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Authors

Myers, William K
Stich, Troy A
Suess, Daniel LM
et al.

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

William K. Myers,^{†,§} Troy A. Stich,^{†,§} Daniel L. M. Suess,[†] Jon M. Kuchenreuther,[†] James R. Swartz,[‡] and R. David Britt^{*†}

[†]Department of Chemistry, University of California, Davis, Davis, California 95616 United States

[‡]Department of Chemical Engineering and Department of Bioengineering, Stanford University, Stanford, California 94305 United States

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}C hyperfine interactions for the two CN^- ligands—a reporter of spin density on their respective coordinating iron ions—collapses from ≈ 5.8 for the H_{ox} form of hydrogenase to < 2 for the CO-inhibited form. Additionally, when the maturation was carried out using ^{15}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 H_2 and thus have received much focus as key elements in biological solar fuel production.¹ The [FeFe] form of hydrogenase (HydA) is particularly active,¹ and its catalytic H-cluster consists of a [4Fe-4S] cluster ([4Fe-4S]_H) linked through a cysteine sulfur to a unique dinuclear iron cluster ([FeFe]_H, Scheme 1).² This subcluster possesses five inorganic ligands—two CN^- and three CO—as well as a bridge recently assigned as dithiomethylamine (DTMA).^{3,4}

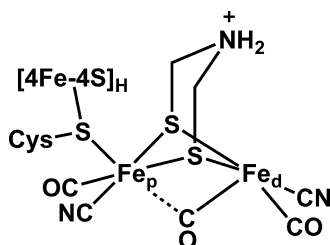
Active HydA can be expressed in *Escherichia coli* only by also adding genes for three Fe-S containing maturase enzymes—HydE, HydF, and HydG—that are required for production of the [FeFe]_H subcluster.⁵ Alternatively, synthetic dinuclear Fe clusters can be transferred to HydA apoprotein (containing only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

enzyme.⁴ 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.⁶ This cell-free biosynthetic method allows for facile and precise isotope incorporation into the [FeFe]_H subcluster.⁷

The Fe-bound CO and CN^- ligands of the [FeFe]_H subcluster are sourced from L-tyrosine (Tyr) and produced by HydG.^{8–10} In the present study, we use the cell-free biosynthetic method along with α - ^{13}C -Tyr ([2- ^{13}C]-Tyr) and ^{15}N -Tyr to specifically label the two CN^- ligands with the magnetic nuclei ^{13}C and ^{15}N ($I = 1/2$).^{11,12} 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 H_{ox} , the [4Fe-4S]_H subcluster is diamagnetic with a formal charge of 2+,¹³ though the [4Fe-4S]_H carries some unpaired density due to the exchange interaction with the [FeFe]_H fragment. [FeFe]_H 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 H_{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]_H subcluster (Fe_d), leaving the proximal Fe ion (Fe_p) in the ferrous oxidation state.¹⁴ However, ^{57}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]_H.¹⁵ Many computational models of the H-cluster have been judged based on the quality of the predicted magnetic parameters. Initially, only the ^{57}Fe HFI were employed as a discriminating constraint.^{14,16} More recently, however, ligand HFI, from either the nearby, naturally abundant ^{14}N nuclei or from ^{13}C nuclei introduced by treatment of HydA with isotopically labeled ^{13}CO gas, have been used to evaluate computer-generated structural models of the H-cluster.^{3,16,17} Unfortunately, in the case of the ^{14}N hyperfine parameters, the assignment of the observed signals to

