Reaction Coordinate Leading to $H_2$ Production in [FeFe]-Hydrogenase Identified by Nuclear Resonance Vibrational Spectroscopy and Density Functional Theory

Vladimir Pelmenschikov,†,# James A. Birrell,§,# Cindy C. Pham,§,# Nakul Mishra,§ Hongxin Wang,§ Constanze Sommer,§ Edward Reijerse,‡ Casseday P. Richers,‖ Kenji Tamasaku,‡ Yoshitaka Yoda,‡ Thomas B. Rauchfuss,‖ Wolfgang Lubitz,† and Stephen P. Cramer*§

†Institut für Chemie, Technische Universität Berlin, Strasse des 17 Juni 135, 10623 Berlin, Germany
‡Max-Planck-Institut für Chemische Energiekonversion, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany
§Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, California 95616, United States
‖School of Chemical Sciences, University of Illinois, 600 S. Mathews Avenue, Urbana, Illinois 61801, United States
JASRI, Spring-8, 1-1-1 Kouto, Mikazuki-cho, Sayo-gun, Hyogo 679-5198, Japan

ABSTRACT: [FeFe]-hydrogenases are metalloenzymes that reversibly reduce protons to molecular hydrogen at exceptionally high rates. We have characterized the catalytically competent hydride state ($H_{hyd}$) in the [FeFe]-hydrogenases from both Chlamydomonas reinhardtii and Desulfovibrio desulfuricans using $^{57}$Fe nuclear resonance vibrational spectroscopy (NRVS) and density functional theory (DFT). H/D exchange identified two Fe–H bending modes originating from the binuclear iron cofactor. DFT calculations show that these spectral features result from an iron-bound terminal hydride, and the Fe–H vibrational frequencies being highly dependent on interactions between the amine base of the catalytic cofactor with both hydride and the conserved cysteine terminating the proton transfer chain to the active site. The results indicate that $H_{hyd}$ is the catalytic state one step prior to $H_2$ formation. The observed vibrational spectrum, therefore, provides mechanistic insight into the reaction coordinate for $H_2$ bond formation by [FeFe]-hydrogenases.

INTRODUCTION

Hydrogenases are enzymes that catalyze the reversible oxidation of molecular hydrogen and reduction of protons with high turnover frequencies under physiological conditions.$^{1,2}$ The [FeFe]-hydrogenases exhibit exceptional activity in both catalytic directions (>10 000 s$^{-1}$ for DdHydAB),$^3$ in part due to the unique structure of their active site cofactor. The active site of [FeFe]-hydrogenase, the “H-cluster”,$^{1,2}$ consists of covalently linked binuclear [2Fe$]_2$ and iron–sulfur [4Fe-4S]$]_H$ subclusters (Figure 1). The Fe$_p$ and Fe$_d$ sites of [2Fe]$_2$H are proximal and distal to [4Fe-4S]$]_H$, respectively bridged by a CO ligand and an azadithiolate (ADT) cofactor, the latter also providing the nitrogen base of the frustrated Lewis pair crucial to $H_2$ conversion processes.$^{5–8}$ Fe$_p$, the site at which hydrogen binds, is the Lewis acid.$^{2,5}$ Many previous experimental and theoretical studies$^{9–12}$ implicate Fe$_p$–H$_x$···H$_x$–N$]_{ADT}$ species as the key intermediate leading to H–H bond formation (Figure 1A). Despite its mechanistic importance, however, experimental characterization of such H···H interactions in the enzyme has not been available so far. Here, we show that under reducing conditions at low pH, the [FeFe]-hydrogenases from Chlamydomonas reinhardtii (CrHydA1) and Desulfovibrio desulfuricans (DdHydAB) both exhibit species with these key Fe$_p$–H$_x$···H$_x$–N$]_{ADT}$ interactions. These turnover conditions give high steady state levels of the terminal hydride bound state, $H_{hyd}$, by maximizing the rate of electron and proton transfer to the hydrogenase, such that the last step in hydrogen formation becomes rate limiting.

