

## The Cyanide Ligands of [FeFe] Hydrogenase: Pulse EPR Studies of $^{13}\text{C}$ and $^{15}\text{N}$ -Labeled H-Cluster

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### S Supporting Information

**ABSTRACT:** The two cyanide ligands in the assembled cluster of [FeFe] hydrogenase originate from exogenous L-tyrosine. Using selectively labeled tyrosine substrates, the cyanides were isotopically labeled via a recently developed *in vitro* maturation procedure allowing advanced electron paramagnetic resonance techniques to probe the electronic structure of the catalytic core of the enzyme. The ratio of the isotropic  $^{13}\text{C}$  hyperfine interactions for the two  $\text{CN}^-$  ligands—a reporter of spin density on their respective coordinating iron ions—collapses from  $\approx 5.8$  for the  $\text{H}_{\text{ox}}$  form of hydrogenase to  $< 2$  for the CO-inhibited form. Additionally, when the maturation was carried out using  $^{15}\text{N}$ -tyrosine, no features previously ascribed to the nitrogen of the bridging dithiolate ligand were observed suggesting that this bridge is not sourced from tyrosine.

Hydrogenases catalyze the redox interconversion of protons and  $\text{H}_2$  and thus have received much focus as key elements in biological solar fuel production.<sup>1</sup> The [FeFe] form of hydrogenase (HydA) is particularly active,<sup>1</sup> and its catalytic H-cluster consists of a [4Fe-4S] cluster ([4Fe-4S]<sub>H</sub>) linked through a cysteine sulfur to a unique dinuclear iron cluster ([FeFe]<sub>H</sub>, Scheme 1).<sup>2</sup> This subcluster possesses five inorganic ligands—two  $\text{CN}^-$  and three CO—as well as a bridge recently assigned as dithiomethylamine (DTMA).<sup>3,4</sup>

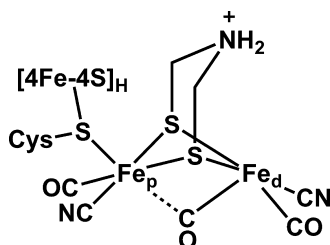
Active HydA can be expressed in *Escherichia coli* only by also adding genes for three Fe-S containing maturase enzymes—HydE, HydF, and HydG—that are required for production of the [FeFe]<sub>H</sub> subcluster.<sup>5</sup> Alternatively, synthetic dinuclear Fe clusters can be transferred to HydA apoprotein (containing only the [4Fe-4S]<sub>H</sub> subcluster) to produce active

enzyme.<sup>4</sup> We are utilizing a different technology: the HydE, HydF, and HydG maturases are added to a solution of apo-HydA for *in vitro* maturation and concurrent activation.<sup>6</sup> This cell-free biosynthetic method allows for facile and precise isotope incorporation into the [FeFe]<sub>H</sub> subcluster.<sup>7</sup>

The Fe-bound CO and  $\text{CN}^-$  ligands of the [FeFe]<sub>H</sub> subcluster are sourced from L-tyrosine (Tyr) and produced by HydG.<sup>8–10</sup> In the present study, we use the cell-free biosynthetic method along with  $\alpha$ - $^{13}\text{C}$ -Tyr ([2- $^{13}\text{C}$ ]-Tyr) and  $^{15}\text{N}$ -Tyr to specifically label the two  $\text{CN}^-$  ligands with the magnetic nuclei  $^{13}\text{C}$  and  $^{15}\text{N}$  ( $I = 1/2$ ).<sup>11,12</sup> The hyperfine interaction (HFI) of these magnetic nuclei with the unpaired electrons distributed over the H-cluster serve as site-specific reporters of its electronic structure, important metrics for evaluating computational models of the H-cluster.

