Identification of Two Important Heme Site Residues (Cysteine 75 and Histidine 77) in CooA, the CO-Sensing Transcription Factor of *Rhodospirillum rubrum*†

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ABSTRACT: The CO-sensing mechanism of the transcription factor CooA from *Rhodospirillum rubrum* was studied through a systematic mutational analysis of potential heme ligands. Previous electron paramagnetic resonance (EPR) spectroscopic studies on wild-type CooA suggested that oxidized (Fe^{III}) CooA contains a low-spin heme with a thiolate ligand, presumably a cysteine, bound to its heme iron. In the present report, electronic absorption and EPR analysis of various substitutions at Cys residues establish that Cys^{75} is a heme ligand in Fe^{III} CooA. However, characterization of heme stability and electronic properties of purified C75S CooA suggest that Cys^{75} is not a ligand in Fe^{II} CooA. Mutational analysis of all CooA His residues showed that His^{77} is critical for CO-stimulated transcription. On the basis of findings that H77Y CooA is perturbed in its Fe^{II} electronic properties and is unable to bind DNA in a site-specific manner in response to CO, His^{77} appears to be an axial ligand to Fe^{II} CooA. These results imply a ligand switch from Cys^{75} to His^{77} upon reduction of CooA. In addition, an interaction has been identified between Cys^{75} and His^{77} in Fe^{III} CooA that may be involved in the CO-sensing mechanism. Finally, His^{77} is necessary for the proper conformational change of CooA upon CO binding.

The small gaseous molecules nitric oxide (NO), O_2, and CO are important regulatory signals in biological systems ranging from procaryotes to mammals. NO is a signaling molecule in mammalian systems that has gained much attention recently, as it plays a role in such diverse processes as vascular relaxation, neurotransmission, and platelet aggregation (*1*, *2*). NO is believed to trigger a conformational change in the enzyme soluble guanylyl cyclase (sGC)[1] by binding to the heme iron; NO binding results in the displacement of the proximal histidine ligand (*3*, *4*). The metabolic activity of bacteria is regulated in a number of different ways by O_2. An example is the rhizobial O_2-sensing protein, FixL, which is thought to be regulated by a conformational change induced by O_2 binding to the heme iron (*5*−*7*). In contrast, little is known about CO-sensing proteins although evidence suggests that CO may play an important role in signaling in mammalian systems (*8*, *9*). It was recently discovered that CO activates CooA (*10*), the CO-sensing transcription factor of the photosynthetic bacterium *Rhodospirillum rubrum*, and that this protein utilizes heme to sense its effector (*10*, *11*). An understanding of the mechanism of CO sensing by CooA should increase our understanding of the possible roles of heme proteins as small molecule receptors.

CooA regulates CO-induced transcription of two operons in *R. rubrum* (*10*, *12*, *13*). These operons, *cooMKLXUH* and *cooFSCTJ* (*13-16*), encode components of an enzymatic CO oxidation system that allows *R. rubrum* to catalyze the anaerobic conversion of CO to CO_2 (*15*, *17*, *18*) and allows the organism to grow on CO as a sole energy source (*19*). *cooA*, which lies 3′ to *cooFSCTJ*, encodes a transcriptional activator that is a member of the CRP (the cyclic AMP receptor protein) family of transcriptional regulators (*20*). In response to CO, CooA binds upstream of the promoter regions of *cooF* and *cooM* (*10*, *12*, *13*). Consistent with its homology to CRP, the DNA binding sites for CooA are highly reminiscent of CRP binding sites (*10*, *12*, *13*).

CooA purified from *R. rubrum* is a homodimeric, protoheme-containing protein that binds CO directly through its heme moiety (*10*). Under anoxic, reducing conditions, CooA exists in the Fe^{II} form, which is competent to bind CO and subsequently bind to its DNA target site (*10*). Exposure of CooA to air promotes the rapid oxidation of the CooA heme to the Fe^{III} form, which is incapable of binding CO (*11*) and is inactive in sequence-specific DNA binding (He et al., unpublished data). The inactivity of Fe^{III} CooA may be

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[1] Abbreviations: sGC, soluble guanylyl cyclase; CRP, cyclic AMP receptor protein; IPTG, isopropyl β-D-thiogalactopyranoside; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; WT, wild type; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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biologically relevant, as exposure of *R. rubrum* to O_2 shuts down the synthesis of the O_2-labile CO oxidation system (*21*). Since *R. rubrum* inhabits aquatic environments where oxygen tension and reduction potential presumably vary, CooA may cycle between the oxidized and reduced forms in vivo.

The heme of CooA is low spin and six coordinate in its oxidized (Fe^{III}) (*22*), reduced (Fe^{II}), and CO-bound (Fe^{II}-CO) states (*10, 23, 24*); therefore, replacement of a heme axial ligand by CO is likely to be the event that triggers high-affinity DNA binding (*10, 23*). Because changes in the heme ligands must lead to the activation of CooA, it is critical to establish the nature and identity of the heme axial ligands in CooA, as well as to characterize the heme environment. Changes in the axial ligand geometry that accompany the binding of small molecule effectors are also hypothesized to be critical in the activation of FixL (*6*) and sGC (*4*).

Studies of CooA, utilizing EPR and electronic absorption spectroscopies, established that the heme of Fe^{III} CooA has a thiolate ligand, presumably a cysteine residue, bound to the heme iron (*22*). Recently, Aono et al. have used mutational and spectroscopic analyses to suggest that Cys^{75} is a ligand to Fe^{III} CooA and that His^{77} replaces Cys^{75} upon reduction to the Fe^{II} form of CooA (*25*). In this paper, we report independent studies that confirm the broad conclusions of that work and provide a stronger basis for, and extend upon, those claims. The present report also describes an interaction between His^{77} and Cys^{75} that may be important for the function of CooA and identifies the importance of His^{77} for the proper conformational change of CooA upon binding CO. On the basis of the results of this study, a unified model of the function of CooA is presented.

EXPERIMENTAL PROCEDURES

Creation of cooA Mutations and Expression of CooA in Escherichia coli. The pKK223-3-derived wild-type *cooA* expression plasmid (designated pCO69, *E. coli* strain UQ1421) was constructed by PCR amplification of *cooA* using upstream (*Eco*RI-containing) and downstream (*Hin*dIII-containing) primers designed according to the *cooA* sequence (*20*) with previously described extensions (*26*). Mutations were introduced by site-directed mutagenesis using the "GeneEditor" (Promega) or "Quick Change" procedures (Stratagene) or by localized random mutagenesis using degenerate oligonucleotide pools (*27*). All PCR reactions employed *Pfu* DNA polymerase (Stratagene). Each mutation was verified by sequencing the entire *cooA* gene.

Construction of a Reporter Strain for CooA Transcription Activity in Vivo. A system for monitoring transcriptional activation in vivo was constructed using the CO- and CooA-dependent promoter upstream of *cooF*, which was fused to a promoterless *lacZ* encoding β-galactosidase. Details of this construct will be included in a future publication (He et al., unpublished data). This construct was then transferred to a bacteriophage λ and integrated into the chromosome of *E. coli* strain XL-1 Blue (Stratagene). *E. coli* strains harboring plasmids mutated in *cooA* were examined for CooA function after anaerobic incubation in the presence of CO (2.5% v/v final concentration of CO in headspace). β-Galactosidase assays were performed as described by Miller (*28*).