The ADT entity is situated at the terminus of a proton transfer pathway (Figure 1B). Protons are relayed to the active site from the protein surface via conserved residues (Glu144/159, Ser189/198, Glu141/156, and finally Cys169/178 respectively in CrHydA1/DdHydAB, here and below).$^{15,16}$ The proton transfer chain is conserved in both algal and bacterial [FeFe]-hydrogenases.$^{16}$ In contrast, the electron transport chains, containing accessory Fe–S clusters leading to [4Fe-4S]$]_H$, vary significantly between organisms. For example, CrHydA1 lacks accessory clusters altogether, while DdHydAB contains two additional [4Fe-4S] clusters, and the
bidirectional hydrogenase from *Clostridium pasteurianum* (CpHydA1) contains one [2Fe-2S] and three [4Fe-4S] clusters.

The thiol of Cys169/178 interacts via hydrogen bonding with the bridgehead N ADT nitrogen of the ADT cofactor. Exchanging this cysteine for another amino acid can abolish or severely reduce the activity of [FeFe]-hydrogenases, as seen with the C299S variant of CpHydA1,16,17 and the C169A and C169S variants of CrHydA1,7,17 whereas C298D of hydrogenase from *Clostridium acetobutylicum* (CaHydA) retains significant activity.18 Modification of the proton transfer pathway by artificial maturation with complexes that substitute the −NH− ADT bridgehead nitrogen NADT with −SC−SC or −O− (PDT) also causes nearly complete loss of activity.19

Along with decreased activity, modification of the proton transfer pathway can stabilize states that are otherwise elusive, including some with unusual IR signatures now assigned as HsredH+ (orange), Hox-CO (gray).

In previous work, we used nuclear resonance vibrational spectroscopy (NRVS) to examine the [257Fe]H-CrHydA1 Hhyd species.21 NRVS is a synchrotron-based X-ray technique that involves observation of vibrational sidebands that occur in combination with nuclear transitions.22−24 57Fe NRVS is particularly useful for vibrational analysis of Fe-containing enzymes because it is only sensitive to normal modes with 57Fe motion. Using the recently developed method of artificial maturation with [257Fe]H-labeling makes the technique completely selective for the normal modes of the subcluster.23−25 For the [257Fe-ODT]H-CrHydA1 HsredH+ samples, under H2/H2O conditions, Fe−H features were observed at 670 and 727 cm−1 (Figure S3) and rationalized by density functional theory (DFT) calculations as [4Fe-4S]+ subcluster and a homovalent Fe(II)Fe(II) binuclear [2Fe]H site, with a hydride Hh bound in the apical position on Fe2.20

[Figure 1. (A) Schematic illustration of the [FeFe]-hydrogenase H-cluster proposed for the Hhyd state. The hydride Hh at the iron site of the [2Fe]H subcluster Feα, distal to [4Fe-4S]H, interacts with the “Axial” proton Hα of the ADT bridgehead nitrogen NADT. The cysteine, shown here as −SC−HC, terminates the proton supply chain and is in contact with NADT. (B) The H-cluster and its protein environment based on the crystal structure (1HFE).13,14 Proposed proton (H+) and electron (e−) transfer pathways are shown in semitransparent gray arrows. Red and blue numbers correspond to the amino acid residues in CrHydA1 and DdHydAB, respectively.]

[Figure 2. FT-IR spectra of the Hhyd state. Samples (10 μL) of 3–5 mM [257Fe]H-CrHydA1 at pH 6 (A) and 2–4 mM [257Fe]H-DdHydAB at pH 4 (B) were reduced with 100 mM sodium dithionite and transferred to the FT-IR cells. The samples were prepared either in H2O under a 2% H2 atmosphere (upper spectra) or in D2O under a 2% D2 atmosphere (lower spectra). Spectra were recorded within 5 min after the initial sample reduction. The peaks are shaded to indicate from which state they derive: Hox (purple), Hoxblue (blue), Hhyd (pink), HsredH+ (green), Hox-CO (gray).]
terminal hydride Fe₄–H₆ bending modes. The corresponding red-shifted Fe₄–D₆ modes were associated with the perturbed Fe₄–H₆=CO bands at 625 and 564 cm⁻¹ under D₂/D₂O conditions. The Fe₄–H₆ vibrations at 670 and 727 cm⁻¹ were assigned, respectively, to H⁺ motion perpendicular to and within the approximate mirror symmetry plane of [2Fe]₆, which passes through Fe₄, Fe₆, and H₆ (Fe₄–Fe₆–H₆ plane). Here we focus on the NRVS spectra for the H₆hyd species from CrHydA1 and DdHydAB artificially maturated with [257Fe]H.

