Characterization of Variants Altered at the N-terminal Proline, a Novel Heme-Axial Ligand in CooA, the CO-sensing Transcriptional Activator

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CooA, the carbon monoxide-sensing transcription factor from Rhodospirillum rubrum, binds CO through a heme moiety resulting in conformational changes that promote DNA binding. The crystal structure shows that the N-terminal Pro2 of one subunit (Met1 is removed post-translationally) provides one ligand to the heme of the other subunit in the CooA homodimer. To determine the importance of this novel ligand and the contiguous residues to CooA function, we have altered the N terminus through two approaches: site-directed mutagenesis and regional randomization, and characterized the resulting CooA variants. While Pro2 appears to be optimal for CooA function, it is not essential and a variety of studied variants at this position have substantial CO-sensing function. Surprisingly, even alterations that add a residue (where Pro2 is replaced by Met1-Tyr2, for example) accumulate heme-containing CooA with functional properties that are similar to those of wild-type CooA. Other nearby residues, such as Phe6 and Asn6, appear to be important for either the structural integrity or the function of CooA. These results are contrasted with those previously reported for alteration of the His77 ligand on the opposite side of the heme.

The sensing of dissolved gas molecules by proteins in biology has recently attracted considerable biochemical interest. The role of nitric oxide in a variety of important biochemical processes (1, 2), and its receptor, soluble guanylyl cyclase (sGC)1, has recently attracted considerable biochemical interest. Numerous studies have clearly demonstrated the physiological importance of CO in a wide variety of processes (9–11), and although sGC has been implicated in sensing CO (12–14), direct evidence of a CO-receptor in eukaryotic signal transduction systems is lacking. CooA senses CO through a heme moiety and represents the current model system for biological CO-sensing. CooA belongs to the cAMP receptor protein (CRP) and fumarate and nitrate reductase activator protein superfamily of transcriptional activators (15). CooA is a non-homodimeric protein, with each monomer possessing a heme that is six-coordinate under all oxidation and ligation states (16). Interestingly, an unusual redox-mediated axial ligand exchange occurs in CooA in that a cysteine ligand (Cys75) in the oxidized (FeIII) form is replaced by a histidine ligand (His77) upon reduction of the heme-iron to the FeII form (17–20). The identification of the axial ligand trans to the Cys75/His77 pair, which is believed to be provided by the protein based on the observation that the FeII and FeIII forms of CooA are six-coordinate and low-spin, had been unclear although spectroscopic studies have suggested a neutral nitrogen ligand (19, 20). Finally, the ligand that is displaced upon binding CO remains speculative.

Recently, the three-dimensional structure of FeII CooA has been solved by x-ray diffraction techniques (21). This report showed that the general folding topology of CooA was indeed similar to that of CRP (22). In addition to the verification of His77 as one of the heme-axial ligands in FeII CooA, inspection of the structure identified the other axial ligand as an N-terminal proline residue (Pro2; Met1 is removed by processing) from the other subunit of the dimer. This structural environment represents an unprecedented axial ligation arrangement for a heme protein.

In a previous study (18), we altered His77 and found that the UV-visual spectra of these variants was normal in the FeII form (when Cys75 is the ligand on that side of the heme) but was perturbed in the FeIII form, when His77 would normally be the ligand. Surprisingly, while the UV-visual absorption spectra of the FeII-CO forms of these His77 variants was similar to...
that of wild type, they were unable to undergo the conformational change in response to CO that is necessary for DNA binding (18). In addition, His$^{77}$ variants in the Fe$^II$ form bind cyanide with high affinity (23). Alteration of His$^{77}$ therefore perturbs the Fe$^II$ form of CooA as well as its ability to properly respond to CO binding. In this present study, we have altered the other side of the heme face in Fe$^III$ CooA, i.e. the Pro$^2$ ligand and contiguous residues, and have found that variants in that region show surprising functional similarity to WT CooA. We then purified a selected variant and characterized its biochemical parameters in detail. Implications concerning the mechanism of activation in the WT CooA protein are discussed.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The construction of *E. coli* strains expressing WT CooA, CooA variants altered in the region of Pro$^2$ by site-directed mutagenesis, and of strains expressing WT CooA and CooA variants in combination with a chromosomally encoded β-galactosidase reporter system have been described previously (18). Site-directed variants were constructed in a pKK233–3-based expression plasmid (24), while region randomization variants were constructed in a pEXT20-based expression plasmid (25) as the screening methodology (described below) depends on the tighter control of CooA expression provided by this vector. In *β-galactosidase Activity Assay—Strains containing the P$_{cooF}$–lacZ reporter fusion were grown in rich medium containing 100 μg/ml ampicillin (18). These inocula were then diluted 40-fold into anaerobic 120-ml stopped serum vials containing 20 ml of MOPS-buffered medium (23) supplemented with 100 μg/ml ampicillin and 25 μg isopropyl-β-D-thiogalactopyranoside. For CO-induced cultures the headspace was made 2% (v/v) with CO gas. Cultures were grown with shaking at 30 °C to mid to late-log phase (A$_{600}$ 0.7 to 1.5), cell pellets were prepared and frozen, and β-galactosidase activities measured (26).