Protein Techniques. Protein concentration was determined by the bicinchoninic acid (BCA) assay (*29*) as modified for dithiothreitol- (DTT-) containing solutions (*30*). Quantitation of bands on Coomassie-stained SDS−PAGE was performed by densitometric scanning.

Enrichment of Variant CooA Proteins. Unless noted, all protein purifications were conducted at 4 °C, and all protein purification buffers were supplemented with 200 μM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 1 mM DTT. The initial analysis of CooA proteins expressed in *E. coli* utilized cell extracts prepared from IPTG-induced cells obtained from a 40 mL culture in 2× LC (*14*) supplemented with 20 μM ferric citrate. Cells were harvested, resuspended in 6 mL of lysis buffer (25 mM MOPS, pH 7.4/0.2 M NaCl), and lysed by passage through a French pressure cell at ∼120 MPa. The crude extract was then centrifuged at 150000*g* for 90 min, and the supernatant was stored at −80 °C. Extracts were diluted to 4 mg of total protein/mL prior to spectral analysis, with subtraction of a background spectrum obtained from a similarly prepared *E. coli* harboring pKK223-3 without *cooA*.

Purification of Wild Type and CooA Variants. Isolation and purification of wild-type and H77Y CooA differed from our previously published procedure (*10*) as follows. Wild-type and H77Y CooA are stable to purification in the presence of air, and therefore the anoxic technique was not required for the isolation of these CooA proteins. Cells of *E. coli* strain UQ1421 (expressing wild-type CooA) or UQ1435 (expressing H77Y CooA) were grown to an optical density at 600 nm of 0.2−0.3 in 2× LC medium supplemented with 160 μM ferric citrate and 50 μg/mL ampicillin. IPTG was added to a final concentration of 0.5 mM, and the incubation was continued at 37 °C under vigorous agitation for 12−15 h.

Cell paste (∼100 g) was washed with 0.1 M NaCl and 25 mM MOPS, pH 7.4, and recentrifuged at 4800*g* for 20 min. The pellets were resuspended in 530 mL of 25 mM MOPS, pH 7.4/10% (v/v) glycerol/0.2 M NaCl, passed through a French pressure cell at ∼120 MPa, and then centrifuged at 6500*g* for 30 min. The resulting cell-free lysate was precipitated with poly(ethylenimine) (0.1% final concentration) with stirring for 30 min and centrifuged again at 6500*g* for 30 min. The pellet was resuspended in 530 mL of 25 mM MOPS, pH 7.4/10% glycerol/0.8 M NaCl and incubated overnight. After centrifugation as described above, ammonium sulfate (85% saturated stock) was added to the supernatant to a final saturation of 35%, and the supernatant was incubated for 1 h and then centrifuged at 6500*g*. To the resulting supernatant was added ammonium sulfate to a final saturation of 50%; the supernatant was incubated for 1 h and centrifuged at 6500*g* for 30 min.

The resulting pellet was dissolved in 250 mL of 25 mM MOPS, pH 7.4/5% glycerol and applied to a 230 mL Q-Sepharose Fast Flow column (Pharmacia). The column was then washed with 1.6 column volumes of 25 mM MOPS, pH 7.4/0.2 M NaCl/5% glycerol. The column was developed with a linear gradient (10 column volumes) of 0.2−0.4 M NaCl, with CooA eluting at approximately 0.35 M NaCl. CooA was further purified using a 230 mL hydroxylapatite HTP column (Bio-Rad) as described (*10*), except the column was washed with 2 column volumes of 40 mM potassium phosphate.

To the resulting CooA-containing fractions was added ammonium sulfate to a final saturation of 55%, and the

solutions were incubated overnight. The solution containing precipitated CooA was centrifuged at 9200*g* for 30 min, and the resulting pellet was resuspended in a minimal volume (~2.7 mL) of 25 mM MOPS, pH 7.4/0.1 M NaCl with no protease inhibitors and desalted using Sephadex G-25 fine (Pharmacia) equilibrated in the identical buffer. Purified CooA isolated by this procedure was determined to be greater than 95% pure, as judged by densitometric scanning of Coomassie-stained SDS−PAGE, and was stored at −80 °C. All purified CooA samples used in this study were tested for CO-dependent, site-specific DNA binding by DNase I footprinting, as described previously (*10*). DNase I protection assays of CooA purified from *E. coli* and *R. rubrum* (*10*) yielded similar results (data not shown).

Enrichment of C75S CooA. Preliminary isolation of the C75S CooA (from *E. coli* strain UQ1493) under aerobic conditions produced a protein that was very unstable and continually lost heme throughout the isolation procedure (see Results). This problem was circumvented by isolation of C75S CooA under anaerobic, reducing conditions. Cells (~100 g) were grown and induced for CooA synthesis as described above. All buffers for cell breakage and poly(ethylenimine) and ammonium sulfate precipitations were exactly as described for the wild-type protein with the exception that the buffers were sparged extensively with nitrogen gas and then degassed and purged with oxygen-scrubbed argon on a manifold prior to the anaerobic addition of sodium dithionite (Fluka) to 1 mM. The ammonium sulfate pellet was resuspended in the hydroxylapatite equilibration buffer and applied to an anoxic hydroxylapatite column. The resultant fractions were ammonium sulfate-precipitated and the pellets resuspended. Under these conditions, heme-containing C75S CooA was enriched to ~80% purity. C75S Fe^{III} CooA was produced by addition of potassium ferricyanide to the ammonium sulfate-precipitated fraction with subsequent removal of excess ferricyanide by Sephadex G-25 gel filtration.

Spectrophotometric Methods. Electronic absorption spectra of CooA were recorded using a Shimadzu UV-1601PC (slit width of 2 nm) or a Cary 4 Bio spectrophotometer (slit width of 0.5 nm). The Fe^{III}, Fe^{II}, and Fe^{II}-CO forms of CooA were generated as described previously (*10*). Heme quantitation was performed by the pyridine hemochromogen assay (*31*). For electronic absorption analyses, CooA was present at ~5 μM in both extracts and enriched proteins.

