Electronic Absorption, EPR, and Resonance Raman Spectroscopy of CooA, a CO-Sensing Transcription Activator from *R. rubrum*, Reveals a Five-Coordinate NO-Heme[†]

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ABSTRACT: Electronic absorption, EPR, and resonance Raman spectroscopies revealed that CooA, the CO-sensing transcriptional regulator from *Rhodospirillum rubrum*, reacts with NO to form a five-coordinate NO-heme. NO must therefore displace both of the heme ligands from six-coordinate, low-spin Fe(II)-CooA in forming five-coordinate Fe(II)CooA(NO). CO, in contrast, displaces a single heme ligand from Fe(II)CooA to form six-coordinate Fe(II)CooA(CO). Of a series of common heme-binding ligands, only CO and NO were able to bind to the heme of wild-type CooA; imidazole, azide anion, and cyanide anion had no effect on the heme absorption spectrum. Although NO binds to the heme and displaces the endogenous ligands, NO was not able to induce CooA to bind to its target DNA. The mechanism of CO-dependent activation of CooA is thus more complex than simple displacement of a ligand from the heme iron since NO does not trigger DNA binding. These observations suggest that the CooA heme site discriminates between NO and the biologically relevant signal, CO.

Gas-sensing heme proteins have received much recent attention because of their role in regulating important biological processes in mammals, plants, and bacteria. Both NO-sensing (*1*−*3*) and O_2-sensing (*4*−*6*) heme proteins are known. The NO-sensing heme protein sGC[1] is found in mammals and is involved in blood pressure regulation, vision, olfaction, and neurotransmission (*7*). The O_2-sensing heme protein FixL regulates gene expression in plant-associated N_2-fixing rhizobia (*6*). These gas-sensing heme proteins are regulated by the interaction of the messenger molecule with the heme cofactor of the protein. CO has also been proposed to act as a physiological regulator of sGC. Heme oxygenase, an enzyme that produces CO as a product of heme degradation, was found in close proximity to sGC in the brain (*8*, *9*). Exogenously applied CO causes smooth muscle relaxation (*10*) and a CO/cGMP pathway is also implicated in biological signaling in the immune system (*11*) and in the inhibition of human platelet aggregation (*12*). There is, however, no direct evidence for a eukaryotic CO receptor.

The only known CO-sensing protein, CooA, was recently purified from the photosynthetic bacterium *Rhodospirillum rubrum* (*13*, *14*). CooA regulates the transcription of the *coo* operon encoding a CO-oxidation system that allows *R. rubrum* to grow with CO as the sole energy source (*15*, *16*). The CooA protein has high sequence similarity with the well-known transcriptional regulators from *Escherichia coli*, CRP,

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[1] Abbreviations: 1MeIm; 1-methylimidazole; cyt *c*, cytochrome *c*; cyt *c*-M80A or cyt *c*-M80C, cytochrome *c* where the methionine ligand is replaced by alanine or cysteine; cyt *c*′ *Alcaligenes*, cytochrome *c*′ from *Alcaligenes* sp. NCIB 11015; CCP, cytochrome *c* peroxidase; CRP, cAMP receptor protein; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DPPH, α,α′-diphenyl-β-picryl hydrazyl; DTT, D,L-dithiothreitol; EPR, electron paramagnetic resonance; equiv, equivalents; FixL, an O_2-sensing heme protein that regulates *nif* gene expression in plant-associated N_2-fixing rhizobia; FixLN, *Rhizobium meliloti* O_2-sensor heme domain (a truncated FixL protein); FNR, fumarate nitrate reduction; H450, hemoprotein 450/cystathionine β-synthase; Hb, hemoglobin; heme, iron(II) protoporphyrin IX.; HRP, horseradish peroxidase; IDO, indoleamine 2,3-dioxygenase; IHP, inositol hexaphosphate; Im, imidazole; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mb, myoglobin; Mb-H93G, Mb-H93Y, Mb-H93C, myoglobin where the proximal histidine is replaced by glycine, tyrosine, or cysteine, respectively; NMR, nuclear magnetic resonance; P420, an inactive form of cytochrome P450; P450, cytochrome P450; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PGHS, prostaglandin H synthase; PPDME, protoporphyrin IX dimethyl ester; PPIX, protoporphyrin IX; py, pyridine; *R. rubrum*, *Rhodospirillum rubrum*; SDS, sodium dodecyl sulfate; sGC_1, soluble guanylyl cyclase isolated from bovine lung containing a mixture of five-coordinate high-spin and six-coordinate low-spin ferrous heme; sGC_2, soluble guanylyl cyclase isolated from bovine lung containing only five-coordinate high-spin ferrous heme; SIR, sulfite reductase; THF, tetrahydrofuran; TPP, tetraphenylporphyrin.

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and FNR (*13*). CooA from *R. rubrum* is a homodimeric protein with subunits of 24.6 kDa. Purified, wild-type CooA contains one heme per monomer (*13*, *17*). CooA responds to CO by binding to specific DNA sequences (*13*); thus, CO-sensing CooA is a new example of a gas-sensing heme protein.

Because the heme of CooA is six-coordinate and low-spin in both the reduced and the CO-bound states, CO must displace an axial ligand from the heme (*17*−*20*). The loss of a heme ligand from Fe(II)CooA is proposed to trigger a conformational change in the protein that leads to site-specific DNA binding (*17*−*21*). A similar mechanism has been proposed for the NO activation of sGC (*22*−*24*), and recent spectroscopic studies with purified sGC revealed that NO displaces the proximal histidine from the heme (*1*, *25*−*29*). In the case of FixL, O_2 binding to the heme iron blocks autophosphorylation via a conformational change regulated by the spin state of the heme (*6*). The manner in which these heme proteins selectively sense NO, CO, or O_2 provides the key to understanding how proteins recognize these messenger molecules. We are therefore interested in understanding how the heme coordination environment is involved in modulating the conformational change in CooA. Toward this goal, we studied the interaction between CooA and the well-known small molecule heme probes NO, CN^-, N_3^-, and Im. We found that only NO and CO were able to bind to the heme of CooA and that only CO, and not NO, was able to cause CooA to bind to its target DNA. These results provide insight into the mechanism of CO activation and suggest that CO is the specific biological messenger for CooA.