"Region Randomization" of the Pro$^2$ Region—To determine the functional importance of residues near the N terminus of CooA, as well as to create some novel variants for future analysis, we employed an unusual mutagenic approach of the complete randomization of certain portions of the cooA gene. This approach will be described in more detail elsewhere, but the salient features are the following. A library of cooA-expressing clones was created, by PCR amplification with an oligonucleotide fragment bearing the randomized region (described below) and then the resulting cooA library was ligated into pEXT20 and transformed into the P$_{cooF}$–lacZ reporter host. The resulting clones were screened for CooA-dependent expression of β-galactosidase. A primer was synthesized with a mixture of all four nucleotides at each position, used in the first round of PCR, a second primer was used with the primers were synthesized with a mixture of all four nucleotides at each position, used in the first round of PCR, a second primer was used with the randomization primer to create a pool of DNA fragments, each completely random at the same multiple specified positions (i.e. the positions coding for residues 2–3 or 2–6) but otherwise encoding a WT portion of CooA; for technical reasons, the template contained a frameshift mutation within the region to be randomized. In the second round of PCR amplification, a second pair of primers was used to produce a DNA fragment bearing the remainder of WT CooA; this fragment overlapped with a WT tail of the randomized fragment. The fragments from the first and second rounds of PCR were then annealed at their overlapping regions, extended, and amplified in a third round of PCR, resulting in a pool of DNA fragments each containing the entirety of cooA with a randomized region of interest. Restriction sites incorporated at the ends of the primer pairs allowed directional ligation into the aforementioned β-galactosidase reporter host (UQ1639). Transformed cells were spread on 1× MOPS plates (23) and active variants were identified after anaerobic growth in the presence of 1% CO at 30 °C and were retested for CO-dependence. The randomized regions of these variants were sequenced.

**Purification of WT and P2Y CooA**—The purification of WT CooA and the P2Y variant (>95% homogeneity) were performed using procedures described previously (18). The heme content of CooA preparations were quantified using the reduced pyridine-hemochromogen method (28). N-terminal analysis of purified P2Y CooA was performed at the Macromolecular Structure Facility of Michigan State University.

**UV-visible Absorption Spectroscopy of Cell-free Lysates**—Preparation of cell-free lysates and their analysis by UV-visible absorption spectroscopy from *E. coli* strains expressing WT and variant CooAs were as described (18).

**Approximate Comparisons of CooA Accumulation**—Cell pellets from 1.5-ml culture were lysed enzymatically at room temperature for 20 min in 1 ml of 4% Triton X-100, 7.5, 10 mM EDTA, 1 mg/ml lysozyme, and subsequently trented with DNase for 10 min in this buffer amended to 100 mM NaCl, 20 mM MgCl$_2$, and 5 μg/ml DNase I. Samples were centrifuged at 20,000 × g for 10 min. The Soret peak intensity, corrected for α -CooA extract blank, was measured in 150 μl of supernatant and compared with the accumulation of WT CooA.

**UV-visible Absorption Spectroscopy of Isolated Proteins**—In this study, all spectra were acquired as described (29). Dependence of the spectra of P2Y Fe$^{III}$ CooA on the pH of the solution were performed using ~4 μM isolated P2Y Fe$^{III}$ CooA in the following buffers: pH 6.5, 7.0, and 7.5; 0.1 M MOPS, pH 8.0, 8.5, and 9.0; 0.1 M Tris HCl, pH 9.5, 10.0; and 0.1 M glycine/NaOH. Absorbance changes as a function of pH in the 388-nm Soret peak (high-spin component) and in the 420-nm Soret peak (low-spin component) were used to estimate the pK$_a$ of the spectral transitions.

**Electron Paramagnetic Resonance Spectroscopy—Isolated P2Y Fe$^{III}$ CooA**—was buffered by mixing 200 μl of the isolated protein (145 μM) with 50 μl of 500 mM buffer (pH 7.0, MOPS, and pH 10.0, glycine/NaOH) to provide a final buffer concentration equal to 100 mM and a final heme concentration ~120 μM. Samples were then degassed on an argon manifold to remove dissolved oxygen, frozen, and stored at 77 K. Spectra were recorded on a Varian E-15 spectrometer, with an Oxford Cryostat 3120 system to monitor and regulate the temperature. The magnetic field was measured using a Varian 929801 gaussmeter with a Tektronix type RM 503 oscilloscope and the microwave frequency (9.2 GHz, X-band) was monitored using a Hewlett Packard 5252A frequency counter. The field at 4 K and 200 microwatt power for the low-field region (30–2030 Gauss), and at 23 K and 50 microwatt power for the high-field region (1850–3850 Gauss). Spectral analysis was performed using a "rombogram" computer program$^2$ for the calculation of theoretical g values.