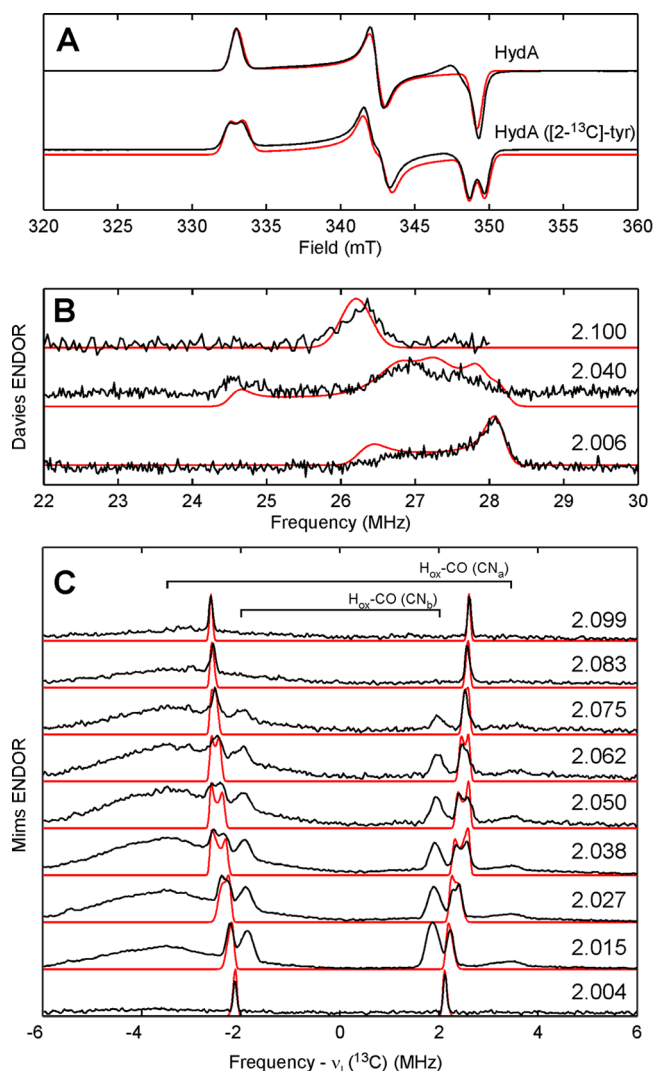
When poised in the active oxidation state known as  $\text{H}_{\text{ox}}$ , the [4Fe-4S]<sub>H</sub> subcluster is diamagnetic with a formal charge of 2+,<sup>13</sup> though the [4Fe-4S]<sub>H</sub> carries some unpaired density due to the exchange interaction with the [FeFe]<sub>H</sub> fragment. [FeFe]<sub>H</sub> itself is in a formally mixed-valence Fe(I,II)  $S = 1/2$  state that is characterized by a rhombic electron paramagnetic resonance (EPR) spectrum (Figure 1A, top). While the overall oxidation state of the  $\text{H}_{\text{ox}}$  form of the H-cluster is widely accepted, the distribution of the valences about the cluster is still debated. One formulation based on results from electronic structure calculations assigns a 1+ oxidation state to the Fe that is distal to the [4Fe-4S]<sub>H</sub> subcluster ( $\text{Fe}_d$ ), leaving the proximal Fe ion ( $\text{Fe}_p$ ) in the ferrous oxidation state.<sup>14</sup> However,  $^{57}\text{Fe}$  electron nuclear double resonance (ENDOR) spectroscopic studies of HydA from *Desulfovibrio desulfuricans* (*DdS*) found that the spin density was shared more-or-less equally over both iron ions of [FeFe]<sub>H</sub>.<sup>15</sup> Many computational models of the H-cluster have been judged based on the quality of the predicted magnetic parameters. Initially, only the  $^{57}\text{Fe}$  HFI were employed as a discriminating constraint.<sup>14,16</sup> More recently, however, ligand HFI, from either the nearby, naturally abundant  $^{14}\text{N}$  nuclei or from  $^{13}\text{C}$  nuclei introduced by treatment of HydA with isotopically labeled  $^{13}\text{CO}$  gas, have been used to evaluate computer-generated structural models of the H-cluster.<sup>3,16,17</sup> Unfortunately, in the case of the  $^{14}\text{N}$  hyperfine parameters, the assignment of the observed signals to

Scheme 1



Received: July 11, 2014

Published: August 15, 2014



**Figure 1.** X-band (9.4 GHz) CW EPR spectra (A) of the  $H_{ox}$  form of HydA matured using natural-abundance Tyr (top) or  $[2-^{13}C]$ -Tyr (bottom). Davies ENDOR spectra (B) of HydA ( $[2-^{13}C]$ -Tyr) collected at 1158, 1192, and 1212 mT (top to bottom). Corresponding  $g$ -values given in figure. Q-band (33.79 GHz) Mims ENDOR spectra (C) of HydA ( $[2-^{13}C]$ -Tyr) collected at 1150, 1157, 1164, 1171, 1178, 1184, 1191, 1198, and 1205 mT (top to bottom). Corresponding  $g$ -values given in figure. Traces of experimental data are shown in black; simulations for the  $H_{ox}$  form are presented in red.

specific nitrogen atoms is ambiguous owing to the high natural-abundance of  $^{14}N$ ; and the  $^{13}CO$ -treatment aids only in characterizing the  $H_{ox}$ -CO form. We therefore reasoned that studies of the electronic structure of  $H_{ox}$  would be aided by selective incorporation of magnetic nuclei into the diatomic ligands of the  $[FeFe]_H$  cluster.