EXPERIMENTAL PROCEDURES

Materials. A NO gas (99.5%) cylinder was purchased from AGA and $Na^{15}NO_2$ (98%) was purchased from Cambridge Isotopes. Im (99%), NaN_3, ascorbic acid, sodium 4-hydroxymercuribenzoate and 1MeIm (99%) were purchased from Aldrich. NaCN was obtained from Mallinckrodt and $HgCl_2$ from J. T. Baker, Inc. The 1MeIm was distilled under vacuum. The NO gas was passed over an anaerobic KOH column to remove higher order nitrogen oxides before use. Other materials were used as purchased.

Expression of CooA in E. 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) (*30*) and downstream (*Hin*dIII-containing) primers designed according to the *cooA* sequence (*31*) with previously described extensions (*32*).

Protein Purification and Methods. Isolation and purification of recombinant CooA was performed as described previously (*19*). CooA isolated by this procedure was stored in 25 mM Mops, 1 mM DTT, and 0.1 M NaCl at −80 °C. Purified CooA was greater than 95% pure as judged by densitometric scanning of Coomassie-stained SDS−PAGE gels. The CooA samples used in these experiments were tested for CO-dependent, site-specific DNA binding by DNase I footprinting, as described previously (*15*). The protein concentration of CooA samples was measured by the bicinchoninic acid (BCA) assay as modified for DTT-containing solutions (*33*). The heme content of each batch of purified CooA was determined using the pyridine hemochromagen assay (*34*).

Spectrophotometric Methods. Electronic absorption spectra were recorded on a Varian Cary 4 Bio spectrophotometer with a slit width of 0.5 nm. The Fe(III), Fe(II), and Fe(II)-CO forms of CooA were formed as previously described (*13*). The Fe(II) NO-adduct was formed by deoxygenating ∼500 μL of Fe(III)CooA (10−20 μM in heme) in a septum-sealed quartz cuvette for 15−20 min under a low flow of Ar, followed by reduction with 2 equiv of sodium dithionite from an anaerobic aqueous stock solution (1 mM). NO gas, from a septum-sealed container of NO, was then added to each Fe(II)CooA sample via a gastight syringe. Typically, ∼100−300 μL of NO gas was required to form the NO adduct. The electronic spectra were then recorded at room temperature after several minutes of incubation.

Stability of NO-Heme in Fe(II)CooA(NO). An aliquot of Fe(III)CooA (100 μL, 50 μM heme) was reduced with a 2-fold excess of sodium dithionite from a 1 mM stock solution and 500 μL of NO gas was added to form Fe(II)-CooA(NO). Formation of Fe(II)CooA(NO) was confirmed by electronic absorption spectroscopy. The protein sample was then passed over a NICK Sephadex G-50 desalting column (Pharmacia) under an Ar atmosphere, and fractions (500 μL) were collected. The fractions were assayed for heme and protein content by electronic absorption spectroscopy.

Ligand-Binding Studies. The ligand-binding experiments were performed by titrating Fe(III)- or Fe(II)CooA samples with aqueous ligand stock solutions. In each titration, four to five additions of ligand were made, with each addition resulting in an order of magnitude increase in the number of equivalents added relative to CooA. The range over which the titrations were carried out were CN^- 1×10^0 to 4×10^4 equiv, N_3^- 2×10^1 to 6×10^3 equiv, Im 2×10^1 to 2.6×10^4 equiv, 1MeIm 6.1×10^4 or 1.8×10^9 equiv, $HgCl_2$ 0.2−20 equiv, and 4-mercuribenzoate 0.2−20 equiv. The experiments were performed by adding 5−10 μL of an appropriately diluted ligand stock solution to a 400 μL aliquot of Fe(III)- or Fe(II)CooA (5−10 μM in heme) in a quartz cuvette. Fe(II)CooA was prepared by anaerobic reduction of Fe(III)CooA by addition of sodium dithionite to 1 mM final concentration. The stock solutions of CN^-, N_3^-, Im, and 1MeIm were prepared in 25 mM Mops and 1 mM DTT at pH 7.4. Solutions of $HgCl_2$ and 4-mercuribenzoate were prepared similarly, but DTT was omitted. (***CAUTION***: Addition of acid to cyanide solutions generates poisonous HCN gas.)

EPR Spectroscopy. Fe(II)CooA(NO) was formed by the addition of 1−2 mL of NO gas to anaerobic Fe(II)CooA samples (500 μL, 50−250 μM in heme), prepared by reduction of Fe(III)CooA with 2 equiv of sodium dithionite, in a septum-sealed vial. Reduction of Fe(III)CooA and formation of Fe(II)CooA(NO) were verified by recording the optical spectra. ^{14}NO gas was collected from an NO gas cylinder attached to a vacuum line, while the ^{15}NO gas was formed under anaerobic conditions by the reduction of an aqueous solution of $Na^{15}NO_2$ with sodium ascorbate. The samples of Fe(II)CooA(NO) were then transferred to Ar-filled EPR tubes, via gastight syringe, and frozen in liquid nitrogen. EPR spectra were recorded on a Bruker ESP 300E spectrometer. The field was calibrated using a Bruker 035 M gaussmeter corrected with a DPPH standard. The microwave frequency was monitored using a model 625A microwave counter. Spectra were obtained at 77 K using a liquid

nitrogen dewar. A background signal due to iron contamination of the DTT-containing buffer was removed from the experimental signals by subtraction. ^{14}NO or ^{15}NO was added to the buffer alone, and the resulting signal was recorded. The signal, attributable to non-heme iron(II) dinitrosyl (g = 2.03) complex of DTT, was then subtracted from the experimental spectra. The resulting spectra of Fe(II)CooA($^{14/15}NO$) were simulated using the WIN-EPR Simfonia software package (version 1.2) from Bruker.