**Fluorescence Polarization Assay for DNA Binding**—We have developed an assay for detecting DNA binding by the CooA-COO complex, based on fluorescence polarization (FP) (30, 31), in order to test quantitatively the activity (i.e. DNA binding in response to CO binding to the heme of CooA) of our CooA protein preparations. The extent of FP of a freely tumbling molecule that has been conjugated with a fluorescent tag is directly related to molecular volume when viscosity and temperature are held constant. The FP technique exploits changes in the molecular volume of a target molecule as a result of macromolecular interactions. Thus, the extent of FP of a fluorescently labeled DNA fragment (containing the promoter sequence of cooF, termed P$^{cooF}$) increases upon binding by active CooA. The value of FP is measured as anisotropy, which is a dimensionless parameter related to the intensity of parallel and perpendicular absorbed light components compared with the total intensity (31). The FP technique has been applied successfully to other DNA-binding protein systems, including CRP (32) and the tryptophan repressor (33). The following conditions were arrived at after testing effects of ionic strength, cation concentration, and protein and probe concentration (data not shown).

Equimolar amounts of two complementary 26-base pair oligonucleotides (containing the cooF promoter, P$^{cooF}$ -5′- AAACTGTCATCTTGCG- CGCACAGCCG-5′), one of which was 5′-end-labeled with Texas Red (Genosys), were mixed and heated at 95 °C for 2 min in a water bath and then allowed to anneal by cooling slowly to room temperature over a 2-h time period. Sufficient hybridization (>90%) was verified by resolving the double-stranded probe using non-denaturing polyacrylamide gel electrophoresis (10% (w/v); in Tris borate-EDTA, pH 8.0) against a 26-base pair single-stranded oligonucleotide control. Binding assays were performed by adding aliquots of protein and their respective final concentrations. Tris-HCl, pH 8.0 (20 mM), CaCl$_2$ (6 mM), KCl (50 mM), glycerol (5% (v/v)) and dithiothreitol (1 mM). The DNA probe (6.4 nM) and varying concentrations of purified CooA samples (~0.5–800 nM) were mixed in 5 × 60-mm test tubes that were fitted

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$^2$ W. R. Hagen, *Rhombus*, version 1.0.
Properties of CooA variants altered in the axial ligands through site-directed mutagenesis

| CooA   | N-terminal sequence | % Accumulation | % β-Gal | (FeIII) Soret (A423/A393) |
|--------|---------------------|---------------|---------|--------------------------|
| pKK23  | PPRFN               | 0             | 1       | 2                       |
| WT     | PPRFN               | 100           | 1       | 2                       |
| P2Y    | VPRFN               | 20            | 3       | 105                      |
| P2H    | MPRFN               | 60            | 5       | 85                       |
| P2Y    | MYPRFN              | 55            | 2       | 76                       |
| P2C P3C| CPRFN               | 5             | 7       | 99                       |
| ΔP3    | PRFN                | 20            | 5       | 107                      |
| ΔP3 ΔR4| PPRFN               | <5            | 1       | 93                       |
| R4A    | PPAFN               | 15            | 7       | 131                      |
| R4S    | PFPFN               | 5             | 2       | 67                       |
| H77F   | PPRFN               | 15            | 2       | 44                       |
| H77Y   | PPRFN               | 15            | 32      | 30                       |
| H77W   | PPRFN               | 15            | 37      | 37                       |
| H77A   | PPRFN               | 15            | 10      | 11                       |
| H77G   | PPRFN               | 15            | 5       | 5                        |
| H77C   | PPRFN               | 15            | 5       | 6                        |
| H77M   | PPRFN               | 15            | 11      | 12                       |
|        |                     | 70            | 12      | 12                       |

- Predicted protein sequence of residues at the N terminus, although the FN residues that are present in all these proteins.
- % accumulation in extracts relative to that of WT CooA based on the reduced spectrum for Pro2 region variants and based on the oxidized spectrum for the His77 variants (rounded to the nearest 5%).
- β-Galactosidase levels are expressed as a percent of that of WT CooA in the presence of CO.
- ND, not determined.
- All His77 variants have the N-terminal of WT CooA.