As we show in this report, the structure of the hydride state(s) in [FeFe]-hydrogenase is extremely sensitive to subtle effects that are not directly at the Fe₄–H₆ center. These remote effects include hydrogen bridging between Fe₄–H₆ and ADT and between ADT and Cys169/178, the very sites that were modified in previous studies that demonstrated the existence of iron hydrides. The overarching question remains: in the absence of such perturbations, what is the structure of an iron-hydride in hydrogenase under turnover conditions? The present paper addresses this question.

## RESULTS AND DISCUSSION

**H₆hyd State Accumulation.** The H₆hyd states of the catalytically active [257Fe]₁₄CrHydA1 and [257Fe]₁₄DdHydAB enzymes were stabilized at low pH values (pH 6 and pH 4, respectively) and with high concentrations of a low potential reductant (sodium dithionite). These turnover conditions give high steady state levels of H₆hyd, which we rationalize as follows. With such a high concentration of reductant, the rate of electron transfer to the hydrogenase is very high. Around neutral pH, proton transfer from the nitrogen base in the H₆redH⁺ state is rate limiting. Protonation at low pH, of the amino acid residues in the proton transfer pathway, increases the rate of proton transfer from the nitrogen base to the open coordination site on Fe₄ forming the H₆hyd state. The rate limiting step then becomes the coupled electron/proton transfer leading to hydrogen formation. Also, under these conditions, an initial burst of hydrogen production saturates the sample with hydrogen, which can rebind to Fe₄ in the oxidized state reforming the H₆hyd state.

**FTIR Spectroscopy.** The FTIR spectra for the H₆hyd state in both [257Fe]₁₄CrHydA1 and [257Fe]₁₄DdHydAB under H₂/H₂O and D₂/D₂O (Figure 2) are very similar to those observed for [257Fe-ODT]₁₄CrHydA1. The characteristic red-shift of the bridging CO band due to H/D exchange is attributed to coupling between the bridging CO vibration and the Fe₄–H₆ vibration. The possibility that this shift is due to H/D exchange in hydrogen bonds to the bridging CO ligand can be excluded because hydrogen bonds to the bridging CO ligand are not indicated crystallographically. The H₆hyd state in [257Fe]₁₄CrHydA1 was formed in high stochiometric yield (≈90%) and appeared to be very stable (Figure S1A).

In DdHydAB, however, lower stability of the H₆hyd state (Figure S1B) due to the much higher activity of this enzyme (≈10 000 s⁻¹ compared with ≈1000 s⁻¹ for CrHydA1) gave slightly lower yields (≈50%) of the H₆hyd state. The major impurities were the Hox state and a protonated form of the Hox state (termed Hox-blue). The Mössbauer (Figure S2A) and EPR (Figure S2B) spectra of CrHydA1 in the H₆hyd state under H₂/H₂O are very similar to those previously published for both wild type CrHydA1 and the C169S mutant.

**NRVS Spectroscopy.** NRVS spectra for the two [FeFe]-hydrogenases under H₂/H₂O and D₂/D₂O conditions are illustrated in Figure 3A. Features in the 150–744 cm⁻¹ range are primarily due to Fe-CO bending and stretching motions, and hence they are relatively unaffected by H vs D conditions. Bands near 450 cm⁻¹ involve mostly Fe-CN motion. Strong bands between 500–600 cm⁻¹ involve mostly Fe-CO bending and stretching character; although their profiles are similar under H vs D conditions, there are indeed subtle changes that derive from coupling with Fe–H/D motion.

