EPR and Electronic Absorption Spectroscopies of the CO-Sensing CooA Protein Reveal a Cysteine-Ligated Low-Spin Ferric Heme†

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Proteins that sense the gaseous molecules NO, O_2, and CO function as cellular regulators in animals, plants and bacteria. These proteins have diverse functions including blood pressure regulation, neurotransmission, control of N_2 fixation, and transcriptional regulation. Although heme proteins that sense NO[1] and O_2[2] have been characterized, little is known about CO-sensing proteins. CO is believed to function as a neurotransmitter in the mammalian brain where it is produced by heme oxygenase;[3] however, the receptor for CO has not been conclusively identified.[4] The first CO-sensing protein, CooA, was purified from the photosynthetic bacterium *Rhodospirillum rubrum* and could serve as a paradigm for other CO-sensing proteins.[5] CooA is a transcriptional regulator and a member of the CRP and FNR family. In the presence of CO, CooA binds to DNA and turns on the expression of a multicomponent CO-oxidation system in *R. rubrum*.[6,7] CooA is a heme-containing, homodimeric protein.[5,8] Because CO binds to ferrous heme, the ligation state of the heme is postulated to play a role in transcriptional regulation by CooA.[5,8] We present herein data from EPR and electronic absorption spectroscopies, revealing that oxidized CooA contains a low-spin ferric heme with a cysteine bound to the heme iron.

The EPR spectrum of Fe(III)CooA[9,11] (Figure 1) has two overlapping sets of rhombic signals centered at $g = 2.25$, characteristic of low-spin heme (Table 1).[12−16] We observed no other EPR signals, and spin quantitation experiments confirmed that all of the heme in Fe(III)CooA is accounted for by these two low-spin components. The finding that Fe(III)CooA contains low-spin heme implies that two axial ligands are bound to the heme iron. The two rhombic signals of Fe(III)CooA were

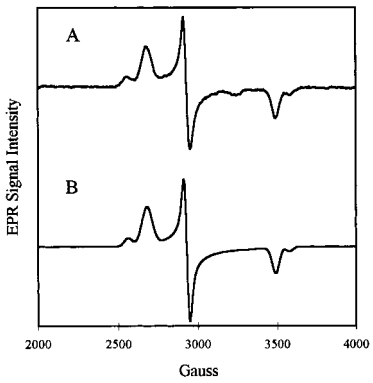

Figure 1. X-band EPR spectrum of Fe(III)CooA (A) and the best-fit simulation (B).[11] (A) The Fe(III)CooA sample contained 119 μM heme, 25 mM MOPS, 1 mM DTT, and 0.1 M NaCl at pH 7.4. The spectrum was recorded at 22.5 K, 200 μW microwave power, 4×10^3 receiver gain, 8 G modulation amplitude, 9.2346 GHz microwave frequency, 100 kHz modulation frequency, and 1 s time constant, using four averaged scans, each containing 2048 data points. (B) The EPR simulation was generated;[17] the best fit was when the more intense signal ($g = 2.46$, 2.25, 1.89) contributed 85% and the less intense signal (g = 2.58, 2.25, 1.84) contributed 15% to the total signal intensity.

Table 1. Comparison of the EPR Spectral Parameters of Fe(III)CooA with Those of Thiolate-Ligated, Low-Spin Heme Proteins and Model Complexes

protein or model	iron ligands	EPR g values[a]			ref
		g_z	g_y	g_x	
Fe(III)CooA (85%), pH 7.4		2.46	2.25	1.89	this work
Fe(III)CooA (15%), pH 7.4		2.58	2.25	1.84	this work
ImH/Fe(III)PPIX/$C_2H_5S^-$	ImH/RS^-	2.45	2.26	1.90	12
Fe(III)Hb + H_2S	His/SH^-	2.46	2.25	1.92	13
		2.56	2.25	1.86	13
Fe(III)P450[b]	Cys/H_2O	2.45	2.26	1.91	14
Fe(III)P450[b] + ImH	Cys/ImH	2.56	2.27	1.87	14
Fe(III)cyt *c*-M80C	His/Cys	2.56	2.27	1.85	15
Fe(III)H450	His/Cys	2.42	2.28	1.91	16
		2.51	2.31	1.87	16

[a] A plot of V/Δ versus Δ/λ places the two Fe(III)CooA signals in the thiolate-ligated category of low-spin heme proteins.[33] [b] P450 CAM from *Pseudomonas putida*.