Scheme 1



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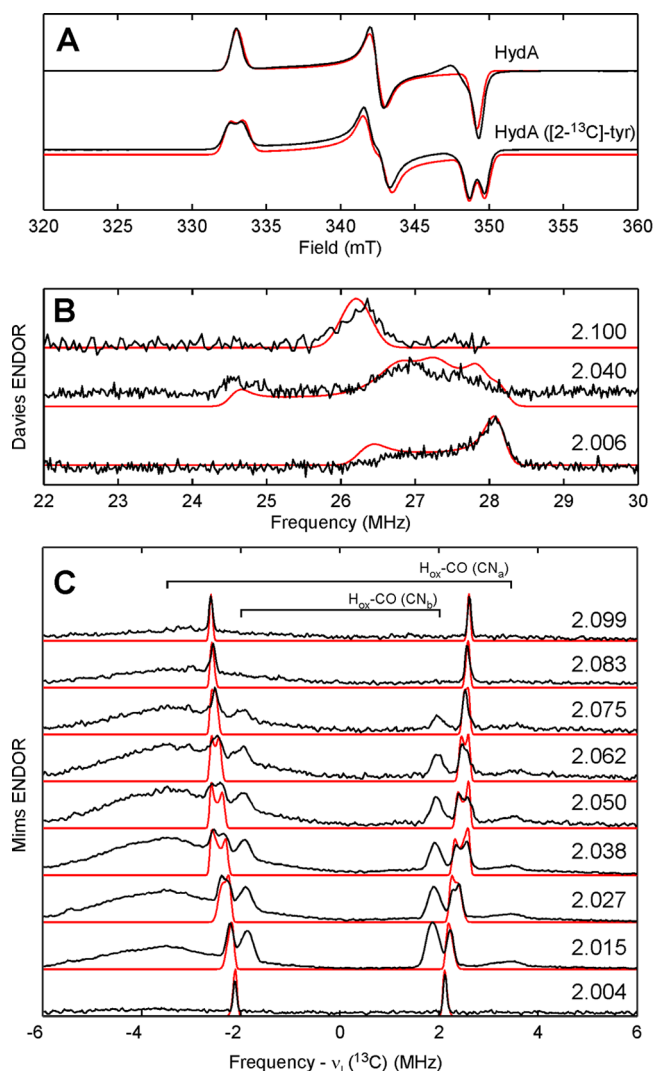


Figure 1. X-band (9.4 GHz) CW EPR spectra (A) of the H_{ox} form of HydA matured using natural-abundance Tyr (top) or $[2-^{13}C]$ -Tyr (bottom). Davies ENDOR spectra (B) of HydA ($[2-^{13}C]$ -Tyr) collected at 1158, 1192, and 1212 mT (top to bottom). Corresponding g -values given in figure. Q-band (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 ≈ 1 mT centered at each g -value of this H_{ox} signal (cf. top and bottom traces in Figure 1A).¹⁸ 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 ≈ 27 MHz that have no counterpart in analogous spectra of HydA matured using natural-abundance tyrosine.¹⁹ 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 ≈ 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 ± 1.8 and ± 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 ± 2.2 MHz in Figure 1C are thus ascribed to the other CN^- ligand in H_{ox} .

Based on the crystallographic results,² 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_{bridge} bond.¹⁴ 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 ≈ 5.8 correlates approximately with the $Fe_d:Fe_p$ ratio of computed Mulliken spin populations.^{14,16} 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.^{14,16} 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.³ 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.⁹ 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}C HFI and ^{15}N HFI for CO and CN Bound to Fe-Centers