Distinct evidence for the presence of an Fe–H/D bond comes in the higher frequency region between 620–750 cm⁻¹ (Figure 3B). In both H₂/H₂O samples there are bands at 675 and 744/747 cm⁻¹, which are replaced by a feature at 629/627 cm⁻¹ in the D₂/D₂O samples. The high frequencies and isotope sensitivity help assign these features as hydride/deuteride bands. The close similarity of the Fe–H/D modes in the two different enzymes suggests nearly identical structures for the H₆hyd state. However, to our initial surprise, the two main hydride bands for the enzyme spectra are upshifted vs those observed for [257Fe-ODT]₁₄CrHydA1 (Figure S3). For both proteins, the [257Fe-ODT]₁₄H 670 cm⁻¹ band is upshifted by 5 cm⁻¹ to 675 cm⁻¹, while the [257Fe-ODT]₁₄727 cm⁻¹ band upshifts by 17/20 cm⁻¹ to 744/747 cm⁻¹ for [257Fe]₁₄H.

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**Figure 3.** NRVS spectra of [FeFe]-hydrogenases. (A) The overall NRVS for samples of [257Fe]₁₄CrHydA1 (top) and [257Fe]₁₄DdHydAB (bottom) prepared under H₂/H₂O (blue) and D₂/D₂O (red). (B) Expansion of the Fe–H/D region of the NRVS for the same samples. Numbers at the top indicate representative peak positions in H₂/H₂O (blue) and D₂/D₂O (red) samples.
CrHydA1/DdHydAB. The obvious question: why should changing N for O in the bridgehead atom, which is at a nonbonding distance of >3 Å from the Fe, yield such significantly different shifts in the Fe–H bending mode vibrational frequencies?

**DFT Calculations.** Although several X-ray crystal structures are available for [FeFe]-hydrogenases, structural studies...
are lacking for the enzyme with a bound hydride. Four schematic alternatives on Hhyd based on earlier proposals are shown in Figure 5F. Two of them, designated Hhyd-A and Hhyd-E, assume an uncharged bridgehead amino group, in either "axial" -N\textsubscript{ADT}H\textsubscript{A}− (H\textsubscript{A} pointing toward Fe\textsubscript{A}) or "equatorial" -N\textsubscript{ADT}H\textsubscript{E}− (H\textsubscript{E} pointing away from Fe\textsubscript{E}) conformation. The other two alternatives H\textsuperscript{+} and H\textsuperscript{H+} assumed a protonated -N\textsubscript{ADT}H\textsuperscript{2+} form wherein both H\textsubscript{A/E} locations are occupied, and the [4Fe-4S]\textsuperscript{H−} [2Fe\textsubscript{H}] subclusters in either [4Fe-4S]\textsuperscript{−}−Fe\textsubscript{E}−(II)Fe\textsubscript{E}−(II) or [4Fe-4S]\textsuperscript{2+}−Fe\textsubscript{E}−(I)Fe\textsubscript{E}−(II) oxidation levels.

DFT modeling of the H\textsubscript{hyd} state, therefore, provides insight into the conformation and the protonation state of the bridging ADT ligand, as well as the electronic configuration at the Fe sites. We started our analysis at a "Small" DFT modeling level called S, including only the [2Fe\textsubscript{H}] subcluster and the Cys421/382 side chain that coordinates Fe\textsubscript{A} (see Figures 4A, S4, and S5, Tables S1 and S2, and Supplementary Discussion in Supporting Information for details on the DFT modeling); earlier, this type of modeling has been applied to rationalize the NRVS Fe−H bands in [2\textsuperscript{57}Fe-ODT]\textsuperscript{H−}CrHydA1. As shown in Figure S10, the S modeling of the four H\textsubscript{hyd} states produced a significantly variable distribution of the Fe\textsubscript{A}−H\textsubscript{A} bands. Only S-H\textsubscript{hyd-A} with its Fe\textsubscript{A}−H\textsubscript{A} modes at 668 and 764 cm\textsuperscript{−1} satisfactorily simulated the observed spread and relative intensities of the two NRVS bands. The "Small" DFT models were thus useful for distinguishing the major protonation and conformational alternatives.