Resonance Raman Spectroscopy. Fe(III)CooA (300 μL, 180 μM in heme) was placed in a septum-sealed 0.5 mm NMR tube, purged with Ar, and reduced by addition of sodium dithionite to a final concentration of 1.7 mM. NO gas was generated by reaction of $Na^{14}NO_2$ or its ^{15}N isotopomer with sodium ascorbate in an anaerobic aqueous solution and added to the Fe(II)CooA sample using a gastight syringe. Absorption spectra of Fe(III)CooA, Fe(II)CooA, and Fe(II)CooA(NO) were obtained on a Hewlett-Packard 8452A diode array spectrophotometer to confirm the reduction and binding of exogenous ligand before obtaining resonance Raman spectra.

Resonance Raman spectra were obtained with an excitation wavelength of 406.7 nm from a Kr^+ laser (Coherent Innova 100-K3) in a backscattering sample geometry. A low incident laser power of ~50 mW was focused with a cylindrical lens onto the sample. The protein samples were kept spinning throughout the laser illumination in order to reduce local heating and to minimize photodissociation of the bound ligands. The scattered light was collected and focused onto a Princeton Instruments Spex 1877 triple spectrograph equipped with a cooled, intensified diode array detector, under computer control. Spectra were calibrated with toluene, indene, acetone, acetonitrile, d_6-DMSO, and CCl_4. All spectral data were imported to and processed with Labcalc software (Galactic Industries Corp.).

DNase I Footprinting. Fe(II)CooA(NO) was formed by adding NO to either Fe(III)CooA or Fe(II)CooA, under the same conditions used to prepare samples for electronic absorption spectroscopy. Initially, the NO concentration was varied from 1 equiv to vast excess, relative to CooA. In further experiments, the level of NO used in the DNA-binding assays was the minimum sufficient to quantitatively form the NO adduct, Fe(II)CooA(NO), as determined by monitoring the visible spectrum. The DNA-binding ability of the Fe(II)CooA(NO) samples was tested using the DNase I footprinting assay previously described (*15*). Care was taken to avoid exposure of the Fe(II)CooA(NO) samples to the air during the DNase I cleavage reaction.

RESULTS

Because CO binds to the heme of CooA, the electronic and structural properties of the heme play a key role in the CO-sensing mechanism. To understand the role of the heme coordination environment in the regulation of CooA DNA-binding activity, we have studied the heme environment of CooA using NO and other well-known small molecule heme ligands. CN^-, N_3^-, Im, and NO were tested for reactivity with Fe(III)CooA since these ligands have a high affinity for ferric heme and have been used to study the ferric state of other heme proteins (*35*−*37*). To probe the ferrous state of CooA, the ligands NO, Im, and 1MeIm were used because of their affinity for ferrous hemes. NO is a particularly useful tool in studying the electronic state and coordination environment of a variety of heme proteins since it has a high affinity for both ferric and ferrous heme (*35*, *38*−*40*). NO also provides a paramagnetic probe of the ferrous state of heme proteins, and in cases where a N-donor ligand is bound trans to NO, EPR spectroscopy can provide conclusive identification of the sixth axial ligand through the observation of a superhyperfine interaction (*39*).

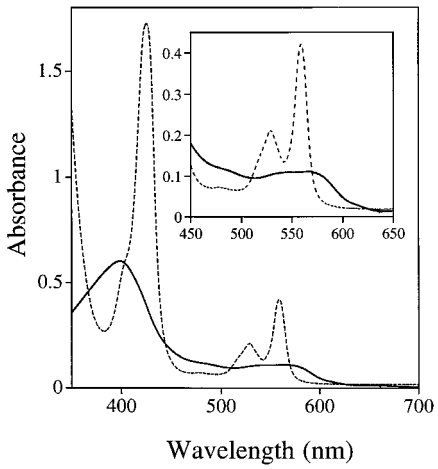

FIGURE 1: Electronic absorption spectra of purified Fe(II)CooA (- - -) and Fe(II)CooA(NO) (—). The sample contained 15 μM heme, 25 mM Mops, 1 mM DTT, and 0.1 M NaCl, at pH 7.4, 25 °C, maintained under an Ar atmosphere.

Characterization of Fe(II)CooA(NO) by Electronic Absorption Spectroscopy. Electronic absorption spectroscopy reveals that NO binds to the heme of CooA displacing both endogenous ligands and forming an NO adduct similar to other five-coordinate NO-hemes. Upon addition of NO to Fe(II)CooA, the Soret band blue-shifted from 425 to 399 nm and decreased in intensity (Figure 1). The α and β bands of Fe(II)CooA also shifted in peak position when NO was added and the peaks became almost equal in intensity. The broad Soret band at ~400 nm for Fe(II)CooA(NO) is typical of five-coordinate NO-hemes (*26*, *41*) and is distinctly different from six-coordinate NO-hemes that have a Soret band at 415−425 nm (Table 1). The electronic spectrum of Fe(II)CooA(NO) is similar to that of known five-coordinate NO-heme models and NO-heme proteins, including NO-sensing sGC (*25*, *26*, *42*) (Table 1). These similarities suggest that Fe(II)CooA(NO) contains a five-coordinate NO-heme. In the reaction of Fe(II)CooA with NO, a six-coordinate NO-heme with a Soret band at 415−425 nm was never observed. The behavior of CooA in response to NO is different than that in response to CO, the natural activator of CooA, which forms a six-coordinate low-spin CO adduct (*13*, *17*, *20*, *21*).