EPR Spectroscopy. Fe^{III} CooA samples were degassed with argon, frozen, and stored at 77 K in 25 mM MOPS, pH 7.4, 1 mM DTT, and 0.1 M NaCl, except where noted. For EPR analyses, final heme concentrations of wild-type, H77Y, and C75S CooA were 364, 190, and 90 μM, respectively. 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 was monitored using a Hewlett-Packard 5255A frequency counter. The spectra were recorded at 23 K and 9.2 GHz (X band); the only EPR signals that were observed between 0 and 4000 G are those reported.

pH Studies. Wild-type and H77Y Fe^{III} CooA EPR samples were set at the appropriate pH by exchanging the protein into new buffer on a Pharmacia NICK desalting column. The buffers (25 mM) used were MES at pH 6.5, EPPS at pH 8.5, and CHES at pH 10.0. The protein was ~40 μM in heme after collection off the column. EPR spectra were obtained at 23 and 77 K as described above. EPR spectral simulations of the two low-spin components were carried out using WIN-EPR Simfonia version 1.2 software (Bruker); the percentage of each component signal was determined from the best fit to the experimental data. The electronic spectra of Fe^{III}, Fe^{II}, and Fe^{II}-CO CooA were recorded between pH 5.5 and pH 11.0 in increments of 0.5 pH unit by diluting the samples from 172 μM heme to 2 μM heme in the appropriate buffer. The buffers used were MES at pH 5.5, 6.0, and 6.5, MOPS at pH 6.5 and 7.0, HEPES at pH 7.0, 7.5, and 8.0, EPPS at pH 7.5, 8.0, and 8.5, CHES at pH 8.5, 9.0, and 10.5, and CAPS at pH 10.0, 10.5, and 11.0. The pH of all samples was confirmed using an Orion model 611 pH meter equipped with a Ross semi-micro temperature compensation electrode.

Native PAGE Analysis of CooA. Wild-type and H77Y CooA were analyzed in the Fe^{III}, Fe^{II}, and Fe^{II}-CO forms by native PAGE. For the Fe^{II} forms, samples were rendered anoxic and then reduced by the addition of sodium dithionite to1.7 mM. Samples were resolved using a 10% nondenaturing gel rendered anoxic by the addition of sodium dithionite to 1.7 mM to nitrogen-sparged buffers. For the Fe^{II}-CO forms, samples were treated identically except CO was added to the headspace of vials to approximately 30% and allowed to incubate at room temperature for 20 min prior to being loaded on native gels that were polymerized under a CO atmosphere. The running buffer was also vigorously sparged with CO gas for approximately 30 min prior to loading of the samples. The gels were then run at 4 °C for a total of 1500 V h, and resolved proteins were visualized by Coomassie Brilliant Blue-R-250 staining. R_f values for CooA proteins under all conditions were calculated with reference to the migration of bovine serum albumin (Sigma).

RESULTS

Analysis of Altered CooA Proteins Expressed in E. coli. CooA purified from *E. coli* was indistinguishable from CooA purified from *R. rubrum*, as judged by the following criteria: (a) the electronic absorption properties of CooA purified from *E. coli* (Table 1) are identical to those of CooA purified from *R. rubrum* (*10*), and (b) CooA purified from *E. coli* was similarly competent for CO- and CooA-dependent DNA binding, as judged by DNase I footprinting (data not shown).

For more rapid screening of CooA in extracts, we expressed CooA variant proteins in *E. coli* and analyzed the electronic absorption spectra following enrichment by ultracentrifugation. The electronic absorption spectra of enriched extracts bearing CooA are virtually identical to those of purified CooA, indicating the validity of screening variant proteins by this method (Table 1).

Cys^{75} Is an Axial Ligand to the Heme in Fe^{III} CooA. Previous EPR studies revealed that a thiolate is bound to the heme iron in CooA (*22*), and the following lines of evidence identify Cys^{75} as the residue that provides that ligand. The five CooA Cys residues were altered, and extracts of CooA variants C35A, C80W, C105A, and C123L/M124L all displayed normal amounts of heme-containing CooA and exhibit electronic absorption spectra of the Fe^{III}, Fe^{II}, and Fe^{II}-CO forms that were indistinguishable from those of the

Table 1: Electronic Absorption Features of Wild-Type and Altered CooA Proteins

	Fe^{III} CooA (nm)			Fe^{II} CooA (nm)				Fe^{II}-CO CooA (nm)		
	Soret γ	β	α[a]	Soret γ	β	α	$A_{\lambda max(FeII)}/A_{\lambda max(FeIII)}$[b]	Soret γ	β	α
purified proteins										
WT	423	540	570	424	528	558	2.3	421	539	568
C75S[c]	422	536	570	423	527	557	1.6	420	538	566
H77Y	423	540	570	419[d]	530[d]	557[d]	0.8	419	540	566
extracts[e]										
WT	423	539	570	424	528	558	2.3	421	540	568
H77C	424	539	570	422	528	557	1.3	420	539	568
H77E	423	538	570	422	527	557	1.4	419	539	566
H77K	423	538	570	422	527	557	1.4	419	538	567
C35A	423	539	570	424	528	558	2.2	421	539	568
C80W	424	539	570	424	529	558	2.0	421	539	567
C105A	423	539	570	424	528	558	2.1	421	539	568
C123L/M124L	423	540	570	424	528	558	2.0	422	540	568
H28C	423	541	570	424	528	558	2.1	421	539	567
H133C	423	541	570	424	528	558	2.3	421	540	568
H146C	423	539	570	424	528	558	2.1	421	540	568
H200C	423	541	570	424	528	558	2.0	421	540	568

[a] The α peak of Fe^{III} CooA is a shoulder with an indistinct maximum. [b] Ratio between reduced and oxidized Soret (γ) band intensities at identical heme concentrations. [c] Purified under anoxic, reducing conditions and chemically oxidized. [d] The precise positions of these α, β, and Soret (γ) peaks are ambiguous because a mixed population is present in dithionite-reduced H77Y CooA. As noted in the text, there is a significant Soret shoulder at 407 nm. [e] Spectra were recorded on extracts enriched for CooA by ultracentrifugation.

wild-type protein (Table 1). In contrast, extracts of strains carrying CooA substituted at Cys^{75} contain substantially less heme-containing CooA protein than the wild-type strain, and these CooA variants were noticeably unstable, losing heme continually during purification (data not shown). Decreased heme binding affinity upon substitution of the native axial ligands in other proteins has been noted previously (*32*−*34*). C75S CooA was chosen for purification and spectroscopic characterization but accumulated to low levels and was unstable unless kept in the reduced state throughout purification; it was therefore isolated anoxically to approximately 80% purity. The stability of C75S Fe^{II} CooA was comparable to that of wild-type Fe^{II} CooA, but upon oxidation in the air, C75S Fe^{III} CooA rapidly lost heme (Figure 1). To examine the unstable oxidized form, C75S Fe^{II} CooA was treated with excess ferricyanide and rapidly exchanged into fresh anoxic buffer by gel filtration. The electronic absorption spectrum of C75S Fe^{III} CooA (Figure 2B) showed features in the 500−700 nm region that differed from those of wild-type Fe^{III} CooA (Figure 2A), consistent with a significant disruption of the heme ligation environment in C75S Fe^{III} CooA. Cys^{75} therefore appears to be a ligand to the Fe^{III} heme, a conclusion consistent with the observations of Aono et al. (*25*), who studied the C75A CooA variant in cell extracts.