RESULTS AND DISCUSSION

Analysis of CooA Variants Altered in the Pro2 Region of CooA—Because of the novelty of the Pro2 ligand, we created a small set of CooA variants that were expected to perturb that ligand. It is known that the terminal Met is removed when the second residue is small, but not when it is large (34), so a variety of changes were made as noted in Table I.

Initial in vivo analyses of these variants, using a CooA-dependent β-galactosidase reporter system, showed that these Pro2 region variants all retained CO-responsive CooA activity (Table I). Because the levels of WT CooA in the reporter strain are in excess of that necessary to fully saturate the reporter, CooA variants with poor accumulation but high specific activity are readily detected. By this assay, all Pro2 region variants with poor accumulation but high specific activity are in excess of that necessary to fully saturate the reporter, (Table I). Because the levels of WT CooA in the reporter strain are in excess of that necessary to fully saturate the reporter, CooA variants with poor accumulation but high specific activity are readily detected. By this assay, all Pro2 region variants with poor accumulation but high specific activity are in excess of that necessary to fully saturate the reporter, (Table I). This spectral feature is further described below with a purified protein.

These results clearly indicate that, while Pro2 appears to be optimal for heme accumulation and activity, a wide variety of alterations of the N terminus allow significant accumulation of heme-containing protein and retention of CO responsive activity. The observation that CooA variants altered at either His77 or Pro2 display some fraction of six-coordinate, low-spin heme, suggests that at least one adventitious ligand is available on each side of the heme and this issue is discussed later.

Functional Requirements for CooA Residues 1–6—The site-directed mutagenesis results provide an insight into the requirements for the N terminus, but were necessarily limited to the analysis of a small number of changes. We therefore employed a different approach, which we term region randomization, in which a library of cooA-containing clones was created in which codons 2–6 (or, in a second analysis, codons 2–3) were completely randomized as described under “Experimental Procedures.” Clones expressing CO-responsive CooA were screened for those that accumulated detectable levels of heme-containing CooA (based on cell pellet color compared with that of WT), and the cooA gene sequenced.

In the randomization of codons 2–6, approximately 39,000 randomized clones were screened and 46 displayed a reasonable level of β-galactosidase in response to CO. These were screened for detectable heme accumulation and the five clones with readily detectable heme-containing CooA are shown in Table II. While screening 39,000 clones does not test all possible amino acid sequences when 5 codons are randomized, it does reveal positions where there are, or are not, strong selections for heme accumulation and CO-responsiveness. In the randomization of codons 2–3, a much smaller number of clones was screened to obtain those in Table II, but technical problems

with rubber septa. Samples were then reduced by the addition of sodium dithionite to 2 mM. Binding assays were initiated by the addition of a small volume of CO-saturated water determined to fully saturate CooA, followed by incubation at room temperature for 5 min to achieve equilibrium. Fluorescence polarization (anisotropy) was measured at 25 °C using a Beacon 2000 fluorescence polarization detector (PanVera Corp., Madison, WI) using 594 nm excitation and 620 nm emission filters designed for measuring the Texas Red fluorescence. Dissociation constants (K_d) were calculated by non-linear curve fitting of the binding data (corrected for quenching) using the scheme of Lundblad et al. (31).
although not critical. We subsequently constructed the nature of the residue at this position is of utility and significant CO responsive activity (Table I), suggested variants). These variants displayed modest heme accumulation and significant heme propionate (21). We therefore anticipated that this residue might simply be a structural requirement. Among randomized variants is unknown and the possibility of more than one such position 6, although we cannot exclude clearly been selected at position 6, although we cannot exclude because of the limited sample size. The reason for the apparent preference for these residues may be structural as the crystal structure (21) shows that position 6 is the start of a helix and the crystal structure suggests that the presence of this residue might simply be a structural requirement. Among randomized variants that had significant function, but relatively less heme, other aromatic residues were common at this position 2 and 3, even those that cause the addition of a residue near the N terminus suggests that this might prevent an estimation of frequency.

The results from both randomizations demonstrate that a variety of possibilities are satisfactory as positions 2 and 3 (Table II). It is important to note that bulky residues at position 2 are also expected to retain the Met1, so that these functional clones differ in N-terminal length as well as specific sequence. The crystal structure shows that Arg4 is about 4 Å from a heme propionate (21). We therefore anticipated that this residue would be important and were surprised by its absence among randomized clones (Table II, 2–6 randomization, and data not shown). We therefore created the variants R4A and R4S (as R4S was fairly common among the functional randomized variants). These variants displayed modest heme accumulation and significant CO responsive activity (Table I), suggesting the nature of the residue at this position is of utility although not critical. We subsequently constructed ΔPro2ΔArg4, whose heme accumulation and activity are consistent with this hypothesis (Table I).