The X-band continuous-wave (CW) EPR spectrum of *in vitro* matured HydA from *Clostridium pasteurianum* (CpI) poised in the  $H_{ox}$  state is consistent with that published previously with  $g = 2.100, 2.040, 1.996$  (Figure 1A). Using  $[2-^{13}C]$ -Tyr in the maturation of HydA leads to a splitting of  $\approx 1$  mT centered at each  $g$ -value of this  $H_{ox}$  signal (cf. top and bottom traces in Figure 1A).<sup>18</sup> Q-band Davies ENDOR spectra acquired at field positions corresponding to each  $g$ -value (Figure 1B) confirm this strong  $^{13}C$  HFI by showing features at  $\approx 27$  MHz that have no counterpart in analogous spectra of HydA matured using natural-abundance tyrosine.<sup>19</sup> The variation in shape and

breadth of these features as a function of resonant field position results from orientation selection, i.e., at certain field positions, a discrete subset of molecular orientations of HydA are probed. Proper simulation of this behavior allows for the orientation of the corresponding  $^{13}C$  hyperfine tensor to be determined relative to the molecular  $g$ -tensor. These parameters are summarized in Table 1. The degree of  $^{13}C$  HFI anisotropy is consistent with that of other Fe-bound cyanides (cf. Table 1).

Orientation-selected Mims ENDOR spectra (Figure 1C) reveal three distinct classes of more weakly coupled  $^{13}C$  nuclei ( $A_{iso} = 3.80, 4.87,$  and  $\approx 7.0$  MHz). These features are centered about the  $^{13}C$  Larmor frequency and split by the magnitude of the HFI. Analogous data sets collected for CO-treated samples (Figures S3 and S4) possess similar features at  $\pm 1.8$  and  $\pm 3.6$  MHz, confirming that they arise from the two cyanide ligands in the  $H_{ox}$ -CO form of hydrogenase (labeled as  $CN_a$  and  $CN_b$  since we cannot distinguish between the  $Fe_p$ -bound and  $Fe_d$ -bound cyanides at this time). Note the absence of contributions from  $H_{ox}$ -CO to the ENDOR spectra acquired at the extreme field positions ( $g = 2.099$  and  $2.004$ ) of  $H_{ox}$  (Figure 1C). This results from the relative narrowness of the  $H_{ox}$ -CO signal. This narrowness is also why we see strong contributions from  $H_{ox}$ -CO even though the contamination is relatively small. The remaining features centered at  $\pm 2.2$  MHz in Figure 1C are thus ascribed to the other  $CN^-$  ligand in  $H_{ox}$ .

Based on the crystallographic results,<sup>2</sup>  $Fe_d$  possesses a square pyramidal local geometry whose  $z$ -axis points along the bond between the  $Fe_d$  ion and the bridging CO. For the six-coordinate  $Fe_p$ , the identity of the local  $z$ -axis is less obvious, but computational results suggest that it is aligned along the  $Fe_p$ -CO<sub>bridge</sub> bond.<sup>14</sup> As the two terminal  $CN^-$  ligands appear to be bound in the same position relative to the local  $z$ -axis of their respective Fe ions, the ratio of the isotropic  $^{13}C$  HFI should serve as a reporter of the relative spin density on each iron. Again, based on earlier computational results, we assign the larger  $^{13}C$  HFI as arising from the distal Fe-bound cyanide of  $H_{ox}$ . For the proximal Fe-bound cyanide, we measure  $A_{iso} = 4.87$  MHz. This ratio of  $\approx 5.8$  correlates approximately with the  $Fe_d:Fe_p$  ratio of computed Mulliken spin populations.<sup>14,16</sup> For  $H_{ox}$ -CO, the  $A_{iso}(^{13}CN_a):A_{iso}(^{13}CN_b)$  ratio drops to  $< 2$  (see magnetic parameters listed in Table 1) indicating a much more even distribution of spin density over the two Fe ions than what was observed for  $H_{ox}$  that is again consistent with computational results.<sup>14,16</sup> Interestingly, the  $^{13}C$  HFI tensors for the two  $CN^-$  ligands in the  $H_{ox}$ -CO form lack significant anisotropy compared to other Fe-bound cyanides (cf. Table 1)