In contrast to the work of Aono et al. (*25*), who reported that C75A CooA was recalcitrant to purification, we noted that the presence of DTT during purification stabilized C75S CooA against denaturation and heme loss when compared to preparations that did not contain DTT (data not shown). This observation facilitated a more detailed spectral analysis of purified C75S CooA. The electronic absorption spectrum of purified, DTT-treated C75S Fe^{III} CooA (Figure 2B) differed significantly from the spectrum of C75A Fe^{III} CooA in the crude extracts reported by Aono et al. (*25*). To determine if these differences were a consequence of the presence of DTT in our samples, we performed a purification of C75S CooA where DTT was omitted in the last step of the isolation procedure. The electronic absorption spectrum

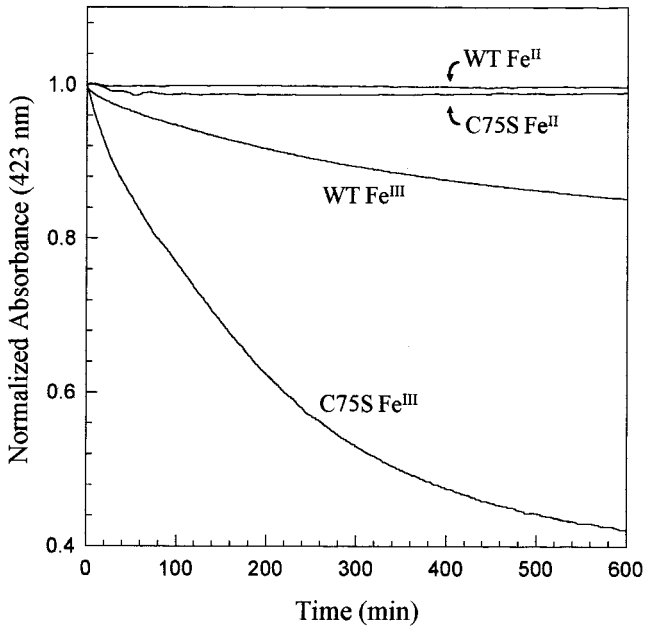

FIGURE 1: Alteration of Cys^{75} dramatically decreases the stability of the heme in Fe^{III} CooA. The change in the absorbance at 423 nm was used to monitor the stability of wild-type and C75S CooA in different oxidation states. Oxidized proteins were exposed to air, whereas reduced proteins were maintained under strict anoxic conditions in the presence of dithionite. Absorbance measurements were obtained every minute for the period shown. The starting heme concentration in each experiment was approximately 10 μM, the buffer was 25 mM MOPS, 1 mM DTT, and 0.1 M NaCl at pH 7.4, and the analysis was performed at room temperature.

of the DTT-deficient C75S Fe^{III}CooA (Figure 2C) was similar to that reported by Aono et al. (*25*). Upon addition of DTT (1 mM) to the DTT-deficient C75S Fe^{III}CooA there was a red shift, from 415 to 423 nm, and an amplitude decrease in the Soret. Changes in the 500−700 nm region and a sharpening of the peak at 360 nm were also observed (Figure 2C). EPR analysis of the DTT-containing and DTT-deficient C75S CooA also revealed significant differences (Figure 3A). The DTT-containing Fe^{III} CooA exhibited a complex low-spin signal, with a minor high-spin component

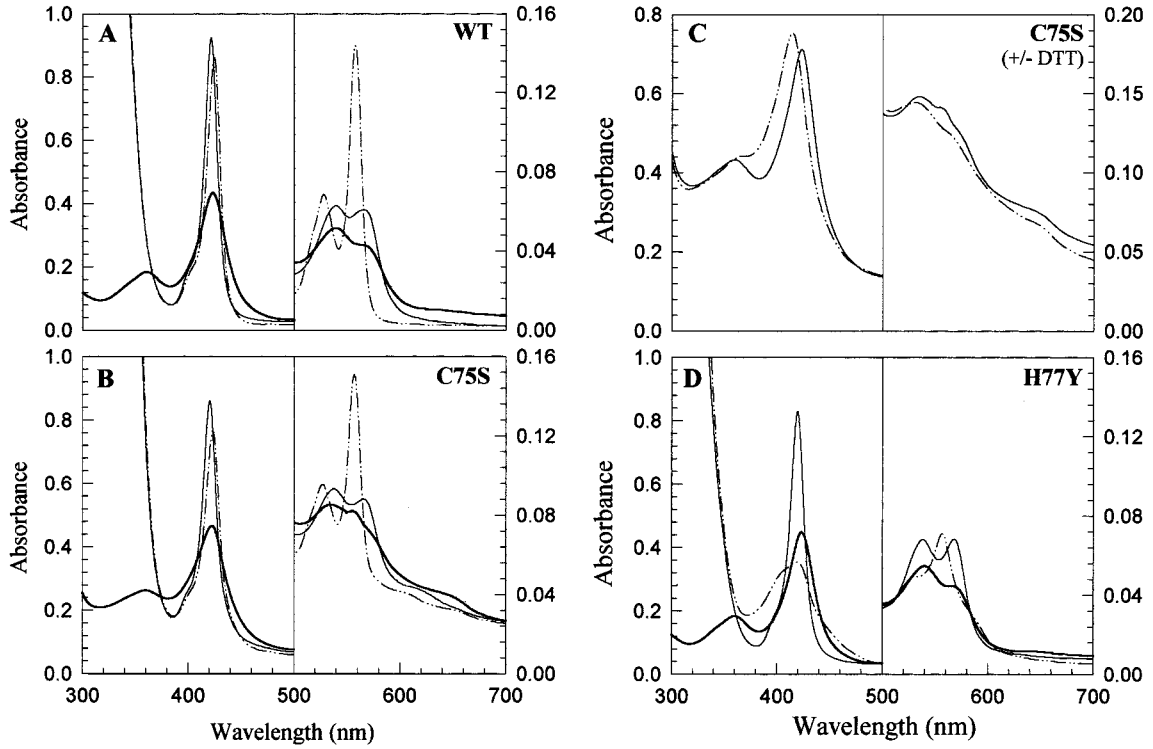

FIGURE 2: Alteration of Cys^{75} and His^{77} perturbs the heme environment of CooA. Electronic spectra of Fe^{III} (thick solid line), Fe^{II} (dot and dashed line), and Fe^{II}-CO (thin solid line) derivatives of the wild type (panel A) and CooA variants (panels B and D) isolated in this study. The heme concentration was approximately 5 μM for each spectrum. For the C75S CooA sample, spectra were obtained before (dot and dashed line) and after (solid line) the addition of DTT to 1 mM (panel C).

at $g = 4.3$, possibly attributable to adventitous iron. In the DTT-deficient sample, the prominent high-spin signals at $g = 6.2$ and $g = 4.3$ are observed in addition to a complex low-spin signal. The spectral data clearly suggest that multiple liganded forms of heme are present in C75S Fe^{III} CooA, in both the presence and absence of DTT. The dramatic spectral changes observed upon addition or removal of DTT suggest that DTT is in fact binding directly to the heme in this CooA variant, consistent with the absence of one of the normal heme ligands.