The loss of both axial ligands upon NO binding may result in loss of the heme from the protein. To determine whether the heme remained bound to CooA, Fe(II)CooA(NO) was passed over a gel filtration column. When the fractions were assayed for NO-heme and protein, by electronic absorption spectroscopy, it was observed that the NO-heme always eluted with the protein. These data indicate that dissociation of NO-heme from CooA does not occur. Uchida et al. (*21*) had reported that Fe(II)CooA(NO) was unstable. Since the heme did not appear to dissociate from the protein, we determined the stability of Fe(II)CooA(NO) over extended times and upon exposure to air. The electronic absorption

Table 1: Comparison between the Electronic Absorption Spectral Parameters of Fe(II)CooA(NO) and Five- and Six-Coordinate Fe(II)Heme(NO) Models and Proteins

protein/compound	Soret (nm)	CT[a] (nm)	β (nm)	α (nm)	refs
	Five-Coordinate Fe(II)Heme(NO), λ_{max} (ϵ, mM^{-1} cm^{-1})				
Fe(II)CooA(NO)	399		544	572	this work
Fe(II)PPIXDME(NO)	400.5 (81.6)	480 sh[b] (11)	550 sh (10)	569.5 (10.5)	*41*
Fe(II)sGC$_1$(NO)	398		NR[c]	NR	*42*, *25*
Fe(II)sGC$_2$(NO)	398 (79)		537 (12)	572 (12)	*27*, *69*
Fe(II)PGHS(NO)	402		536	567	*47*
Fe(II)Mb-H93G(NO)	400		NR	NR	*46*
Fe(II)cyt *c*′(NO)	397 (78.9), 415 sh	485 (9.8)	541 (10.4)	565 sh (10)	*49*
	Six-Coordinate Fe(II)Heme(NO), λ_{max} (ϵ, mM^{-1} cm^{-1})				
Fe(II)PPDME(NO)(1MeIm)	418.5 (118)		546.5 (11.5)	576.5 (10.6)	*41*
Fe(II)IDO(NO)	418.5 (127)		544 (12.2)	574 (12.5)	*70*
Fe(II)cyt *c*(NO)	411 (127)		538.5 (10.2)	567 (9.8)	*71*
Fe(II)Mb(NO)	420 (127)		548 (11.3)	579 (10.1)	*51*
Fe(II)CCP(NO)	421 (99)		542 (12)	572 (11)	*39*
Fe(II)Hb(NO), R state	418		545	573	*72*

[a] CT, charge-transfer band. [b] sh, shoulder. [c] NR, not reported.

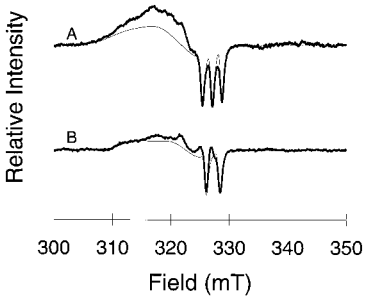

FIGURE 2: EPR spectra of Fe(II)CooA(^{14}NO/^{15}NO). (A) X-band EPR spectrum of Fe(II)CooA(^{14}NO) (thick line) and the best-fit simulation (thin line). The Fe(II)CooA(^{14}NO) sample contained 50 μM heme, 25 mM Mops, 1 mM DTT, 100 μM dithionite, and 0.1 M NaCl at pH 7.4. The spectrum was recorded at 77 K, 9.2201 GHz microwave frequency, 2.53 mW microwave power, 1×10^6 receiver gain, 2.09 G modulation amplitude, 100 kHz modulation frequency, 0.66 s time constant, using 64 added scans each containing 2048 data points. The background was corrected and the best-fit simulation was generated as described in the Experimental Procedures. (B) X-band EPR spectrum of Fe(II)CooA(^{15}NO) (thick line) and the best-fit simulation (thin line). The Fe(II)CooA(^{15}NO) sample contained 100 μM heme, 25 mM Mops, 1 mM DTT, and 0.1 M NaCl at pH 7.4. The spectrum was recorded at 77 K, 9.2223 GHz microwave frequency, 50.5 mW microwave power, 8×10^5 receiver gain, 2.09 G modulation amplitude, 100 kHz modulation frequency, 0.66 s time constant, using 32 added scans, each containing 2048 data points. The background was corrected and the best-fit EPR simulation was generated as described in the Experimental Procedures.

spectrum of Fe(II)CooA(NO) did not change even after 3 h at 25 °C, as long as the samples were kept strictly anaerobic. Exposure of dithionite-free Fe(II)CooA(NO) [prepared by addition of NO to Fe(III)CooA] to the atmosphere resulted in the formation of Fe(III)CooA, as revealed by the electronic absorption spectrum (data not shown).

EPR Spectroscopy of Fe(II)CooA(NO). EPR spectroscopy provides a more definitive method than electronic spectroscopy for determining the coordination state of ferrous NO-hemes. The EPR spectrum of Fe(II)CooA(NO) (Figure 2a) is characteristic of a five-coordinate NO-heme, confirming the conclusion based on the optical spectrum. Fe(II)CooA(^{14}NO) exhibits the classic axially symmetric three-line spectrum characteristic of five-coordinate NO-heme proteins and model complexes (*40*). The hyperfine triplet arises from coupling to the single $I = 1$ ^{14}N nucleus of the bound NO; the EPR spectrum of Fe(II)CooA(^{15}NO) (Figure 2b) exhibited a hyperfine doublet (^{15}N, $I = 1/2$) consistent with this assignment. The g values and the hyperfine coupling constants measured for Fe(II)CooA(^{14}NO/^{15}NO) are comparable to those of other five-coordinate nitrosyl hemes (Table 2). The EPR spectral characteristics of Fe(II)CooA(NO) are most similar to those of NO adducts of Hb in the presence of SDS or a T-state stabilizing allosteric regulator (*43*−*45*), Mb-H93G (*46*), FixLN (*5*), PGHS (*47*, *48*), Cyt *c*′ (*49*), P420 (*50*, *51*), and CCP and HRP at pH 3 (*52*). All of these heme proteins are believed to contain either a substantially weakened or ruptured iron−proximal histidine bond when NO is bound. Six-coordinate nitrosyl heme proteins, such as the NO-adducts of CCP or HRP at neutral pH (*39*), exhibit rhombic EPR spectra with nine-line signals due to additional superhyperfine coupling between the unpaired electron of NO and the nitrogen of the proximal histidine ligand (Table 2). Since Fe(II)CooA(NO) contains a five-coordinate NO-heme, indicating that both of the heme ligands present in six-coordinate Fe(II)CooA are displaced upon NO binding, we were unable to identify any endogenous heme ligands by EPR spectroscopy.