Phe5 was clearly selected for in the codons 2–6 randomization and the crystal structure suggests that the presence of this residue is less likely in these variants.

Spectral Analysis of FeII and FeII-CO P2Y CooA. Spectra of FeIII forms (panel A), FeII forms (panel B), and FeII-CO forms (panel C) of proteins were acquired according to “Experimental Procedures.” Protein concentrations were ~5 μM in heme.

### Table II

| N-terminal sequence | % Accumulation | % β-Gal | −CO | +CO |
|---------------------|----------------|---------|-----|-----|
| pEXT20 (−c ooA)     | 0              | 1       | 1   |     |
| WT (PRFN)           | 100            | 1       | 100 |     |
| TS(RFN)             | 40             | 2       | 88  |     |
| MRS(RFN)            | 10             | 1       | 56  |     |
| VS(RFN)             | 5              | 2       | 89  |     |
| MLS(RFN)            | 30             | 1       | 83  |     |
| MIK(RFN)            | 15             | 3       | 120 |     |
| PM(RFN)             | 5              | 1       | 80  |     |
| MHEFS               | 15             | 2       | 93  |     |
| GPEFS               | 5              | 11      | 136 |     |
| VCSFN               | 5              | 1       | 74  |     |
| GSFN                | 40             | 3       | 98  |     |
| GHI F               | 5              | 51      | 141 |     |

* Predicted protein sequence of residues the N terminus. For most variants, residues 2–6 are shown, but Met1 is shown where the sequence predicts its retention. The randomization of codons 2–3 (middle data set) does not perturb positions 4–6 and these are shown in brackets.

**% accumulation relative to that of WT CooA based on the reduced spectrum for Pro2 region variants.**

β-Galactosidase levels are expressed as a percent of that of WT CooA in the presence of CO.
Pro2 region variants have substantial functionality

WT FeII-CO CooA is lower than that of (cAMP) 1-CRP (the same conditions (data not shown). Although the affinity of in vivo in previous DNase I footprinting experiments (18) and is not

In this experiment, target DNA → 6.4 nm. Line represents data fitted to an equation described in Lundblad et al. (31). Circles represent WT CooA and squares represent P2Y CooA.

FeIII P2Y is some other unknown protein ligand.

Functional Analysis of FeIII-CO P2Y—Because the various Pro2 region variants have substantial functionality in vivo, yet have certainly been perturbed in the vicinity of the heme, we analyzed the functional properties of purified P2Y in greater detail, using a quantitative assay for CooA binding to the PcooF promoter DNA sequence based on fluorescence polarization. When very low amounts of purified WT FeII-CO CooA, which had been shown previously to be active in DNase I footprinting experiments (16), are added to the assay, the value for anisotropy is relatively low (Fig. 2). Upon addition of higher concentrations of WT FeII-CO CooA to the assay, CooA-DNA interactions increase the molecular volume of the complex and the value of anisotropy increases until saturation. The intensity of the Texas Red-labeled DNA samples decreased slightly during the assay with increasing addition of FeII-CO CooA (15% at saturating protein levels; data not shown), which was attributed to fluorescence quenching. Therefore, we included a quench correction factor into our non-linear curve fitting equation according to Lundblad et al. (31). By this analysis, WT FeII-CO CooA binds one of its cognate promoters (PcooF used in this study) with a reasonable affinity (\(K_a = 12.7 \pm 2.3 \text{ nM}, K_d = 7.9 \times 10^7 \text{ M}^{-1}\)), while FeII-CO P2Y CooA has an order of magnitude decrease in its affinity (\(K_a = 102 \pm 3.2 \text{ nM}, K_d = 9.8 \times 10^6 \text{ M}^{-1}\)).

Analysis of P2Y FeIII CooA—Although the FeIII form of CooA is not involved in DNA binding, its analysis will be important as it is critical for the proper redox-dependent ligand switch in CooA (18). The perturbation of the UV-visible spectra (Tables I and II, Fig. 1) is also consistent with the view that changes in the Pro2 region perturb FeIII CooA.

In the UV-visible absorption spectrum of P2Y FeIII CooA at pH 7.4 (Fig. 1A), a spin equilibrium is evident in that there is a split Soret peak at 388 and 423 nm, denoting five-coordinate, high-spin and six-coordinate, low-spin fractions, respectively. In addition, there is an enhanced peak with the FeIII P2Y CooA variant at 640 nm (relative to that of WT CooA), often associated with high-spin, five-coordinate hemes. Low-spin FeII heme proteins, such as cyano-Met sperm whale Mb (35) and b-type cytochromes (36) typically have Soret peaks from 422 to 429 nm, while high-spin FeIII heme proteins, such as those found in catalase (37) and FixL (38) have Soret peaks from 391 to 417 nm. While the data suggests that some fraction of the P2Y FeIII CooA exists as a high-spin, five-coordinate thiolate linkage, other hypotheses are possible and this issue is pursued below with EPR spectroscopy.