It is well-known, however, that the [4Fe-4S]\textsubscript{H} subcluster and the protein environment surrounding the H-cluster play crucial roles in the enzyme’s properties, and H-bonding between ADT protons and the neighboring Cys169/178 residue are also important. We, therefore, proceeded to explore the four H\textsubscript{hyd} alternatives using a "Large" modeling level L that, beyond S, integrated protein side chains surrounding the [2Fe\textsubscript{H}] subcluster (see Figures 4A and B, S4 and S6). A second large model, labeled L’ and additionally included the [4Fe-4S]\textsubscript{H} subcluster, was evaluated (see Figure S7).

The 600–900 cm\textsuperscript{−1} “active window” is uniquely sensitive to Fe−H/D bending motions. As shown in Figure 5A–E, model L-H\textsubscript{hyd-A} is the only simulation that reasonably replicates the positions and intensities of the two observed Fe−H bands. The other L-H\textsubscript{hyd} models predict either too many bands (L-H\textsubscript{hyd-E}), only a single band (L-H\textsuperscript{+}H\textsuperscript{H+}), or hardly any bands.
(L-H_{hyd}H^+)\), with intensity levels above the experimental noise. Interestingly, the total intensity concentrated in the calculated Fe_{L-H} bands decreases in the sequence H_{hyd,E} > H_{hyd,A} > H_{hyd,E} > H_{hyd,H} \), which correlates with the strength of the ADT-hydride interaction, as reflected by the H_{L-H} distances collected in Table S3. Only for the H_{hyd,E} alternative that lacks the H_{A} proton, modeling S vs L predict essentially the same set of Fe_{L-H} bands (Figure S10C). For other H_{hyd} alternatives the H_{L-H_{A}} hydride interaction with ADT is communicated further to Cys169/178 only in the L models, and S vs L band positions generally diverge (Figure S10B, D, E). For the best-fit hydride state, inclusion of Cys169/178 in the L-H_{hyd,A} model leads to an improvement in the higher frequency Fe_{L-H} mode position by 15 cm^{-1} (764 in S-H_{hyd,A} and 749 in L-H_{hyd,A} vs 744/749 cm^{-1} observed, see Figure S10B). We note also that our preference for H_{hyd,A} is supported by its \(\approx 6-9\) kcal/mol lower energy vs H_{hyd,E} (Table S5). Simulations for the entire spectral range reported in Figures S9 and S11-S14 provide additional support for H_{hyd,A} and unequivocally indicate the benefits of the large DFT models for reproducing NRVS bands <400 cm^{-1}.

The motion of the various nuclei that is the source of the calculated intensities for the L-H_{hyd,A} bands (Figure S5B) are illustrated in Figure 6A and animated vibrational modes available in Supporting Information. The mode calculated at 665 cm^{-1} and associated with the band observed at 675 cm^{-1} involves essentially pure H_{A} motion normal to the Fe_{L-H} plane. In contrast, the three higher frequency modes are in-plane H_{B} motions becoming increasingly less pure and more strongly coupled to the ADT bridgehead, Cys169/178, and further molecular fragments. The in-plane modes can be characterized in terms of in/out of phase motion of the hydride, ADT, and cysteine H nuclei: \([\text{H}_{A} \cdot \text{H}_{A} \cdot \text{H}_{C_{1}}] \) at 749, \([\text{H}_{B} \cdot \text{H}_{A} \cdot \text{H}_{C_{1}}] \) at 766, and \([\text{H}_{C_{1}} \cdot \text{H}_{A} \cdot \text{H}_{C_{1}}] \) at 788 cm^{-1}. Among the in-plane modes, the one at 749 cm^{-1} has sufficient \(^{57}\text{Fe}\) motion to be detected in the NRVS experiment and associated with the H_{hyd} band observed at 744/747 cm^{-1}. Transition from the S to L modeling for the H_{hyd,A} spectral simulations reveals that the \textit{intrinsically} decoupled out-of-plane hydride mode hardly changes its position (Figure S10B, 668 to 665 cm^{-1}) upon the protein environment inclusion. In contrast, the coupled in-plane mode shifts by \(-15\) cm^{-1} as discussed above. Here, S vs L modeling replicates the behavior of the \([^{57}\text{Fe-ODT}]_{H} \) vs \([^{57}\text{Fe}]_{H} \) protein systems on the two Fe–H band shifts (see the NRVS section above); only in the \([^{57}\text{Fe}]_{H} \) systems the H_{L-H_{A}} interaction is present, and the higher energy Fe_{L-H} mode becomes delocalized.