Cys^{75} Is Not a Ligand to Fe^{II} or Fe^{II}-CO CooA. Taken together, the following lines of evidence demonstrate that Cys^{75} is not a ligand to the heme in the Fe^{II} and Fe^{II}-CO CooA: (i) The electronic absorption spectra of C75S Fe^{II} and Fe^{II}-CO CooA are similar to those of wild-type CooA (Figure 2B and Table 1). (ii) The heme in C75S CooA is greatly stabilized upon reduction (Figure 1), which is most easily rationalized if Cys^{75} is not a ligand to heme in the Fe^{II} and Fe^{II}-CO forms. (iii) The electronic spectra of wild-type Fe^{II} and Fe^{II}-CO CooA were unchanged between pH 6.5 and pH 11.0 (data not shown); the absence of any titratable proton in this range suggests that a thiol is not bound to the heme iron of either reduced or CO-bound CooA. For comparison, the heme protein H-450 exhibits a dramatic red shift of the Soret for the Fe^{II} form as the pH is increased, implicating a transition from a thiol to a thiolate bound heme (*35*). (iv) C75S and C75A alterations of CooA have no significant effect on in vivo CO-dependent transcription (see later), compared to wild-type CooA, revealing that Cys^{75} is not critical for this functional response. These results demonstrate that Cys^{75} is not a heme ligand in the Fe^{II}- and Fe^{II}-CO-bound forms and suggest that Cys^{75} is replaced by another ligand upon reduction of CooA. In addition, the absence of pH-dependent spectral changes also suggests that water is not an axial heme ligand in Fe^{II}- and Fe^{II}-CO-bound CooA.

Substitutions of His^{77} Perturb the Heme Environment of Fe^{II} and Fe^{II}-CO CooA. The spectral characteristics of wild-type CooA in the reduced and CO-bound forms are consistent with the presence of a histidine ligand (*10*, *23*); therefore, we carried out systematic substitution of the five histidine residues in CooA to determine if any of these residues were ligands to the heme. Extracts of strains with alterations at positions 28, 133, 146, and 200 (where histidine was substituted by cysteine) contain levels of CooA comparable to those in wild-type extracts and exhibit electronic absorption spectra that are indistinguishable from those of wild type in the oxidized, reduced, and CO-bound forms (Table 1). Of note, although the oxidized extracts containing His^{77} variants appear spectrally identical to those of wild type (Figure 2D and Table 1), the reduced extracts are notably different (Figure 2D). Addition of CO to dithionite-containing extracts of position 77 CooA variants resulted in the formation of CO adducts with spectral properties similar to those of the wild-type protein.

To examine the role of His^{77} in more detail, one variant protein, H77Y, was selected for purification and detailed spectroscopic study. Purification of H77Y CooA resulted in a heme-containing protein of approximately 95% purity. The protein was isolated in the oxidized form and exhibited an A_{423}/A_{280} (λ_{max} Fe^{III} CooA/λ_{max} protein) ratio of 2.2, which was nearly identical to that of the wild-type protein ($A_{423}/A_{280} = 2.3$). Electronic absorption spectra of isolated H77Y Fe^{III} CooA were indistinguishable from those of wild-type Fe^{III} CooA (Figure 2D). Purified H77Y Fe^{III} CooA was difficult to reduce; addition of up to 1 mM dithionite resulted

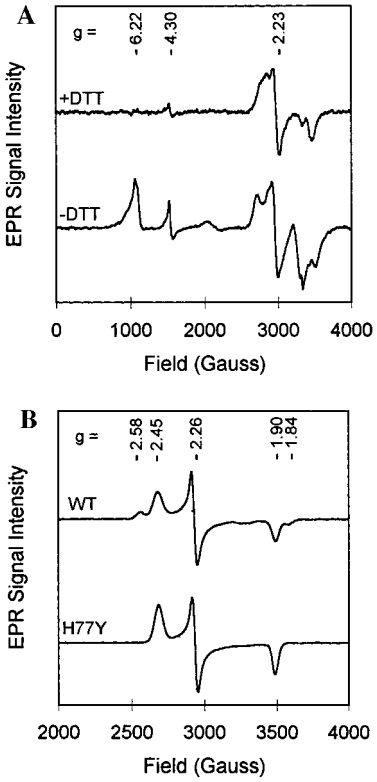

FIGURE 3: EPR spectroscopy confirms that alterations of Cys^{75} and His^{77} affect the heme environment of CooA. The EPR samples of wild-type CooA and the C75S and H77Y CooA variants contained 25 mM MOPS, 1 mM DTT, and 0.1 M NaCl at pH 7.4. EPR spectra were recorded at 23 K, 200 μW microwave power, 8 G modulation amplitude, 9.2 GHz microwave frequency, 100 kHz modulation frequency, and a 1 s time constant. (A) X-band EPR spectra of (top) C75S Fe^{III} CooA with DTT present (90 μM heme) and (bottom) C75S Fe^{III} CooA where DTT was omitted in the last purification step (60 μM heme). The receiver gain was 4×10^4, and the number of averaged scans was 4 (top) and 32 (bottom). The samples were prepared as described in Experimental Procedures. The spectra of DTT-containing C75S Fe^{III} CooA from two different protein preparations were not identical, although both spectra contained a perturbed $g = 2$ low-spin signal different from that of wild-type Fe^{III} CooA. One such spectrum is shown. (B) X-band EPR spectra of (top) wild-type Fe^{III} CooA (100 μM heme) and (bottom) H77Y Fe^{III} CooA (364 μM heme). The spectra reported were recorded at a receiver gain of 1.2×10^4 (top) and 3.2×10^3 (bottom) averaged over 16 scans (top) and 4 scans (bottom), with each scan containing 2048 data points.

in a spectrum suggestive of incomplete reduction and an admixture of multiple reduced species. Notably, neither the Soret nor the β band increased in intensity, as is typically observed upon reduction. Instead, a split Soret with apparent maxima at 407 and 419 nm was observed, the former peak being substantially blue shifted relative to what is observed for wild-type Fe^{II} CooA (Figure 2A). A split Soret band and less facile reduction were also observed for H77K (Table 1) and H77M CooA. Incomplete reduction of H77Y upon dithionite treatment was confirmed by a recent resonance Raman study, in which a split oxidation state marker band (ν_4) was observed at 1362 and 1372 cm^{-1} (*24*), as well as a recent magnetic circular dichroism study (Dhawan et al., unpublished data). These observations are in conflict with those of Aono et al., who suggested that H77Y CooA could be fully reduced upon dithionite treatment (*25*).