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

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

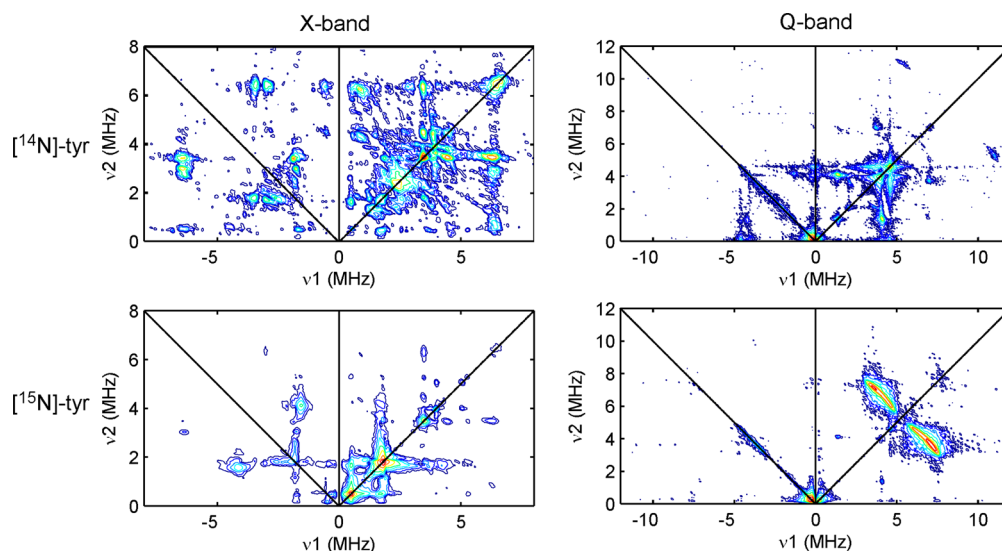


Figure 2. X-band (left) and Q-band (right) HYSCORE spectra of the H_{ox} form of HydA matured using natural-abundance ($[^{14}\text{N}]\text{-Tyr}$, top) or with $[^{15}\text{N}]\text{-Tyr}$ (bottom).

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.²⁰ Analysis of ^{14}N HYSCORE spectra of *DdS* HydA poised in the H_{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).³ By scaling this reported ^{14}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}N -labeled (see Figures S6 and S7). The predicted correlation ridges corresponding to the ^{15}N -DTMA nitrogen are not found in the experimental HYSCORE spectrum of H_{ox} ($[^{15}\text{N}]\text{-Tyr}$) suggesting either that tyrosine is not the source of the DTMA nitrogen or that the previously reported ^{14}N HFI parameters for *DdS* HydA are not appropriate for CpI H_{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}C and ^{15}N . The signals we observe from ^{15}N are unambiguously attributed to the nitrogen of an Fe-bound cyanide. Further, comparison of the two cyanide ^{13}C couplings is consistent with just one of the Fe ions (Fe_{d}) of $[\text{FeFe}]_{\text{H}}$ carrying the majority of unpaired electron spin in the H_{ox} state. As such, the relatively large rhombicity of the H_{ox} EPR signal can be understood as arising from the asymmetry in the equatorial ligand set for the low-spin $3d^7$ Fe_{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.²⁴ If we orient the g -tensor for H_{ox} as follows: g_z is oriented along z -axis of Fe_{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}C hyperfine tensor for CN_{d} is found to point approximately along the $\text{Fe}_{\text{d}}\text{-}$

CN_d bond, as expected (Figure S8).²⁵ This finding supports our electronic structure description of H_{ox}; namely, that the unpaired electron largely resides in a molecular orbital of 3d_z² character centered on the Fe_d ion.

Based on the similar magnitudes of the ¹³CN 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_A cluster in nitrous oxide reductase is understood as a weighted sum of the hypothetical mononuclear *g*-matrices of each Cu site.²⁶ In the case of H_{ox}-CO, we do not know the values for the intrinsic *g*-matrix for the two Fe ions. However, we can use the H_{ox} *g*-values as a first estimate. Upon forming H_{ox}-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⁻ ligands in H_{ox}-CO result from this same mechanism of anisotropy cancellation. These findings are in agreement with earlier computational models^{14,16} that indicate a dramatic delocalization of unpaired spin density in going from the H_{ox} form to H_{ox}-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>.

■ AUTHOR INFORMATION

Corresponding Author

rdbritt@ucdavis.edu

Author Contributions

[§]These authors contributed equally.

Notes

The authors declare no competing financial interest.

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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_{ox}-CO was removed by subtraction. H_{ox}-CO contamination is common and can be seen by other methods such as infrared absorption spectroscopy.⁹

(19) This ENDOR transition at 27 MHz is approximately equal to twice the ¹³C 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 ¹³C spin-flip transitions are observed in the Q-band HYSCORE spectrum (Figure S2).

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