Resonance Raman Spectroscopy of Fe(II)CooA(NO). The NO-bound form of wild-type Fe(II)CooA was further characterized by resonance Raman spectroscopy. The resonance Raman spectrum of Fe(II)CooA(NO) is shown in Figure 3. The elevated frequencies of ν_3 (1506 cm^{-1}) and ν_{10} (1641 cm^{-1}), porphyrin vibrational modes that are sensitive to the heme coordination and spin state (*53*), are consistent with the presence of a five-coordinate NO-heme (*25*, *54*). The five-coordinate model complex Fe(II)PPIX(NO) (*55*) and the NO-adducts of sGC (*1*, *25*, *28*, *54*), Mb with the denaturant SDS (*25*) and Mb-H93Y (*25*), exhibit similarly elevated ν_3 and ν_{10} bands. Six-coordinate NO-hemes, in contrast, have lower ν_3 and ν_{10} frequencies (*56*). The NO stretching modes of Fe(II)CooA(NO), ν(Fe−NO), and ν(N−O), were at 523 and 1672 cm^{-1}, respectively, as determined by isotopic substitution (Figure 3). These values are typical of five-coordinate NO-hemes and are substantially different from those of six-coordinate NO-hemes (Table 3). The resonance Raman spectral data confirms the electronic absorption and EPR spectroscopic identification of a five-coordinate NO-heme in Fe(II)CooA(NO).

Table 2: Comparison between the EPR Spectral Parameters of Fe(II)CooA(NO) and Those of Five- and Six-Coordinate Fe(II)Heme(NO) Models and Proteins

protein/complex	g_1	g_2	g_3	A_3[a] (Gauss)	refs
Fe(II)CooA(^{14}NO)	2.106	2.050	2.015	16.0	this work
Fe(II)CooA(^{15}NO)	2.100	2.045	2.015	21.8	this work
		Five-Coordinate Fe(II)Heme(NO)			
Fe(II)PPDME(NO),	2.102	2.058	2.010	16.7	*73*
Fe(II)Hb(NO) + IHP	2.070	2.04	2.008	16.5	*45*
Fe(II)cyt *c′*(NO)	2.106	2.058	2.010	16.0	*49*
Fe(II)Mb-H93G(NO)	NR[b]	NR	~2.01	~17	*46*
Fe(II)sGC$_2$(NO)	2.076	2.029	2.005	16.0	*26*
Fe(II)FixLN(NO)	NR	NR	2.0	17	*5*
Fe(II)PGHS(NO)	NR	NR	2.00	17.0	*47*
		Six-Coordinate Fe(II)Heme(NO)			
protein/complex	g_1	g_2	g_3	A_1/A_2[c] (Gauss)	refs
Fe(II)PPDME(NO)(Im)	2.072	1.971	2.004	21.7/6.9	*74*
Fe(II)PPIX(NO)(Im)	2.077	2.003	1.968	21/7	*40*
Fe(II)HRP(NO)	2.080	1.955	2.004	20.5/6.5	*39*
Fe(II)CCP(NO)	2.080	1.96	2.004	21/6.4	*39*
Fe(II)Hb(NO)	2.075	1.975	2.005	d	*39*
Fe(II)Mb(NO)	2.08	1.998	1.979	d	*40*

[a] A_3, the hyperfine coupling constant arising from NO (^{14}N, $I = 1$; ^{15}N, $I = 1/2$). [b] NR, not reported. [c] A_1/A_2, the hypefine coupling constants from NO and an additional nitrogen atom (histidine). [d] Hyperfine coupling was present but not resolved.

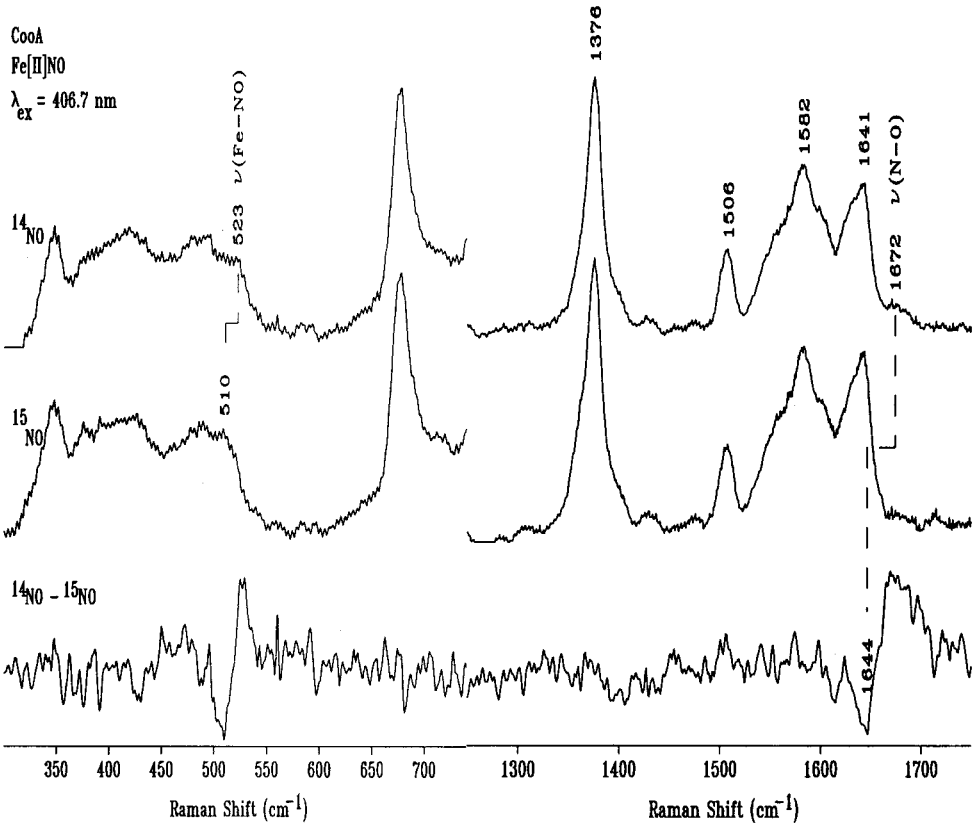

FIGURE 3: Resonance Raman spectra of the $^{14/15}$NO adducts of CooA and the ^{14}NO minus ^{15}NO difference spectrum identifying the ν(Fe−NO) and ν(N−O) bands. The CooA samples contained 180 μM heme, 25 mM Mops, 1 mM DTT, 1.7 mM dithionite, and 0.1 M NaCl at pH 7.4.