Because Aono et al. used imidazole in their purification of CooA (*25*), we considered the possibility that imidazole

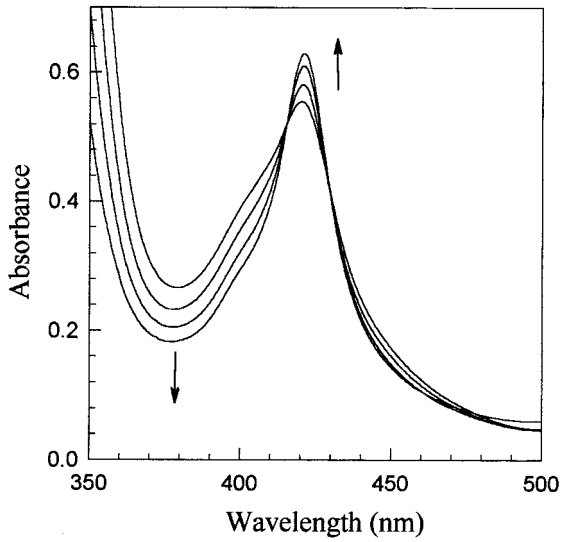

FIGURE 4: The heme binding pocket of the Fe^{II} H77Y CooA variant is accessible to imidazole. Optical absorption spectra of Fe^{II} H77Y CooA with increasing concentrations of exogenous imidazole in 25 mM MOPS pH 7.4, and 0.1 M NaCl are shown. Arrows indicate spectral changes upon addition of exogenous imidazole; imidazole levels are 0, 2.5, 5.0, and 7.5 mM.

may have been present in the samples and might be the basis for the different behavior. Spectral changes were observed upon addition of imidazole at pH 7.4 in an ~500−1000-fold molar excess relative to purified H77Y Fe^{II} CooA (Figure 4), consistent with the binding of imidazole to the Fe^{II} heme. The intensity of the Soret band (419 nm) increased at the expense of the broad absorption at 407 nm. Under identical conditions neither wild-type Fe^{II} CooA nor H77Y Fe^{III} CooA showed detectable spectral changes (data not shown). These data suggest that the heme of H77Y Fe^{II} CooA is more accessible to imidazole than that of the wild-type protein, consistent with the loss of a normal heme ligand in H77Y Fe^{II} CooA. Although the levels of imidazole in the preparations of Aono et al. (*25*) are unreported, the relatively high concentrations of imidazole required to elicit a spectral change suggest that residual imidazole is not the basis of differences between our results and theirs.

His^{77} and Cys^{75} Interact in Fe^{III} CooA. Substitutions at His^{77} resulted in slight perturbations of the heme environment of Fe^{III} CooA, suggesting that His^{77} and Cys^{75} interact. The EPR spectrum of H77Y Fe^{III} CooA shows a single rhombic EPR signal ($g = 2.46, 2.25, 1.89$) (Figure 3B). The g values corresponded exactly to the major component signal observed in wild-type Fe^{III} CooA, with a g-value anisotropy characteristic of low-spin thiolate-bound heme (*22*). The minor component signal, which invariably accounted for 15% of the heme in wild-type Fe^{III}CooA, was not observed in the H77Y variant. Spin quantitation reveals that 100% of the heme in H77Y Fe^{III}CooA is accounted for in the one signal; therefore, we conclude that the heme has been completely converted to the major component form present in wild-type CooA. Although Aono et al. (*25*) report similar Fe^{III} CooA EPR spectra, they do not comment on the presence of the minor component in the wild-type CooA and its absence in H77Y CooA. We have observed that active, wild-type Fe^{III} CooA always contains two overlapping rhombic signals (*22*). Since only a small perturbation of the EPR spectrum and no perturbation of the electronic spectrum are observed in H77Y

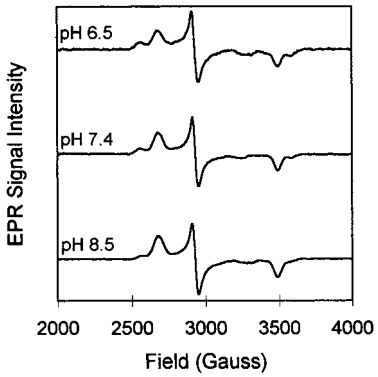

FIGURE 5: The ratio of major to minor signals in the EPR spectrum of wild-type CooA is pH dependent. EPR spectra of wild-type Fe^{III} CooA in 25 mM MES, pH 6.5 (top), 25 mM MOPS, pH 7.4 (middle), and 25 mM EPPS, pH 8.5 (bottom) at 23 K are shown. All the samples contained 40 μM (in heme) CooA and were prepared as described in Experimental Procedures. The spectral parameters were identical to those parameters listed in Figure 3, except the receiver gain was 3.2×10^4 (top) and 1.6×10^4 (bottom), and the number of averaged scans was 26 (top) and 20 (bottom). The middle spectrum, pH 7.4 in MOPS, is a replot of that in Figure 3B.

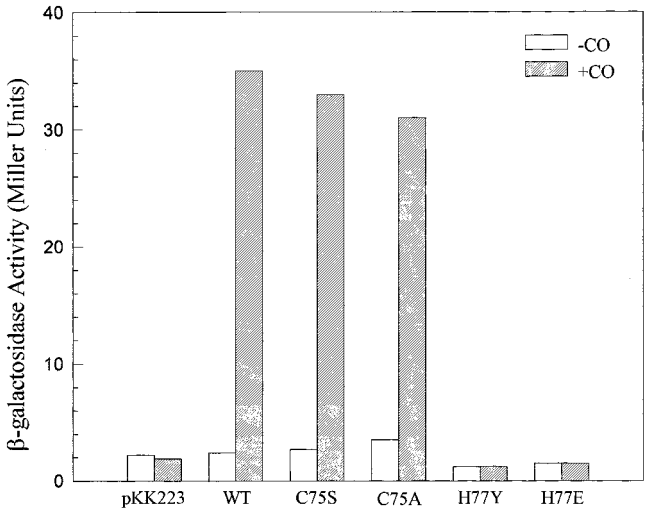

FIGURE 6: Substitutions at Cys^{75} have a negligible effect on CooA activity, while substitutions at His^{77} have a dramatic effect. This assay measures the functionality of CooA variants in vivo by their ability to activate transcription of *lacZ* (encoding β-galactosidase) in the presence or absence of CO. Values are expressed as an average of duplicate assays.

Fe^{III} CooA, in contrast to the large perturbation of both EPR and electronic absorption spectra seen in C75S Fe^{III} CooA, we conclude that Cys^{75} remains bound as the axial ligand in H77Y Fe^{III} CooA. It appears improbable that both amino acids could serve simultaneously as axial ligands, as His^{77} is only two amino acids removed from Cys^{75}. Thus, the fact that substitution at His^{77} does perturb the EPR spectrum of Fe^{III} CooA suggests that His^{77} interacts with Cys^{75} in Fe^{III} CooA. A plausible explanation for the presence of two low-spin EPR signals in the spectrum of wild-type Fe^{III} CooA is the presence of both thiol and thiolate bound to the heme iron; the absence of one signal in the EPR spectrum of H77Y Fe^{III} CooA suggests that His^{77} may partially protonate Cys^{75}.

To test the hypothesis that His^{77} serves to hydrogen bond to Cys^{75}, the electronic and EPR spectra were taken of wild-type and H77Y Fe^{III} CooA at a variety of pH values. The electronic spectra of wild-type and H77Y Fe^{III} CooA did not change between pH 6.5 and pH 11.0 (data not shown). Although the *g* values of the two components in wild-type CooA remain the same at the different pH values, the shapes and intensities of the EPR signals change (Figure 5). The relative amount of the minor component shows a decrease with increasing pH; the minor component represents 25%, 15%, and 5% of the total signal intensity at pH 6.5, 7.4, and 8.5, respectively. As electronic spectra fail to resolve two species of wild-type CooA, it is unsurprising that the method is unable to detect the pH-dependent minor component seen in EPR. These observations are consistent with the minor species representing an H-bonded, thiol-bound heme and the major species representing a fully deprotonated, thiolate-bound heme. Consistent with the hypothesis that the minor species represents partial protonation of Cys^{75} by His^{77}, the EPR spectrum of H77Y CooA was identical at pH 6.5, 7.4, and 8.5, displaying only the majority species under all pH conditions (data not shown).