X- and Q-band HYSCORE spectra for natural-abundance  $H_{ox}$  (Figure 2, top) are essentially identical to those obtained earlier by Silakov et al.<sup>3</sup> When the *in vitro* maturation of HydA is performed with  $^{15}N$ -labeled tyrosine ( $[^{15}N]$ -Tyr), the nitrogens of the cyanide ligands become selectively isotopically labeled.<sup>9</sup> The corresponding HYSCORE data are strikingly different from those of natural-abundance  $H_{ox}$  (cf. top and bottom plots in Figure 2) signaling that the majority of features arise from tyrosine-derived nitrogens. The correlation ridges in the Q-band spectrum of  $H_{ox}$  ( $[^{15}N]$ -Tyr) are well-simulated with the hyperfine parameters  $A(^{15}N) = [0.8, 6.3, -1.2]$  MHz (Figure S5). Given the rather large magnitude of  $A_{iso}(^{15}N)$ , this nitrogen is likely that in the  $Fe_d$ -bound cyanide. We observe no  $^{15}N$ -derived features that we could assign to cyanides in the  $H_{ox}$ -CO form.

Table 1.  $^{13}\text{C}$  HFI and  $^{15}\text{N}$  HFI for CO and CN Bound to Fe-Centers

species	$A^{13}\text{C}$ (MHz)	$[\alpha, \beta, \gamma]$ (deg) <sup>a</sup>	assignment	reference
CpI $\text{H}_{\text{ox}}$ ( $[2\text{-}^{13}\text{C}]\text{-Tyr}$ )	[30.9, 23.3, 30.2]	[60, 120, 170]	$\text{CN}_{\text{d}}$	this work
	[5.22, 5.24, 4.16]	[30, 90, 0]	$\text{CN}_{\text{p}}$	this work
CpI $\text{H}_{\text{ox}}\text{-CO}$ ( $[2\text{-}^{13}\text{C}]\text{-Tyr}$ )	[7.0, 7.0, 7.2]	[0, 0, 0]	$\text{CN}_{\text{a}}$	this work
	[3.75, 3.75, 3.90]	[0, 0, 0]	$\text{CN}_{\text{b}}$	this work
<i>DdS</i> $\text{H}_{\text{ox}}\text{-}^{13}\text{CO}$	[15.6, 16.6, 19.2]		$\text{CO}_{\text{ext}}$	17
	[8.5, 9.8, 3.9]		$\text{CO}_{\text{bridge}}$	17
	[3.2, 3.7, 4.4]		$\text{CO}_{\text{d}}$	17
Mb- $^{13}\text{CN}$	[-23.0, -27.6, -28.7]		Fe(III)-CN	21
<i>Pf</i> Fd- $^{13}\text{CN}$	[-4.5, -4.5, +0.1]		$[\text{4Fe-4S}]^+\text{-CN}$	22
species	$A^{15}\text{N}$ (MHz)	$[\alpha, \beta, \gamma]$ (deg)	assignment	reference
CpI $\text{H}_{\text{ox}}$ ( $[^{15}\text{N}]\text{-Tyr}$ )	[0.8, 6.3, -1.2]	[45, -20, 0]	$\text{CN}_{\text{d}}$	this work
<i>DdS</i> $\text{H}_{\text{ox}}$	[2.1, 5.3, -0.6] <sup>b</sup>	[41, 24, 0]	$\text{CN}_{\text{d}}$	3
	[1.4, 2.7, 2.0] <sup>b</sup>	[40, 25, 0]	DTMA	3
	[-3.4, 2.0, -1.0] <sup>b</sup>	[0, 4, 20]	Lys	3
<i>DdS</i> $\text{H}_{\text{ox}}\text{-CO}$	[0.56, -0.28, 0.79] <sup>b</sup>	[0, -10, 0]		17
Mb- $^{15}\text{N}$	[n.d., n.d., 5.25]		Fe(III)-CN	23
<i>Pf</i> Fd- $^{15}\text{N}$	[+1.8, +1.0, -2.4]		$[\text{4Fe-4S}]^+\text{-CN}$	22

<sup>a</sup>Euler angles are relative to  $g$ -frame defined by  $g_1 < g_2 < g_3$ . For  $\text{H}_{\text{ox}}$ , this corresponds to  $g_z < g_y < g_x$  as we assign the local  $z$ -axis of  $\text{Fe}_{\text{d}}$  to the  $\text{CO}_{\text{bridge}}$  bonding vector. <sup>b</sup>Determined by scaling the experimentally determined  $^{14}\text{N}$  HFI by the ratio of the  $^{15}\text{N}/^{14}\text{N}$  Larmor frequencies (1.4028). <sup>c</sup>Abbreviations: Mb = myoglobin; *Pf* Fd =  $[\text{4Fe-4S}]$  ferredoxin from *Pyrococcus furiosus*; n.d. = not determined.