**Mechanistic Proposal.** The details of the catalytic mechanism of \([\text{FeFe}]\)-hydrogenases have been debated for decades. Past proposals for the catalytic mechanism can be divided into one set involving a bridging hydride between Fe_{A} and Fe_{C} (Mechanism I as described by Trohalaki and Pachter\(^{11}\)) and another set involving a terminal hydride on Fe_{C} (Mechanism II).\(^{35}\) We see no evidence that H_{hyd} involves a bridging hydride. Notably, in contrast to the \([^{57}\text{Fe}]_{H} \) - CrHydA1/DhHydAB samples and the best-fit H_{hyd,A} state producing \textit{two} (Fe–H bending) bands in the NRVS \textit{"active window"} (Figure S5A and B), bridging Ni(II)(\(\mu\)-H)\(^{57}\text{Fe}(II)\) hydrides in [NiFe]-hydrogenase and a model complex produced only a \textit{single} (Ni–H–Fe wagging) band.\(^{35}\) A qualitatively similar result displaying strong deviations to the \textit{observed} \textit{H}_{hyd} \textit{spectra} is \textit{obtained from a} \([^{57}\text{Fe}(II)(\mu-H)\text{Fe}(II)]\) DFT candidate called L-H_{L-hyd,A} (see Figures S8 and S15 and Supplementary Discussion in Supporting Information). Other reports also disfavor bridging hydride-based mechanisms on a variety of grounds.\(^{5,12,15,38}\) We thus consider the implications of the NRVS results on mechanisms involving a terminal hydride. For this case, a consensus has emerged that proton transfer from an \(-\text{NH}_{2}\) – form of the bridgehead nitrogen to Fe_{A} hydride is the key step in H_{2} formation.

**Figure 6.** DFT-calculated normal modes and possible mechanism of H_{2} formation in \([\text{FeFe}]\)-hydrogenase. (A) The unscaled arrow representation of relevant atoms motion in the normal modes at 665, 749, 766, and 788 cm^{-1} for model L-H_{hyd,A} (cf. Figure S5B). Contributions from the H_{B} and \(^{57}\text{Fe}\) nuclei to the vibrational energies are given. (B) Proposed sequence of events leading to H_{2} formation: H_{hyd} becomes protonated leading to H_{hyd}H^+, and subsequently bond formation occurs giving the H_{2} product complex. H_{2} release generates the H_{ox} state of the enzyme, which can be reduced and protonated to form H_{hyd} again.
We note that model L-H_{hyd-A} predicts a H···H distance of 2.01 Å (Table S3), a similar Fe···H···N distance of 1.88 Å observed in the \([\text{[(H)}\text{Fe}_2(\text{adt-NH}_2)(\text{CO})_2(\text{dpv})_2](\text{BF}_4)_2]\) complex by X-ray crystallography.\(^9\) This distance is still quite a bit longer than the \(\approx 1.5\) Å H···H distance reported by Bullock and coworkers for a strong Fe···H···N dihydrogen interaction.\(^{10}\)

The long Fe···H···H···N distance in the complex is, however, a consequence of hydrogen bonding to the BF\(_4^-\) anion.\(^{39}\) Therefore, model L-H_{hyd-A} probably represents motions of the nuclei related to a step prior to the last step leading to H\(_2\).