His^{77} Affects the Ability of CooA To Undergo a Conformational Change upon CO Binding. To test the importance of His^{77} in CO-activated transcription of CooA, we analyzed CooA His^{77} variants in vivo with the *lacZ* reporter system. This system utilized the *cooF* promoter fused to a promoterless *lacZ* gene and provides CO- and CooA-dependent β-galactosidase activity, which are at levels 15−20-fold over negative controls lacking either CooA or CO. In the presence of CO, H77Y, H77C, and H77E CooA were unable to stimulate detectable increases in transcription, as judged by accumulated levels of β-galactosidase (Figure 6). In contrast, CooA variants with substitutions at Cys^{75} were normal in their response to CO. This result indicates that H77Y CooA is defective in at least one of the following properties: proper conformational change upon binding CO, interaction with DNA, or interaction with RNA polymerase.

To examine these possibilities, purified H77Y CooA was tested in vitro for CO-dependent DNA binding using DNase I footprinting. With wild-type CooA purified from *E. coli*, CO-dependent DNase I protection was observed at approximately 180 nM CooA, consistent with results obtained for wild-type CooA purified from *R. rubrum* (*10*). In contrast, no DNase I protection was observed with H77Y CooA at concentrations up to 710 nM (data not shown). These results indicate that H77Y CooA is defective either in its conformational response to CO or in interactions with DNA. To separate these two possibilities, we examined wild-type and H77Y CooA by native PAGE, which is highly sensitive to changes in protein conformation. Under aerobic conditions, both wild-type and H77Y Fe^{III} CooA migrated identically, suggesting similar conformations (data not shown). However, under reducing conditions, there was a clear difference in the migration characteristics of wild-type and H77Y Fe^{II} CooA, with R_f values (using the migration of a BSA standard as a reference) of 0.66 and 0.71, respectively (Figure 7, left panel), consistent with some difference in the Fe^{II} forms of these proteins. More importantly, upon ligation of CO, there was a dramatic increase in the R_f value of wild-type Fe^{II} CooA ($R_f = 0.75$), suggesting that a conformational change takes place upon binding the natural effector. In contrast, there was no discernible change in the R_f value of H77Y CooA in the presence of CO ($R_f = 0.71$; Figure 7, right panel). Because H77Y CooA binds CO effectively, this result

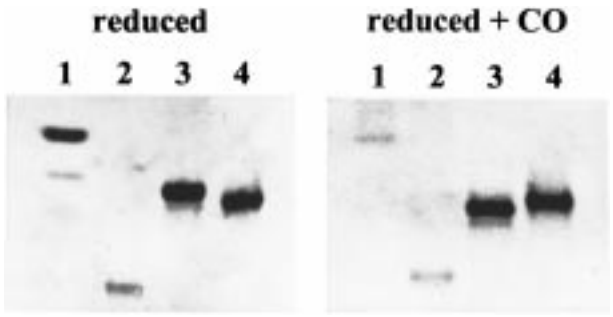

FIGURE 7: His[77] is required for eliciting a conformational change in CooA upon CO binding. Coomassie-stained 10% anoxic native PAGE of wild-type and H77Y CooA under reducing conditions (left panel) and reducing + CO conditions (right panel) was performed as described in Experimental Procedures. Lane assignments are identical for each gel. Lane 1, carbonic anhydrase (2 mg); lane 2, bovine serum albumin (2 mg); lane 3, wild-type CooA (4 mg); lane 4, H77Y CooA (4 mg). R_f values were calculated with reference to the migration of bovine serum albumin.

strongly implies that His[77] is involved in eliciting the conformational change that is necessary for CooA to become competent to bind DNA and thus activate transcription.

DISCUSSION

Fe[III] CooA: Ligation by Cys[75]. The data presented in this study identify Cys[75] as an axial ligand in Fe[III] CooA, consistent with the conclusion of Aono et al. (*25*), which was based on mutational and spectroscopic analyses. However, these authors were unable to purify C75A CooA due to its instability, and our analyses of enriched preparations for C75S CooA provide more compelling evidence. Heme stability is compromised upon alteration of the axial Cys ligand of other proteins, such as nitric oxide synthase (*33*), and the availability of an open coordination position in DTT-free Fe[III] C75S CooA, as indicated by the DTT addition experiments, is also consistent with the alteration of an axial ligand.

Cys[75] and His[77] Interact in Fe[III] CooA. The EPR spectrum of wild-type CooA is similar to that of H77Y CooA, indicating that thiolate ligation is retained in this variant and suggesting that His[77] is not a ligand to the oxidized form of CooA; similar results were obtained by Aono et al. (*25*). However, we note that the minor signal in the wild-type CooA EPR spectrum, which may originate from a thiol ligand (*22*), is not observed in the H77Y EPR spectra. The loss of this species can be rationalized if His[77] normally interacts with Cys[75], leading to partial protonation of the cysteinate side chain. Our studies of the pH effects on the EPR spectra are consistent with this hypothesis. In wild-type CooA the minor component is affected by pH, while in the H77Y variant, which has no minor component and where the presumed proton donor has been mutationally eliminated, there were no detectable pH effects on the EPR signal. The interaction between His[77] and Cys[75] may affect the redox properties of wild-type CooA, as hydrogen bonding of amino acid residues to thiolate ligands has been proposed to modulate the redox properties of other heme proteins, such as NO synthase (*36, 37*) and chloroperoxidase (*37, 38*) by reducing charge density on the thiolate heme ligand of these proteins. In addition, if His[77] and Cys[75] are in close proximity to one another, this positioning would facilitate the proposed ligand switch discussed below.

Fe[II] Form: Reduction of CooA Apparently Leads to His[77] Ligation. The stabilization of the heme of C75S CooA upon reduction, the similarity of the electronic absorption spectra of the Fe[II] and Fe[II]-CO forms of C75S and wild-type CooA, and the absence of any pH-dependent changes in the electronic absorption spectra of Fe[II] and Fe[II]-CO CooA all suggest that Cys[75] is replaced by another ligand upon heme reduction. Additional support for this hypothesis comes from a recent resonance Raman study by Vogel et al., where the ν_{11} marker, a vibrational mode that is sensitive to the nature of the heme ligands, was observed at similar positions for C75S and wild-type Fe[II] CooA, a result that would be surprising if Cys[75] was retained as a ligand to Fe[II] CooA (*24*). Taken together, the data indicate a switch between Cys and a new ligand in Fe[II] CooA.