We examined if this spin-mixture equilibrium could be perturbed by the pH of the solution. Fig. 3 shows that upon raising the pH of the solution, there is a decrease in the Soret ABS 388 nm (high-spin species) and a concomitant increase in the Soret ABS 423 nm (low-spin species). However, even at pH 10.5, a significant fraction of the five-coordinate, high-spin species remained. The pH titration spectra display an isosbestic point at 410 nm, consistent with a simple two-state transition as a function of pH. We could not measure the effect of pH changes in P2Y FeIII CooA below pH 6.5 as the protein becomes unstable and begins to aggregate (data not shown). These results suggest a simple water-to-hydroxide ligation transition as a function pH in P2Y FeIII CooA. An alternative explanation for the pH dependence of P2Y FeIII CooA is that there is a depopulation of some residue near the vicinity of the FeIII heme that can now act as a ligand in the absence of the natural Pro2 ligand. However, the fact that the curve fitting above pH 8 is relatively poor implies that there is apparently another unknown transition in this region (see below).

EPR spectroscopy of P2Y FeIII CooA corroborated the results with UV-visible spectroscopy. An increase in the pH of the solution resulted in a dramatic decrease in the intensity of high-spin forms of P2Y FeIII CooA and a concomitant increase in the spin-quantity of the low-spin forms (as determined quantitatively by comparison to a CuEDTA standard) from ~0.2 to ~0.8 spins/heme (Fig. 4). Inspection of the high-spin region of
the spectrum of P2Y Fe\textsuperscript{III} CooA revealed the presence of two distinct S = 5/2 systems. Analysis of the g values using a rhombogram analysis (29) for an S = 5/2 system with g = 2 indicates the spectrum is comprised of a system with theoretical g values of \(g_x = 1.63\) (assumed; see below), \(g_y = 3.59\), and \(g_z = 8.07\), and a system with theoretical g values of \(g_x = 1.95\) (assumed; see below), \(g_y = 5.04\), and \(g_z = 6.89\) (Table III). The high-spin system has unusual relaxation properties in that it is only observable at relatively high power and very low temperature (20 microwatts and 4 K). Under these conditions, the low-spin (S = 1/2) features are saturated and appeared as a dispersion line shape that dominated the high-field signals arising from the S = 5/2 system (data not shown). At lower powers and higher temperature (20–50 microwatts, 23 K), the low-spin system can be observed as well defined derivative\-shaped features, although the high-spin features are completely unobservable under these conditions. Therefore, the \(g_x\) values from the high-field region (S = 5/2) systems can only be assumed from the rhombogram analysis.

The EPR data of P2Y Fe\textsuperscript{III} CooA in the low-field region can be interpreted as a mixture of two major ligation states. The g values observed for the two high-spin systems are absent in WT CooA (18) (Table III). The g values for one of the major high-spin systems (\(g_x = 1.63, g_y = 3.59,\) and \(g_z = 8.07\)) are similar to those that are found in Fe\textsuperscript{III} high-spin, five-coordinate thiolate-ligated hemes such as P-450\textsuperscript{cam} (39), H93C myoglobin (39, 40), and the H175C/D235L double mutant of cytochrome c peroxidase (41). The g values for the other major high-spin system (\(g_x = 1.95, g_y = 5.04,\) and \(g_z = 6.89\)) are similar to those found in Fe\textsuperscript{III} high-spin, five-coordinate histidine-ligated hemes such as FixL (38) and sGC (42, 43) and five-coordinate tyrosine-ligated hemes, such as H93Y myoglobin (44) and catalase (37) at low temperatures.

Both of these high-spin signals decrease as a function of pH. At neutral pH, the five-coordinate neutral nitrogen species represents a higher fraction when compared with the five-coordinate thiolate species. However, at high pH, these two species are roughly equivalent. This observation implies that during the pH titration, the five-coordinate thiolate species may be an intermediate to the formation of the low-spin species that predominates at high pH. Because Cys\textsuperscript{75} is the normal ligand in WT Fe\textsuperscript{III} CooA, it is very likely that this residue is the source of the thiolate signal.

