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Vibrational Perturbation of the [FeFe] Hydrogenase H-cluster Revealed by a $^{13}\text{C}^2\text{H}$ -ADT Labeling

Vladimir Pelmeshnikov,[§] James A. Birrell,^{†*} Leland B. Gee,[‡] Casseday P. Richers,[#] Edward J. Reijerse,[†] Hongxin Wang,[§] Simon Arragain,[⊥] Nakul Mishra,^{||} Yoshitaka Yoda,[¥] Hiroaki Matsuura,[¶] Lei Li,[£] Kenji Tamasaku,[±] Thomas B. Rauchfuss,^{**} Wolfgang Lubitz,[†] and Stephen P. Cramer^{§*}

[§]Institut für Chemie, Technische Universität Berlin, 10623 Berlin, Germany

[†]Max Planck Institute for Chemical Energy Conversion, 45470 Mülheim an der Ruhr, Germany

[‡]Department of Chemistry, Stanford University, Stanford, California 94305, United States

[#]School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801, United States

[§]SETI Institute, Mountain View, California 94043, United States

[⊥]IFP Energies nouvelles, 92852 Rueil-Malmaison, France

^{||}Department of Chemistry, University of California, Davis, California 95616, United States

[¥]Precision Spectroscopy Division, SPring-8/JASRI, Sayo, Hyogo 679-5198, Japan

[¶]Life Science Research Infrastructure Group, Advanced Photon Technology Division, RIKEN/SPring-8 Center, Sayo, Hyogo 679-5148, Japan

[£]Hyogo Science and Technology Association, Synchrotron Radiation Research Center, Tatsuno-shi, Hyogo 679-5165, Japan

[±]Research and Utilization Division, SPring-8/JASRI, Sayo, Hyogo 679-5198, Japan

SUBJECTS: *hydrogenase, 2-azapropane-1,3-dithiolate, catalysis, NRVS, DFT*

ABSTRACT: [FeFe] hydrogenases are highly active catalysts for the interconversion of molecular hydrogen with protons and electrons. Here, we use a combination of isotopic labeling, ^{57}Fe nuclear resonance vibrational spectroscopy (NRVS), and density functional theory (DFT) calculations to observe and characterize the vibrational modes involving motion of the 2-azapropane-1,3-dithiolate (ADT) ligand bridging the two iron sites in the $[\text{2Fe}]_{\text{H}}$ subcluster. A $^{-13}\text{C}^2\text{H}_2$ -ADT labelling in the synthetic diiron precursor of $[\text{2Fe}]_{\text{H}}$ produced isotope effects observed throughout the NRVS spectrum. The two precursor isotopologues were then used to reconstitute the H-cluster of [FeFe] hydrogenase from *Chlamydomonas reinhardtii* (CrHydA1), and NRVS was measured on samples poised in the catalytically crucial H_{hyd} state containing a terminal hydride at the distal Fe site. The $^{13}\text{C}^2\text{H}$ isotope effects were observed also in the H_{hyd} spectrum. DFT simulations of the spectra allowed identification of the ^{57}Fe normal modes coupled to the ADT ligand motions. Particularly, a variety of normal modes involve shortening of the distance between the distal Fe-H hydride and ADT N-H bridgehead hydrogen, which may be relevant to formation of a transition state on the way to H_2 formation.

Molecular hydrogen is viewed as an ideal carbon-free energy carrier that could be part of a transition to a sustainable economy without CO_2 emissions.¹⁻² At the moment, the majority of industrial hydrogen is produced by high-temperature steam reforming of natural gas which leads to release of at least one molecule of CO_2 for every 4 H_2 produced.³ Ideally, electrochemical energy from solar, wind, or other carbon-free sources could be used to drive the water-splitting or ‘hydrogen evolution reaction’ (HER) without CO_2 release.^{1, 4} Highly efficient catalysts with low overpotentials are essential for electrochemical conversions of hydrogen, and the high prices and scarcity of the current Pt or other noble metal HER catalysts has led to the search for systems that use Earth-abundant materials.⁵⁻⁷ One source of inspiration driving this search is Nature, which uses prolific transition metals Fe or Fe with Ni in the active sites of hydrogenases.⁸⁻⁹