A five-coordinate NO-heme has been examined in several heme proteins, permitting comparison with Fe(II)CooA(NO). A correlation diagram of ν(Fe−NO) versus ν(N−O) shows that Fe(II)CooA(NO) falls on the back-bonding line for five-coordinate NO-heme proteins and models (Figure 4) (*57*). The CooA point is close to that of five-coordinate Mb(NO) adducts, prepared either by lowering the pH to 4.0 or by changing the His^{93} proximal ligand to weakly coordinating tyrosine (*58*). There is no indication of a distal perturbation of the bound NO in CooA, as there is for sGC$_1$, one of the sGC isoforms (*25*). The vibrational frequencies observed for Fe(II)CooA(NO) are consistent with a five-coordinate NO-heme in an unperturbed Fe−NO environment (*25*).

Only CO and NO Bind to the Heme of CooA. The heme environment of CooA is clearly different from other gas-sensing heme proteins, providing no site for the interaction

Table 3: Comparison between the Vibrational Frequencies of Fe(II)CooA(NO) and Five- and Six-Coordinate Fe(II)Heme(NO) Models and Proteins

protein/compound	frequency (cm^{-1}) ν(Fe−NO)	ν(N−O)	refs
Fe(II)CooA(NO)	523	1672	this work
Five-Coordinate Fe(II)Heme(NO)			
Fe(II)sGC$_1$(NO)	NR[a]	1660	*25*
Fe(II)sGC$_1$(NO)	520	NR	*28*
Fe(II)sGC$_2$(NO)	525	1677	*1*
Fe(II)sGC$_2$(NO)	521	1681	*27*
Fe(II)TPP(NO)	525	1681	*75*
Fe(II)Mb(NO) + SDS	525	1675	*25*
Fe(II)Mb-H93Y(NO)	524	1672	*25*
Fe(II)FixL(NO)	525	1675	*4*
Fe(II)FixLN(NO)	525	1676	*4*
Six-Coordinate Fe(II)Heme(NO)			
Fe(II)Hb(NO)	551	1615	*55*
Fe(II)Mb(NO)	554	1624	*55*
Fe(II)P450(NO)	554	1591	*76*
Fe(II)SIR(NO)	558	1555	*77*

[a] NR, not reported.

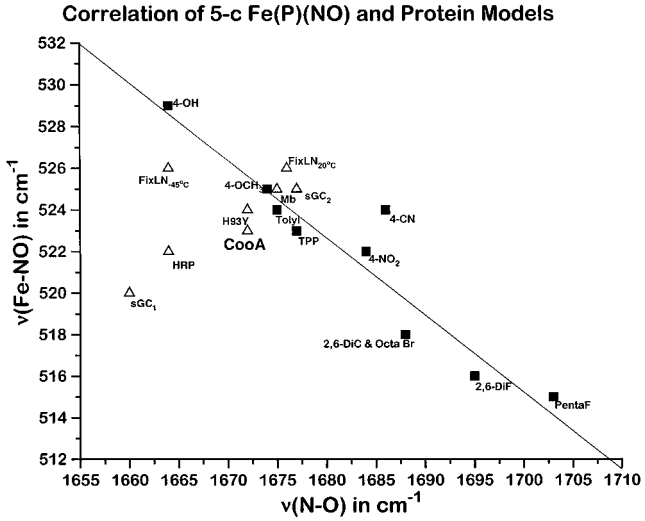

FIGURE 4: A correlation diagram of vibrational frequencies for five-coordinate Fe(II)heme(NO) models and proteins. The triangles designate the NO-adducts of heme proteins (see abbreviations)[1] while the squares designate phenyl-modified derivatives of Fe(II)-TPP(NO). The model metalloporphyrin complexes are denoted by the identity of the functional group(s) on the phenyl rings of TPP; an exception is the 2,6-DiC & Octa Br, which bears chlorine substituents on the 2 and 6 positions of the phenyl rings and bromine substituents on all the pyrrole rings.

of typical heme ligands, with the exception of CO and NO. The binding of CN^-, N_3^- and Im to the heme of Fe(III)-CooA was monitored by electronic absorption spectroscopy. Addition of CN^- (4×10^4 equiv), N_3^- (6×10^3 equiv), Im (2.6×10^4 equiv), and 1MeIm (6.1×10^4 equiv) caused no changes in the electronic absorption spectrum of Fe(III)CooA (data not shown). These results suggest that the heme of Fe-(III)CooA is either inaccessible to these small molecules or that two ligands are bound tightly to the heme. Fe(II)CooA was also unable to bind exogenous ligands. Neither addition of 1MeIm (1.8×10^9 equiv) nor Im (1×10^3 equiv), typical ferrous heme ligands, resulted in any change in the electronic absorption spectrum of Fe(II)CooA (data not shown). Prior studies suggested that both Fe(III)CooA and Fe(II)CooA contain six-coordinate low-spin heme with two endogenous

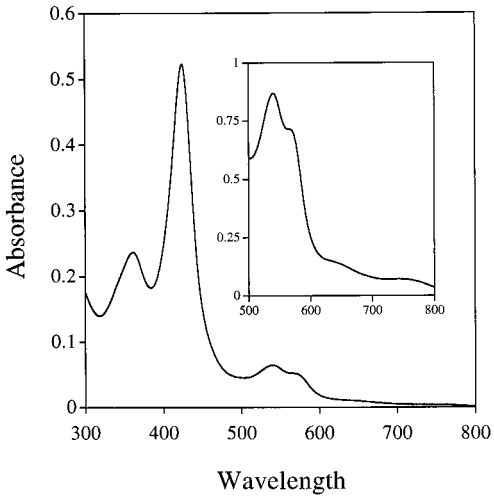

FIGURE 5: The electronic absorption spectrum of Fe(III)CooA showing the thiolate sulfur-to-Fe(III) charge-transfer bands (inset). The Fe(III)CooA samples contained 10.2 or 102 μM heme (inset) and 25 mM Mops, 1 mM DTT, and 0.1 M NaCl at pH 7.4.

protein ligands bound to the heme iron (*13*, *14*, *17*−*21*); these ligands are apparently not displaced by exogenous heme ligands with moderate binding affinities.