The six-coordinate, low-spin system exists in equilibrium with the two five-coordinate, high-spin systems and is evident even at neutral pH. This signal increases with pH and represents a thiolate/strong field ligation, based on the g values that are identical to those of WT CooA (Table III). The identity of the strong-field ligand trans to thiolate can either be H\textsubscript{2}O or hydroxide, which is observed in P-450 (45), Tyr\textsuperscript{2}, or presumably a neutral nitrogen donor ligand provided by another residue in P2Y Fe\textsuperscript{III} CooA. Based on these observations, we believe that there are a number of different liganding forms that exist in equilibrium in P2Y Fe\textsuperscript{III} CooA. While the specific ligands remain unresolved in many of these forms, the results strongly support the notion of extreme flexibility in the heme:protein contacts, as suggested by the analysis of the crystal structure (21).

The spectral data of P2Y Fe\textsuperscript{III} CooA suggested that a portion of the material exists as a five-coordinate species, and therefore

![Fig. 4. EPR spectra of P2Y Fe\textsuperscript{III} CooA at neutral and basic pH. The left panel depicts the low-field region and the right panel depicts the high-field region examined under conditions described under “Experimental Procedures.” Protein concentrations were ~120 μM in heme.](image)

![Fig. 5. UV-visible absorption spectral changes in P2Y Fe\textsuperscript{III} CooA as a result of binding exogenous imidazole. Protein concentration was ~5 μM in heme. Arrows depict spectral changes from increasing additions of imidazole concentrations ranging from 1 to 10 mM.](image)

| Table III | EPR parameters of selected heme proteins |
|-----------|------------------------------------------|
| Protein   | Low-field | High-field | Reference  |
|           | \(g_x\)  | \(g_y\)  | \(g_z\)  | \(g_x\)  | \(g_y\)  | \(g_z\)  |
| P2Y CooA  | 1.62\*   | 3.59     | 8.07     | 1.90     | 2.26     | 2.45     | This work |
| WT CooA   | 1.95\*   | 5.04     | 6.89     | 1.91     | 2.26     | 2.46     | 18        |
| P-450     |           |          |          | 1.91     | 2.25     | 2.44     | 29        |
| HMB H93C  | 1.60     | 3.20     | 8.40     | 39       |
| P-450\textsuperscript{cam} | 1.80     | 4.00     | 7.90     | 40       |
| FixL      | 1.99     | 5.75     | 6.16     | 38       |
| sGC       | 2.00     | 5.16     | 6.36     | 42, 43   |
| H93Y myoglobin | 1.98   | 5.01     | 6.63     | 44       |
| Catalase  | 2.03     | 5.40     | 6.60     | 37       |

* These g values are assumed from the rhombogram analysis (see “Results”).
might be able to coordinate exogenous ligands, which should provide some insight into that open coordination position. Upon addition of exogenous imidazole to ~1000-fold molar excess, there were substantial spectral changes, with an isobestic point at ~410 nm, from a signature of a five-coordinate, high-spin heme to that of a six-coordinate, low-spin heme, indicative of imidazole binding to the heme of P2Y FeIII CooA (Fig. 5). However, the complete conversion of P2Y FeIII CooA to the imidazole-adduct was not observed even at high concentrations (~2000-fold molar excess) as judged by the remaining fraction of the five-coordinate, high-spin form. Interestingly, P2Y FeIII CooA failed to coordinate either cyano or azide even under large molar excess of ligand (1000-fold) and after long (1 h) incubation times (data not shown). Not surprisingly, the P2Y FeIII CooA was inactive in the FP assay in the presence or absence of imidazole (data not shown). This differential binding result is interesting in light of the fact that FeIII P-450cam and FeIII FixL are high-spin, five-coordinate hemeproteins that contain cysteine and histidine ligands, respectively, and bind both cyano and azide (42, 43, 45). These results suggest that charge repulsion by an anionic environment might be the mechanism preventing cyanide and azide binding. However, because we do not know which side of the heme is bound by imidazole in these experiments, it is premature to speculate further on this observation. To address the concern that the five-coordinate form of P2Y FeIII CooA might be an intermediate form that is in the process of losing its heme, stability experiments were performed and the heme of P2Y showed no detectable loss from the FeIII, FeII, or FeII-Co forms (data not shown).

Conclusions—The major conclusions from this work are the following (i) Pro3 has a non-critical role in the response of CooA to CO, as an unknown adventitious ligand can apparently serve as a reasonable substitute. (ii) There is great flexibility in the ligation state of the heme, as revealed by the complex properties of P2Y FeIII CooA, where at least three forms are detected in EPR analysis. (iii) Because of the perturbation of the P2Y FeIII CooA in the Pro3 region variants, it is highly likely that Pro2 serves as the normal ligand in WT FeIII CooA. (iv) While other residues in the vicinity of the N terminus appear to be important for function, such as Phe5 and perhaps Asn6, it is our hypothesis that they are involved in creating a stable heme-containing protein rather than in the actual response to CO; indeed, stabilizing the heme might be a major role of the N terminus of CooA. The results are consistent with the hypotheses proposed with the recent x-ray crystal structure of WT FeII CooA (21), whereby the positioning of the heme upon CO binding is critical for a CO response.