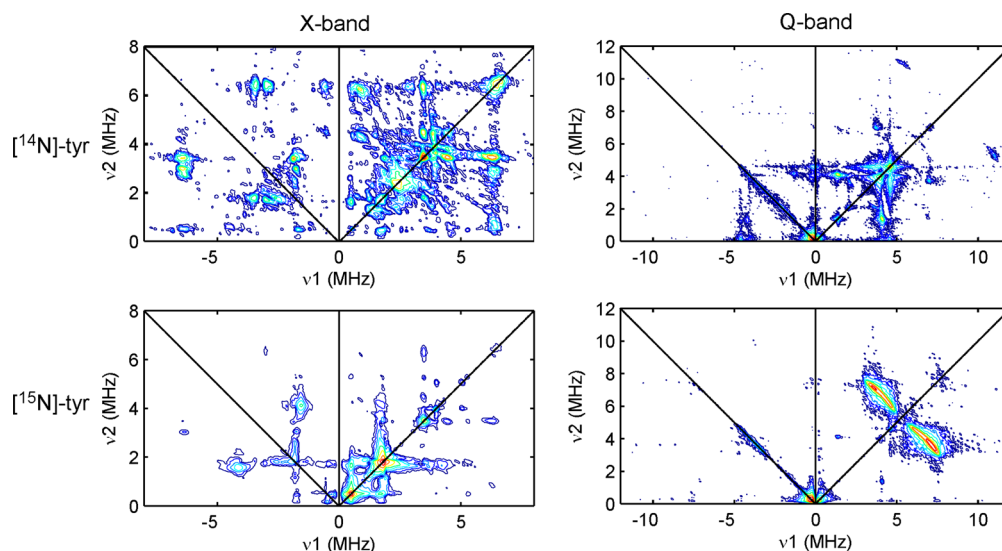


Figure 2. X-band (left) and Q-band (right) HYSCORE spectra of the  $\text{H}_{\text{ox}}$  form of HydA matured using natural-abundance ( $[^{14}\text{N}]\text{-Tyr}$ , top) or with  $[^{15}\text{N}]\text{-Tyr}$  (bottom).

The biosynthetic origin of the putative DTMA bridge is presently unknown. One proposal suggests that HydG can assemble this bridging ligand from two molecules of tyrosine.<sup>20</sup> Analysis of  $^{14}\text{N}$  HYSCORE spectra of *DdS* HydA poised in the  $\text{H}_{\text{ox}}$  state led to the assignment of a set of correlation ridges to the DTMA amino nitrogen ( $A(^{14}\text{N}) = [1.0, 1.9, 1.4]$  MHz).<sup>3</sup> By scaling this reported  $^{14}\text{N}$  HFI by the ratio of the  $^{15}\text{N}/^{14}\text{N}$  Larmor frequencies, we can simulate the X-band HYSCORE spectrum as if the DTMA had been  $^{15}\text{N}$ -labeled (see Figures S6 and S7). The predicted correlation ridges corresponding to the  $^{15}\text{N}$ -DTMA nitrogen are not found in the experimental HYSCORE spectrum of  $\text{H}_{\text{ox}}$  ( $[^{15}\text{N}]\text{-Tyr}$ ) suggesting either that tyrosine is not the source of the DTMA nitrogen or that the previously reported  $^{14}\text{N}$  HFI parameters for *DdS* HydA are not appropriate for CpI  $\text{H}_{\text{ox}}$ .