We also studied the interaction of NO with Fe(III)CooA. NO, unlike CO, binds to the ferric state of heme proteins (*35*, *38*). Upon addition of NO to an anaerobic solution of Fe(III)CooA, the 426 nm Soret band of Fe(III)CooA blue-shifted to 400 nm; the new spectrum was identical to the spectrum of Fe(II)CooA(NO) previously observed. Thus, NO reacts with Fe(III)CooA to form the same five-coordinate NO-heme that was observed in the reaction of NO with Fe-(II)CooA. The heme of Fe(III)CooA is therefore similar to that of several heme proteins, including Hb, and model ferric porphyrins, which are reduced by NO to form ferrous NO-hemes (*38*). Fe(III)CooA is dissimilar to heme proteins such as Mb (*38*), PGHS (*47*), and the nitrophorins (*59*, *60*), which can reversibly bind NO in the ferric state. Most importantly, these results reinforce the conclusion that only very high affinity ligands such as NO and CO are able to displace the endogenous ligands from the heme iron of CooA.

The interaction of thiol-modifying agents with Fe(III)CooA was studied because Fe(III)CooA contains a cysteine thiolate ligand (*17*−*20*, *62*). The electronic absorption spectrum of Fe(III)CooA is similar to that of other low-spin, thiolate-bound heme proteins (Figure 5, Table 4). Interestingly, in neither Fe(II)CooA nor Fe(II)CooA(CO) is the heme iron bound to a thiolate ligand, as evidenced by the spectral properties of these forms of the protein (Table 4) and the effects of site-directed mutagenesis of Cys[75] (*17*, *19*). When Fe(III)CooA was treated with an equimolar amount of a thiol-modifying agent, either $HgCl_2$ or 4-mercuribenzoate, the electronic absorption spectrum was significantly altered. The Soret absorption decreased in intensity, broadened, and blue-shifted to approximately 400 nm. The product spectrum appears to be that of a high-spin Fe(III) species. Although the thiol-modifying reagents may interact with the other cysteine residues either inside or outside the heme pocket, the change in the electronic absorption spectrum suggests that the reagents have direct access to the heme pocket of Fe(III)CooA and are reacting directly with a ligand to the heme. Together, our results suggest that the heme site of

Table 4: Comparison of the Electronic Absorption Spectral Parameters of CooA Derivatives with Those of Thiolate-Ligated Heme Proteins and Model Complexes[a]

protein	iron ligands	δ (nm)	Soret (nm)	β (nm)	α (nm)	LMCT[b] (nm)	refs
			Fe(III) Derivatives				
Fe(III)CooA		362	424	540	574	649, 750	*c*
Fe(III)P450(Im)	ImH/Cys	358	425	542	574	638, 753	*78*
Fe(III)cyt *c*-M80C	His/Cys[d]	355	416	540	570	635, 734	*78*
Fe(III)cyt *c*-M80A(CH_{3S}^-)	His/RS^-	355	416	535	566	648, 763	*78*
Fe(III)H450	His/Cys	360	428	550	NR[e]	NR	*79*
Fe(III)Mb + H_{2S}	His/SH^-		425	545	570	625	*80*
Fe(III)P420	*f*	367	422	541	566	651	*81*
			Fe(II) Derivatives				
Fe(II)CooA			425	529	559		*c*
Fe(II)P450(py)	Cys/py		444	538	566		*82*
Fe(II) cyt c-M80C	*d*		416	520	550		*83*
Fe(II)H450, pH 6.0	His/CysH		425	530	558		*79*
Fe(II)H450, pH 8.0	His/Cys		448	540	571		*79*
Fe(II)P420	*f*		424	530	558		*81*
Fe(II)Mb-H93C	*g*		429	NR	558		*84*
			Fe(II)CO Derivatives				
Fe(II)CooA(CO)			422	540	569		*c*
Fe(II)Mb(CO)	His	346	423	540	579		*78*
Fe(II)H450(CO)	His		420	540	570		*79*
Fe(II)P420(CO)	*f*		420	540	570		*81*
Fe(II)Mb-H93C(CO)	*g*		422	541	571		*84*
Fe(II)P450(CO)	Cys	366	446	551			*82*

[a] Peak positions are reported in nanometers. [b] LMCT, ligand-to-metal charge-transfer band. [c] This work, spectra were recorded at 25 °C in 25 mM Mops buffer, 1 mM DTT, 0.1 M NaCl, pH 7.4. [d] Cyt *c*-M80C contains a His/Cys ligated heme in the ferric form but the ligation state of the reduced and CO bound states is not known (*78*, *83*, *85*). [e] NR, not reported. [f] The ligation state of the heme in the oxidized, reduced and CO-bound states is not known (*81*). [g] The ligation state of the heme in the reduced and CO-bound states is not known (*84*, *86*).

CooA is accessible to exogenous ligands, since thiol-modifying agents affect the heme electronic spectrum, but only high-affinity heme ligands, such as CO and NO, are able to displace an endogenous ligand and bind to the heme iron.

Effect of NO on CooA Binding to Its Target DNA. Since NO has a high affinity for ferrous heme and is a cellular signaling agent in eukaryotes, we wished to determine whether NO could cause CooA to become competent to bind to DNA. The effect of NO was tested on Fe(III)CooA and Fe(II)CooA; the protein samples were tested for their ability to bind to the target DNA sequence using the DNase I footprinting assay (*13*). NO did not induce DNA binding on exposure to either oxidized or reduced CooA. When tested side by side, CO induced protection of the expected target sequence while NO produced no detectable footprint (data not shown). It is impossible to completely rule out an experimental artifact as the source of this negative result; however, we note that NO does not cause CooA denaturation or heme loss under the conditions used (vide supra). We were also unable to detect NO-dependent DNA binding by CooA using a fluorescence polarization assay.[2] Thus, it appears that CO is the only messenger molecule that induces CooA to bind its target DNA.