As argued in the paper presenting the crystal structure of WT FeII CooA, it appears highly likely that activation in response to CO involves a movement of the two CooA monomers with respect to each other through a repositioning of the two long helices (termed the “C” helices by analogy with CRP). This repositioning is presumably stimulated in some way by repositioning of the heme of CooA after CO binding. At present, it is unclear whether CO binding displaces His77 or Pro2, but the moderate impairment of function of the CO response of P2Y CooA seen with FP can be rationalized as follows. If His77 is normally displaced, then the adventitious ligand in the Pro2 region variants must be slightly defective in positioning the heme to “signal” the C helix. On the other hand, if Pro2 is normally displaced, then either the adventitious ligand in the Pro2 region variants interferes with the CO-bound heme to prevent proper positioning or the released Pro2 region itself normally affects the C helix and the Pro2 region variants are altered in their ability to perform this interaction.

Acknowledgments—We thank Melissa Killen and John Beck for technical assistance.

REFERENCES

1. Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., and Snyder, S. H. (1993) Science 259, 381–384
2. Brede, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
3. Bursted, J. N., Yu, A. E., Dierks, E. A., Hawkins, B. K., and Dawson, J. H. (1996) Biochemistry 34, 5896–5903
4. Marletta, M. A. (1994) Cell 78, 927–930
5. Gilles-Gonzales, M. A., Ditta, G. S., and Heinlinski, D. R. (1991) Nature 350, 170–172
6. Lukat-Rodgers, G. S., and Rodgers, K. R. (1997) Biochemistry 36, 4178–4187
7. Delgado-Nixon, V. M., Gonzalez, G., and Gilles-Gonzales, M. A. (2000) Biochemistry 39, 2685–2695
8. Kerby, R. L., Ludden P. W., and Roberts, G. P. (1995) J. Bacteriol. 177, 2241–2244
9. Morita, T., Perrella, M. A., Lee, M., and Kourinemans, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1475–1479
10. Morita, T., Mitsialis, S. A., Kioke, H., Liu, Y., and Kourinemans, S. (1997) J. Biol. Chem. 272, 32804–32809
11. Zakhary, B., Poss, K. D., Jaffrey, S. R., Ferris, C. D., Tegnaw, S., and Snyder, S. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14844–14853
12. Denninger, J. W., Schleis, J. P., Brandish, P. E., Zhao, Y., Babcock, G. T., and Marletta, M. A. (2000) Biochemistry 39, 4191–4198
13. Vogel, K. M., Sprio, T. G., Dierks, E. A., and Bursten, J. N. (1999) J. Biol. Inorg. Chem. 4, 804–813
14. Stone, J. R., and Marletta, M. A. (1998) Chem. Biol. 5, 255–261
15. Shevler, D., Kerby, R. L., He, Y., and Roberts, G. P. (1997) J. Bacteriol. 177, 2157–2163
16. Shevler, D., Kerby, R. L., He, Y., and Roberts, G. P. (1999) Biochemistry 38, 2689–2697
17. Shivker, D., Thorsteinsson, M. V., Kerby, R. L., Chung, S. Y., Roberts, G. P., Reynolds, M. F., Parks, R., and Bursten, J. N. (1999) Biochemistry 38, 8284–8290
18. Brosius, J., and Messing, J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6929–6933
19. Dyckhoon, D. M., St. Pierre, R., and Linn, T. (1996) Gene (Amst.) 177, 132–136
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1995) in Short Protocols in Molecular Biology, 2nd ed., pp. 945–963, ASM Press, Washington, D. C.
21. Antonini, E., and Brunori, M. (1971) J. Biol. Chem. 246, 749–795
22. Lundblad, J. R., Laurance, M., and Goodman, R. H. (1996) J. Mol. Biol. 270, 1057–1072
23. Grillo, A. O., Brown, M. P., and Royer, C. A. (1999) J. Mol. Biol. 297, 876–890
24. Kolb, A., Bushy, S., Buc, H., Garges, S., and Adhya, S. (1993) Annu. Rev. Biochem. 62, 749–795
25. Winter, J. (1996) in Bacterial Display libraries, in Protein Engineering, Principles and Practice (Cleland, J. L., and Criak, C. S., eds) pp. 349–367, John Wiley-Liss, Inc., New York
26. DeDuve, C. (1948) Acta Chem. Scand. 2, 264–268
27. Sono, M., Andersson, L. A., and Dawson, J. H. (1982) J. Biol. Chem. 257, 8308–8320
28. Cechovic, W. J., Bolger, R. E., and Burke, T. (1995) Trends Biochemistry 20, 5496–5502