Using isotopically labeled tyrosine substrates in conjunction with the *in vitro* biosynthetic route to generate the H-cluster

gives us the flexibility to site-specifically label the cyanide ligands with  $^{13}\text{C}$  and  $^{15}\text{N}$ . The signals we observe from  $^{15}\text{N}$  are unambiguously attributed to the nitrogen of an Fe-bound cyanide. Further, comparison of the two cyanide  $^{13}\text{C}$  couplings is consistent with just one of the Fe ions ( $\text{Fe}_{\text{d}}$ ) of  $[\text{FeFe}]_{\text{H}}$  carrying the majority of unpaired electron spin in the  $\text{H}_{\text{ox}}$  state. As such, the relatively large rhombicity of the  $\text{H}_{\text{ox}}$  EPR signal can be understood as arising from the asymmetry in the equatorial ligand set for the low-spin  $3d^7$   $\text{Fe}_{\text{d}}$  spin center. Thus, the difference in  $g$ -shifts for  $g_y$  and  $g_x$  (0.0367 vs 0.0947) is attributed to the difference in the energies of the  $\text{Fe}_{\text{d}}\text{-}3d_{xz} \rightarrow \text{Fe}_{\text{d}}\text{-}3d_{z^2}$  and the  $\text{Fe}_{\text{d}}\text{-}3d_{yz} \rightarrow \text{Fe}_{\text{d}}\text{-}3d_{z^2}$  transitions, respectively.<sup>24</sup> If we orient the  $g$ -tensor for  $\text{H}_{\text{ox}}$  as follows:  $g_z$  is oriented along of  $z$ -axis of  $\text{Fe}_{\text{d}}$ , and  $g_x$  and  $g_y$  are made to bisect the  $\text{Fe}_{\text{d}}\text{-S}$  and  $\text{Fe}_{\text{d}}\text{-S}$  bonding vectors and the  $\text{Fe}_{\text{d}}\text{-CO}_{\text{d}}$  and  $\text{Fe}_{\text{d}}\text{-CN}_{\text{d}}$  bonding vectors, respectively; then the unique axis of the  $^{13}\text{C}$  hyperfine tensor for  $\text{CN}_{\text{d}}$  is found to point approximately along the  $\text{Fe}_{\text{d}}\text{-}$



CN<sub>d</sub> bond, as expected (Figure S8).<sup>25</sup> This finding supports our electronic structure description of H<sub>ox</sub>; namely, that the unpaired electron largely resides in a molecular orbital of 3d<sub>z<sup>2</sup></sub> character centered on the Fe<sub>d</sub> ion.

Based on the similar magnitudes of the <sup>13</sup>CN HFI, the electron spin becomes distributed more evenly over both iron ions after inhibition with free CO. This more delocalized spin topology leads to a collapse of the *g*-matrix rhombicity. Analogously, the rather narrow EPR signal for the formally mixed-valence Cu(I,II) Cu<sub>A</sub> cluster in nitrous oxide reductase is understood as a weighted sum of the hypothetical mononuclear *g*-matrices of each Cu site.<sup>26</sup> In the case of H<sub>ox</sub>-CO, we do not know the values for the intrinsic *g*-matrix for the two Fe ions. However, we can use the H<sub>ox</sub> *g*-values as a first estimate. Upon forming H<sub>ox</sub>-CO, delocalization of the unpaired electron spin cancels out some of the anisotropy from each site-specific *g*-matrix, leading to the axial (*g* = 2.072, 2.006, 2.006), molecular *g*-matrix. The nearly isotropic HFI tensors for the two CN<sup>-</sup> ligands in H<sub>ox</sub>-CO result from this same mechanism of anisotropy cancellation. These findings are in agreement with earlier computational models<sup>14,16</sup> that indicate a dramatic delocalization of unpaired spin density in going from the H<sub>ox</sub> form to H<sub>ox</sub>-CO

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Details of experimental procedures and data analysis methods. Supplemental EPR spectra and corresponding simulations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was funded by National Institutes of Health (GM104543 to R.D.B.) and the Division of Material Sciences and Engineering (J.R.S. award no. DE-FG02-09ER46632) of the Office of Basic Energy Sciences of the U.S. Department of Energy. D.L.M.S. acknowledges support from the National Institutes of Health (F32GM111025 from the NIGMS).

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(18) A modest (20% of overall spectral intensity) of the axial signal (*g* = 2.072, 2.006, 2.006) arising from H<sub>ox</sub>-CO was removed by subtraction. H<sub>ox</sub>-CO contamination is common and can be seen by other methods such as infrared absorption spectroscopy.<sup>9</sup>

(19) This ENDOR transition at 27 MHz is approximately equal to twice the <sup>13</sup>C Larmor frequency at this field; therefore the ENDOR transition in other spin manifold is expected at <1 MHz though it is not evident in our ENDOR data. However, both <sup>13</sup>C spin-flip transitions are observed in the Q-band HYSCORE spectrum (Figure S2).

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