DISCUSSION

Spectroscopic properties of the CooA heme determined to date suggest that different amino acids are coordinated to the heme iron in the Fe(III), Fe(II), and Fe(II)-CO states, but the identities of all the heme ligands are not yet known. EPR and electronic absorption spectroscopic studies reveal that oxidized Fe(III)CooA contains a low-spin, cysteine-bound heme (Table 4) (*17*−*20*, *62*). The cysteine ligand was identified as Cys^{75} through site-directed mutagenesis (*17*, *19*); replacement of cysteine with serine in an Fe(III)CooA-C75S variant resulted in a protein with a labile, high-spin heme (*19*, *62*). Heme lability has also been observed in cysteine thiolate bound heme proteins such as nitric oxide synthase (*63*, *64*) and cytochrome P450 (*65*), when the cysteine ligand is altered. Interestingly, there is no evidence for a thiolate-bound heme in Fe(II)CooA or Fe(II)CooA(CO) (Table 4). Mutagenesis combined with spectroscopic studies indicated that a nearby residue, His^{77}, might replace the Cys^{75} residue upon reduction of the CooA heme (*17*, *19*, *20*). The heme of Fe(II)CooA(CO) is low-spin with spectroscopic properties that are similar to CO-bound Hb and Mb (Table 4), an observation most consistent with a histidine ligand trans to the CO (*13*, *20*, *21*). It is not known conclusively whether CO displaces His^{77} or the as yet unidentified sixth ligand from the heme (*17*, *19*, *62*), but clearly the retained ligand has donor characteristics similar to histidine.

NO often binds to the same position as CO and, by virtue of the coupling of its unpaired electron to the nuclear spin of an additional axial ligand, may report on the ligand trans to CO. CooA, however, forms a five-coordinate NO-heme adduct, in which both endogenous ligands are displaced. The electronic absorption, EPR, and resonance Raman studies presented reveal that NO binds to either Fe(III)- or Fe(II)-CooA to form a five-coordinate nitrosyl ferrous heme. Relatively few heme proteins form five-coordinate NO-hemes since the axial ligand is often held in close proximity to the heme by the protein fold. Proteins that do form five-coordinate NO-hemes include the α subunits of the T state of Hb (the state of lower oxygen affinity) (*66*) and NO-sensing sGC (*26*). In these cases, it is believed that there is

[2] Thorsteinsson et al., unpublished results.

strain on the proximal iron−histidine bond. The formation of five-coordinate NO-heme is thermodynamically preferred as NO exerts a strong trans influence (*22*, *67*); however, this effect is commonly overwhelmed by the forces holding the protein in its native conformation. The fact that two ligands can be easily displaced in CooA suggests that the CooA heme may be in a conformationally labile environment.

Further evidence for conformational mobility and its importance to CooA function is revealed in the differences between the response of the protein to CO and NO. The formation of the five-coordinate Fe(II)CooA(NO) from either Fe(III)- or Fe(II)CooA is not instantaneous, but is complete in a matter of minutes. This result is surprising since there are apparently two protein-derived ligands that are bound to the heme, and both ligands must be displaced by NO. A more typical ferrous six-coordinate heme protein, Fe(II)cyt *c*, reacts very slowly with NO (*68*). In the reaction of CO with CooA, only one endogenous ligand is displaced; the displacement of even a single ligand is unexpected and suggests that the heme-binding environment of CooA is uniquely flexible. Furthermore, CO activates the protein for DNA binding but NO does not. Since both CO and NO displace one endogenous ligand, either the loss of this ligand is necessary but not sufficient to achieve activation or the NO-induced displacement of the second ligand negates the effect of displacement of the first.

It is presently uncertain which of the heme ligands is displaced by CO to form the active, DNA-binding state of CooA, and it is not clear whether NO binds to the same side of the CooA heme as CO (*20*, *21*). The bound CO in Fe(II)CooA(CO) is clearly in an environment of negative polarity (*20*). In contrast, the bound NO in Fe(II)CooA(NO) appears to be in a neutral electrostatic environment since the NO stretching position is at a normal position on the back-bonding correlation, close to that of five-coordinate Mb-H93Y(NO) adduct. One plausible explanation for the difference between the NO and CO adducts of CooA is that NO may not induce the same conformational changes upon binding as CO. Resonance Raman studies of Fe(II)CooA(CO) (*20*) imply that CO binding shifts a proton from a nearby donor to a His^{77}-imidazolate, either because this is the ligand displaced by CO or because of a protein conformational change accompanying the displacement of the trans ligand whose identity is unknown. NO may not induce these changes and thus may be bound in a different local environment than CO. Alternatively, NO and CO may bind on opposite sides of the heme, thus being located in different environments.

Remarkably, only CO and NO, of the small molecule probes tested, were able to bind to the heme of CooA, suggesting that the heme site is able to discriminate between ligands. The typical ferric heme ligands CN^-, N_3^-, and Im were unable to bind to the heme of Fe(III)CooA. Similarly, only CO and NO, but not Im and 1MeIm, were observed to bind to Fe(II)CooA. These responses are in marked contrast to those of other gas-binding heme proteins such as Mb (*35*), Hb (*35*), or FixL (*4*, *36*, *37*), which contain a high-spin ferric or ferrous heme and readily bind exogenous heme ligands. Because a thiol-modifying agent was able to perturb the spectrum of Fe(III)CooA and NO was able to react with both Fe(III)- and Fe(II)CooA, it appears unlikely that the heme site is physically inaccessible from the exterior of the protein. Rather, it appears that the heme is firmly bound to two endogenous amino acid ligands and that only the strongest ligands are able to effectively compete with the endogenous ligands for the heme. This proposal is consistent with electronic absorption (*13*, *17*, *18*), EPR (*18*), and resonance Raman (*20*, *21*) spectroscopic studies which indicate that both Fe(III)- and Fe(II)CooA contain only low-spin heme. The heme site of CooA thus appears to be distinctly different from other gas-sensing heme proteins since the heme is six-coordinate, low-spin and the binding of heme ligands is limited. The details of how the heme environment of CooA is specifically tuned to sense CO remain to be determined.

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