furthermore, given the fact that Ala is statistically the best helix former (33), but is the second best in terms of CO-responsiveness, the role of Gly117 being a “helix breaker” seems unlikely.

Fig. 7 provides a schematic representation of our proposed ligation states of WT, G117I, and ΔP3R4 CooA. As noted in the figure, ΔP3R4 CooA has a mixture of five- and six-coordinate hemes in both the Fe(II) and Fe(III) states, but the identity of the residues that provide the sixth ligand is unknown; it might be Pro itself, the N terminus, or some adventitious ligand. In contrast, the Fe(III) form of G117I CooA contains only five-coordinate high spin heme with Cys75 as one axial ligand. Upon reduction, G117I CooA undergoes a ligand switch in which His77 replaces Cys75. Given that Pro2 ligation is totally precluded in Fe(III) G117I CooA, Pro2 is not critical for the ligand switch in G117I CooA. In this regard, the relatively low rhombicity of high spin signals in the EPR spectrum of Fe(III) G117I CooA is intriguing. Other high spin thiolate-ligated hemes such as P-450cam, endothelial nitric oxide synthase, and chloroperoxidase display higher rhombicity than G117I CooA and do not undergo any ligand switch. The low level of interaction between the sulfur of Cys75 and iron valence orbitals indicated by the low rhombicity of Fe(III) G117I CooA may make the ligand switch possible upon reduction, but the paucity of other ligand switch examples makes this difficult to test. The Fe(II) form of G117I CooA also contains a significant amount of five-coordinate heme with the Pro2 ligand being highly perturbed. Taken together, the G117I substitution perturbs only the Pro2 ligand and is therefore superficially similar to the situation in ΔP3R4 CooA.

Despite these similarities, the functional properties of G117I and ΔP3R4 CooA are significantly different. Although the G117I CooA shows DNA-binding activity in the Fe(III) form, it does not undergo CO-dependent activation. On the other hand, ΔP3R4 CooA appears to be functionally rather normal in vitro (i.e. only active in the Fe(II)-CO form), even though its DNA-binding affinity in vitro is approximately ten times lower than that of WT CooA. It is therefore unlikely that the lack of CO-sensing function in G117I CooA originates from merely perturbing the Pro2 ligation.

The strictly five-coordinate high spin heme in Fe(III) G117I CooA and the inability of any adventitious/exogenous ligand to access the open coordination site of Fe(III) G117I CooA indicates that the substituted Ile is very close to the heme iron. We assume that this close proximity is the basis for the surprising DNA-binding activity of Fe(III) G117I CooA. It is our hypothesis that in the Fe(III) state of G117I CooA, the heme/C-helix interaction, partially mimics that of heme-CO/C-helix in WT CooA, although by a different specific mechanism. In contrast, the steric interaction of the substitution Ile at position 117 with the CO-bound heme is presumably responsible for the lack of CO-sensing function of G117I CooA.

Smaller substitutions such as Ala or Ser at 117 position do not perturb the ligation states of CooA based on UV-visible spectra. However, the G117A substitution does exert a severe effect on CO-sensing function of CooA as demonstrated by the absence of noticeable DNA binding up to 1 μM heme in the fluorescence polarization assay (data not shown). It is possible that Ala117 of G117A CooA might actually be closer to the heme in the Fe(II)-CO form than in the Fe(II) form and that steric hindrance between the substituted Ala and the heme is responsible for the functional perturbation of G117A CooA. The apparently distinct effects of the G117A substitution on different ligation states of CooA would not be surprising, because it is well established in WT CooA that the heme must move with respect to the protein matrix upon reduction (8).

Taking these results together, we suggest that (i) CO binding is followed by heme or/and the protein matrix movement toward each other, and that the interaction of the heme with the C-helix is crucial for CooA activation; and (ii) this event occurs only when there is a sufficient heme pocket afforded by Gly117. This hypothesis is consistent with the previous prediction based on the structural comparison between effector-free CooA and effector-bound CRP that the repositioning of the two C-helices might be required for CooA activation (8). This proposed close proximity of the heme to the C-helix in the Fe(II)-CO form might create the small CO pocket suggested by the rapid recombination rate of CO rebinding after CO photolysis (34).

The results with G117H CooA are surprising. Although this variant is functionally similar to G117I CooA (Table I), it is spectrally distinct, showing apparently normal UV-visible spectra under all conditions. Although we do not know the basis for this difference, it is our working hypothesis that His117 is serving as a ligand in this variant, a function that would not be possible for the similarly sized Ile117.

In summary, this study shows that the heme pocket afforded by Gly117 is crucial for proper ligation and activity of CooA. The molecular basis for the functional perturbation in G117I CooA is probably steric interaction between the substituted Ile and heme, which might hinder heme movement and proper interaction with the C-helix required for CooA activation after CO binding. It remains to be determined if the CO bound to the heme is directly involved in such proper interaction.

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