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† Abbreviations: CRP, cAMP receptor protein; cAMP, adenosine 3′,5′-cyclic monophosphate; FNR, fumarate nitrate reduction; EPR, electron paramagnetic resonance; P450, cytochrome P450; cyt *c*, cytochrome *c*; heme, iron(II) protoporphyrin IX; ImH, imidazole; Mb, myoglobin; Hb, hemoglobin; H or His, histidine; C or Cys, cysteine; M, methionine; py, pyridine; LMCT, ligand-to-metal charge-transfer; DTT, dithiothreitol; MOPS, 3-[N-morpholino]-propane-sulfonic acid; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethane-sulfonic acid]).

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(9) *Escherichia coli* strain UQ1421, harboring plasmid pCO69, a pKK223-3[10] derivative with *cooA* fused to the *tac* promoter, was grown to an optical density at 600 nm of 0.2−0.4, induced with 0.5 mM IPTG, and harvested after 14−16 h of aerobic growth. CooA was purified as previously reported.[5] Electronic spectra of recombinant CooA purified by this protocol were identical to those of the protein purified from *R. rubrum*.[5] CooA purified by this procedure was greater than 93% pure by densitometric scanning of Coomassie-stained SDS gels and was active in sequence-specific, CO-dependent DNA binding by DNAse I footprinting.[5]

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(11) Fe(III)CooA samples were degassed with argon, frozen, and stored at 77 K. The EPR spectra were recorded on a Varian E-15 spectrophotometer, and an Oxford Cryostat 3120 system was used to monitor and regulate the temperature. The magnetic field was measured using a Varian 929801 gaussmeter and a Tektronix type RM 503 oscilloscope. The only EPR signals observed between 0 and 4000 G are those shown in Figure 1.

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simulated (Figure 1);[17] the best fits were obtained when the major signal contributed 85% and the minor signal contributed 15% to the total intensity. Both low-spin components were seen in independently isolated protein samples and in different buffers,[18] suggesting that the two signals are intrinsic to the Fe(III)CooA protein. Thus, the EPR spectrum of Fe(III)CooA reveals a low-spin heme with two axial ligands and two distinct subpopulations.

The *g* values obtained from the EPR spectrum of Fe(III)CooA identify a cysteine-thiolate as one of the two ligands bound to the low-spin heme iron. The two sets of *g* values observed for Fe(III)CooA are similar to the *g* values of low-spin, thiolate-ligated heme proteins and model complexes (Table 1).[12−16] Low spin hemes containing a single thiolate bound to the heme iron (Table 1) have *g* values that are tightly spaced around $g = 2.25$, distinct from other types of low-spin hemes.[12] Two overlapping sets of signals, as observed in Fe(III)CooA, have been seen in the EPR spectra of other heme-proteins containing a single thiolate ligand bound to the heme iron.[13,14,19] It was proposed that the two signals arose from either a mixture of thiolate, and thiol bound to the heme iron or to two different orientations of the heme-bound thiolate imposed by the protein environment.[19] The EPR spectrum of Fe(III)CooA is thus consistent with two different populations of cysteine-ligated heme iron.