Furthermore, the Ha distance in the Bullock complex, and it is thus even shorter than in the Bullock complex, and it is thus an H\(_{hyd}\), yields H\(_{hyd}\)H\(_+\), which can directly yield an Fe

DFT modeling has been directly identified for the enzyme

CONCLUSIONS

The key hydride intermediate in \([\text{FeFe}]-\)hydrogenase catalysis has been directly identified for the first time in two native enzymes using selective vibrational spectroscopy. The NRVS vibrational data are consistent with a terminal hydride at the distal iron of the \([\text{2Fe}]\)H subsite interacting with the amine proton to subsequently yield an Fe

DFT optimizations and subsequent normal mode calculations were performed using GAUSSIAN 09 Revision D.01\(^{46}\) based on the densities extracted from single point calculations performed by JAGUAR 9.4\(^{47}\) that provided high-quality initial guess. The BP86\(^{48,49}\) functional and the LACVP^**\(^{50}\) basis set as implemented in JAGUAR were employed, unless otherwise mentioned. For the first- and second-row elements, LACV5P^**\(^{51}\) triple \(\xi\) basis sets including polarization functions. For the Fe atoms, LACV5P^**\(^{52}\) consists of a triple \(\xi\) basis set for the outermost core and valence orbitals, and the quasi-relativistic Los Alamos effective core potential (ECP) for the innermost electrons. The molecular systems environment was considered using a self-consistent reaction field (SCRF) polarizable continuum model and integral equation formalism (IEFPCM)\(^{53}\) as implemented in GAUSSIAN 09, with the static dielectric.

METHODS

Sample Preparation. \([\text{2}^{57}\text{Fe}_{\text{II}}\text{I}]\text{H-HydA1} and [\text{2}^{57}\text{Fe}_{\text{II}}\text{I}]\text{D-HydA2} were prepared as described previously,\(^{40,41}\) but using an \(\text{57}^{1}\)Fe-labeled azadithiolate cofactor.\(^{25}\) All sample preparation steps were performed in an anaerobic glovebox (Coy) under 2% H\(_2\) (or D\(_2\))/98% N\(_2\). Samples of \([\text{2}^{57}\text{Fe}_{\text{II}}\text{I}]\text{H-HydA1} (60 \mu\text{L of 3--5 mM}) in pH 6 buffer (50 mM MES, 50 mM HEPES, 150 mM NaCl, either in H\(_2\)O or D\(_2\)O) and \([\text{2}^{57}\text{Fe}_{\text{II}}\text{I}]\text{D-HydA2 (60 \muL of 2--4 mM)} in pH 4 buffer (100 mM sodium acetate, 150 mM NaCl, either in H\(_2\)O or D\(_2\)O) were reduced with 100 mM sodium dithionite, 50 mM were transferred to each NRVS cell and frozen in liquid nitrogen, and the remaining 10 \(\mu\text{L were loaded into an FTIR cell and measured within 5 min.}\)
constant set to $\varepsilon = 4.0$ as often used for proteins, and the remaining IEF-PCM parameters at their default values for water.

Our attempts to vary the DFT methodology described above using alternative functionals (specifically nonhybrid PBE,\textsuperscript{11} hybrid PBE0,\textsuperscript{56} and hybrid B3LYP\textsuperscript{114-116} including variations on the exact exchange admixture) did not produce any better correspondence between the DFT-calculated and NRVS-observed vibrational data. When applicable, the B3LYP functional results are for single-point calculations only.

Computational schemes were furthermore tested (i) excluding and (ii) including two-body D3 empirical dispersion correction by Grimme et al. in its original formulation,\textsuperscript{57} as well as (iii) D3 reformulated with (ii) including two-body D3 empirical dispersion correction by Grimme only.

DFT-calculated and NRVS-observed vibrational data. When applying admixture) did not produce any better correspondence between the DFT-calculated and NRVS-observed vibrational data. When applicable, the B3LYP functional results are for single-point calculations only.

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ASSOCIATED CONTENT
Supporting Information
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Supplementary FTIR, EPR, Mössbauer, NRVS, and DFT figures and tables; Supplementary Discussion on the DFT models (PDF)
Coordinates of the DFT models as XYZ files (ZIP)
Animated vibrational normal modes as GIF files (ZIP)

AUTHOR INFORMATION
Corresponding Author
*spjcramer@ucdavis.edu
ORCID
Thomas B. Rauchfuss: 0000-0003-2547-5128
Wolfgang Lubitz: 0000-0001-7059-5327

Author Contributions
V.P., J.A.B., C.C.P. contributed equally to this work.

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