In vivo and in vitro CooA activity, electronic absorption, and EPR analyses of H77Y CooA are all consistent with His[77] as a ligand to Fe[II] CooA. More direct evidence has been provided by resonance Raman analyses of wild-type and H77Y Fe[II] CooA, which identified a signal in wild-type Fe[II] CooA that is consistent with imidazolate model compounds, yet is absent in H77Y Fe[II] CooA (*24*). Aono et al. (*25*) have also concluded that His[77] may be a ligand to Fe[II] CooA on the basis of the electronic spectrum of dithionite-treated CooA. These authors suggest that H77Y CooA can be fully reduced by dithionite treatment, though their electronic spectra are similar to ours. In contrast, we observe that treatment of H77Y Fe[II] CooA with dithionite results in a mixture of Fe[II] and Fe[III] species; this admixture has been detected by resonance Raman spectroscopy (*24*). The Aono conclusion may actually reflect the binding of CO to H77Y Fe[II] CooA, shifting the equilibrium in the mixture of Fe[II] and Fe[III] H77Y CooA by simple mass action. These results strongly suggest that His[77] may play a role in controlling the redox properties of wild-type CooA.

Fe[II]-CO CooA. A sixth ligand (a ligand trans to His[77]) must be present in Fe[II] CooA as Fe[II] and Fe[II]-CO CooA hemes are low spin in nature (*23, 10, 24*). Mutational studies by Aono et al. (25) and ourselves (unpublished) on various candidate residues have thus far been unproductive in identifying this sixth ligand. Indeed, available data cannot rule out the possibility of the sixth ligand being exogenous. The two possible models are presented in Figure 8. The first possibility is that CO displaces the His[77] and an unknown ligand X remains bound as the ligand trans to CO. Consistent with this model, resonance Raman data suggest that in Fe[II]-CO CooA a negatively charged residue (i.e., imidazolate) is present in proximity to the CO molecule (*24*). In contrast, Uchida et al. (*23*) have obtained resonance Raman evidence that suggests an apolar environment of the CO of Fe[II]-CO CooA, an observation that is in conflict with our results. The origin of this discrepancy is not clear at present, although the protein preparations of Uchida et al. (*23*) have not been reported to be active in sequence-specific DNA binding and may have changed during purification.

The second possible model is that CO binding displaces the ligand trans to His[77]. Uchida et al. have concluded that His[77] is the trans ligand on the basis of a modest (2 nm) shift in the electronic spectrum of H77Y Fe[II]-CO CooA relative to that of wild type, a lack of in vivo activity of H77Y CooA, and resonance Raman data that indicate a neutral ligand trans to CO (*23*). However, a variety of

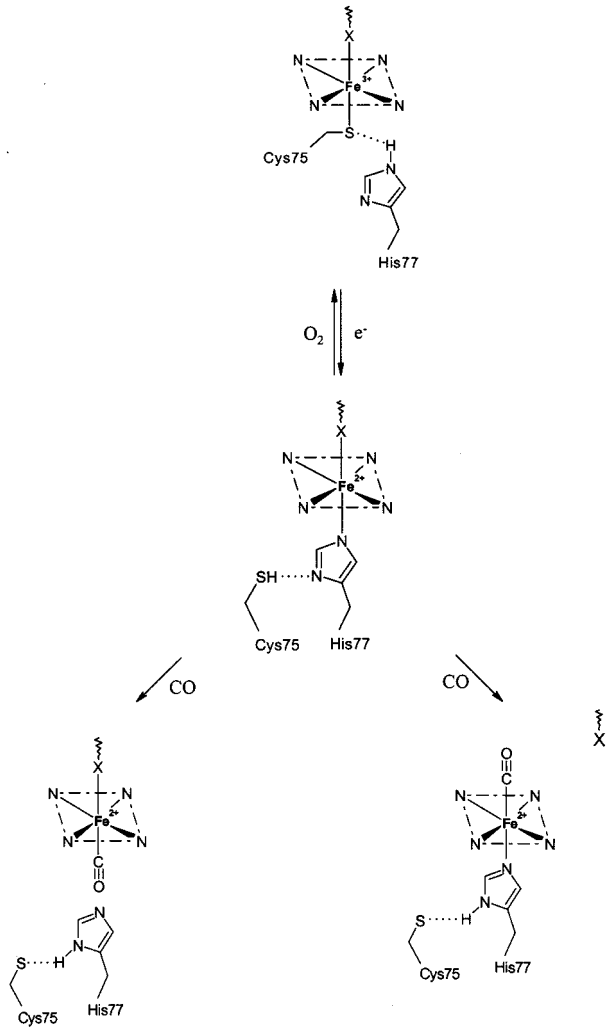

FIGURE 8: A working model for the CO-mediated activation mechanism of CooA involves a redox-mediated ligand switch. Fe^{III} CooA possesses a thiolate ligand identified as Cys^{75} in this paper. Cys^{75} and His^{77} are suggested to interact. Upon reduction, there is apparently a ligand switch to His^{77}. The identity of the ligand (X) trans to Cys^{75} or His^{77} in the oxidized or reduced forms, respectively, remains unknown, as does the side of the heme that CO binds to. In either case, the displacement of the axial ligand apparently causes a conformational change that induces CooA to bind its target site in a site-specific manner.

alternative ligands trans to CO give electronic and resonance Raman spectra similar to those of the histidine adduct. Thus, we believe that it is premature to assign the side of the heme to which CO binds, and this will remain an important question for future studies.

His^{77} is clearly a residue important for the CO activation of CooA. The results presented in this study demonstrate that although H77Y CooA has altered redox properties, its defect in transcription activation is due to an inability to undergo a conformational change upon CO binding. The negative charge present on the CO-displaced His^{77} imidazolate side chain may result in a charge repulsion with an undefined nearby residue, setting in motion movement of the polypeptide chain. An alternative model has been proposed in which His^{77} is the heme ligand retained upon CO binding, and the perturbation of this residue leads to an inactive protein through an unspecified mechanism (*23*, *24*). Both models appear to be reasonable possibilities given the currently available data.

Few naturally occurring proteins are known to undergo redox-mediated ligand switches under physiological conditions. An example of such a protein is cytochrome *cd* nitrite reductase (*39*), although a number of variant proteins are thought to undergo such a switch. Interestingly, several of these heme proteins are Cys ligated in the Fe^{III} form and are thought to lose their cysteine ligand upon reduction. In the hemoglobin variant H93C, it has been proposed that another histidine displaces the Cys^{93} upon reduction (*40*). A similar ligand switch has been proposed for the semisynthetic M80C variant of cytochrome *c*, which is thought to undergo a ligand switch from Cys^{80} to an unknown residue upon reduction (*41*, *42*). A switch from the naturally occurring thiolate ligand to an alternative ligand, possibly a histidine, has also been proposed to explain the unusual spectroscopic properties of cytochrome P420 (*43*), an inactive form of cytochrome P450, and of a similar inactive form of chloroperoxidase, C420 (*44*).

A ligand switch between the Fe^{III} and Fe^{II} forms of CooA may be physiologically relevant, as the reducing potential of the aquatic environment of *R. rubrum* is likely to fluctuate. Exposure of *R. rubrum* to O_2 (*21*) or to an oxidizing environment is known to prevent expression of the *coo* region, and this may be mediated by CooA, which does not bind CO in its oxidized form (*20*). By this mechanism, rapid inactivation of CooA in the presence of O_2 could be accomplished.

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