The greatest similarity exists between the EPR spectrum of Fe(III)CooA and that of proteins in which the sixth axial ligand is histidine or another neutral donor (Table 1); however, the spectra are dominated by the thiolate ligand, and assignment of the sixth ligand is equivocal.[14] The electronic absorption spectrum of Fe(III)CooA[5,8] (Supporting Information) exhibits unique features characteristic of thiolate-ligated, low-spin heme with a sixth neutral donor ligand.[13,14,16] The positions and intensities of the δ, Soret, α, and β bands in Fe(III)CooA are similar to those of complexes of Fe(III)P450 where the ligand trans to the cysteinate is a nitrogen donor.[14] Furthermore, two heme proteins that are proposed to contain cysteine and histidine as the axial heme ligands, Fe(III)H450[16] and Fe(III)cyt *c*-M80C,[20] exhibit spectra that are similar to that of Fe(III)CooA. The low energy region of the Fe(III)CooA electronic absorption spectrum contains two bands at 648 and 748 nm that are observed in thiolate-ligated heme proteins where imidazole is bound trans to the thiolate: Fe(III)P450 with imidazole and Fe(III)cyt *c*-M80C.[20] Absorption bands at these positions have been previously assigned to thiolate sulfur-to-Fe(III) charge-transfer (p_y-d shell and p_z-d shell) transitions.[22,23] Thus, the features of the electronic absorption spectrum of Fe(III)CooA are consistent with the EPR assignment of a low-spin, thiolate-ligated heme protein and support the hypothesis that the sixth ligand is histidine or an alternate neutral donor.

The results from our spectroscopic studies of Fe(III)CooA provide insight into the changes that occur at the heme site upon reduction and CO activation. The electronic absorption spectrum of Fe(II)CooA[5,8] is similar to that of other low-spin, ferrous heme proteins,[16,21,22] suggesting that two ligands are bound to the heme iron in Fe(II)CooA. However, low-spin thiolate-ligated Fe(II) heme proteins typically exhibit Soret absorptions near 450 nm,[21] whereas Fe(II)CooA has a Soret band at 425 nm.[5,8] One explanation for the blue-shifted Soret band is that the thiolate-iron bond is weakened upon reduction, possibly by protonation of the thiolate to form a thiol. A Soret absorption near 420 nm is observed for the Fe(II) low-spin, heme proteins, Fe(II)H450[16] at pH 6.0 and Fe(II)cyt *c*-M80C,[20] where the sulfur−iron bond is weakened due to protonation of the heme-bound thiolate.[24−26] Fe(II)P420[27] and Fe(II)Mb-H93C[28,29] also show spectra similar to Fe(II)CooA, and both are proposed to contain a weakly bound or displaced cysteine ligand. Although the electronic spectrum of Fe(II)CooA is consistent with a thiol or thiolate weakly bound to the heme iron of Fe(II)CooA, replacement of the cysteine ligand upon reduction cannot be ruled out.

Addition of CO to Fe(II)CooA perturbed the electronic spectrum, confirming that CO binds to the heme iron.[5,8] The Soret, α, and β bands of Fe(II)CooA(CO)[5,8] are similar to those of low-spin, carbonyl adducts of hemoglobin and myoglobin[20] and are different from the carbonyl adducts of thiolate-ligated model complexes and proteins.[30,31] The conversion from low-spin Fe(II)CooA to low-spin Fe(II)CooA(CO) requires that CO displace one of the two axial ligands bound to the heme iron. The observation that the electronic spectrum of Fe(II)CooA(CO) is similar to that of histidine-ligated heme proteins is consistent with the displacement of the cysteine ligand from the heme iron by CO. The electronic spectrum of Fe(II)CooA(CO) is also similar to that of the heme proteins Fe(II)H450(CO),[16] Fe(II)-P420(CO),[27] and Fe(II)Mb-H93C(CO),[28] all of which are thiolate-ligated in the ferric state. CO has a high affinity for five-coordinate Fe(II)heme proteins and could displace a weakly bound thiol or thiolate from the heme iron; however, it is also possible that a cysteine-thiol remains weakly bound to the heme iron while the other ligand is displaced by CO.

CooA is believed to undergo a conformational change upon CO binding to the heme iron.[5,8] This proposal is based on the similarity between CooA and the well characterized transcriptional regulator, CRP;[6,7] the binding of cAMP to CRP activates DNA binding via a conformational change.[32] Small molecules such as NO and O_2 are known to trigger conformational changes in heme proteins by binding to the heme iron.[1,2] Thus, displacement of a protein ligand by CO could trigger a conformational change in CooA producing the active, DNA-binding state.

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Supporting Information Available: Figure of electronic absorption spectrum and table of parameters (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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