Hydrogenases are enzymes that catalyze the reversible interconversion of molecular hydrogen with protons and electrons: $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$. [FeFe] hydrogenases contain an active site ‘H-cluster’ consisting of a $[\text{4Fe-4S}]_{\text{H}}$ cluster linked *via* a cysteine residue to a unique $[\text{2Fe}]_{\text{H}}$ subcluster (**Figure 1a**).¹¹ This subcluster carries a 2-azapropane-1,3-dithiolate (ADT) ligand bridging a pair of CO and CN^- ligated Fe ions. The ADT bridgehead nitrogen has been implicated as part of a proton transfer relay extending through a neighboring cysteine.¹²⁻¹⁶ In the *Chlamydomonas reinhardtii* [FeFe] hydrogenase (CrHydA1), the conserved relay consists of C169, a water molecule, and oxygens from E141, S189, and E144 residues.

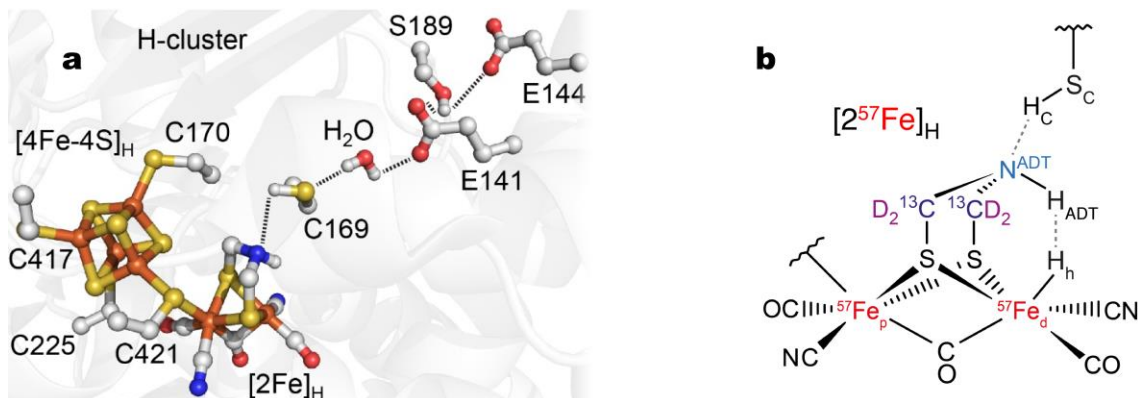


Figure 1. (a) [FeFe] hydrogenase active site including key amino acids in the proton transfer pathway (based on the PDB 4XDC¹⁰ structure of the Cpl enzyme from *Clostridium pasteurianum*, but using CrHydA1 sequence numbering). **(b)** Schematic structure of the [2Fe]_H subcluster in the H_{hyd} state, showing the isotopically labelled nuclei ⁵⁷Fe, ¹³C, and D(=2H). The important hydrogens, H_h (catalytic hydride at the distal Fe_d iron), H_{ADT} (at the ADT N_{ADT} nitrogen), and H_c (at the S_c C169 sulfur), are shown.

An iron hydride form of [FeFe] hydrogenase, H_{hyd}, is a key intermediate of the catalytic cycle, and it has been studied by multiple spectroscopic and molecular modeling techniques.^{17–24} The H_{hyd} species contains a terminal Fe_d-H_h hydride at the [2Fe]_H iron site distal to [4Fe-4S]_H (**Figure 1b**), with a [4Fe-4S]_H⁺-Fe_p(II)Fe_d(II) redox state for the H-cluster, along with the -N_{ADT}H_{ADT}- amine form of the ADT bridgehead.^{18–21}

Nuclear resonance vibrational spectroscopy (NRVS) has become a popular technique for elucidating the element-selective normal modes of appropriate Mössbauer isotopes.^{25–31} In previous work on the CrHydA1 and DdHydAB (from *Desulfovibrio desulfuricans*) enzymes,^{20–22} we have shown that ⁵⁷Fe_d-H_h bending modes can be observed using ⁵⁷Fe-NRVS for the H_{hyd} species, and that these modes exhibit peak positions that are characteristic of the local environment. To better define additional normal modes of H_{hyd}, we proceeded to label the [2Fe]_H subcluster not only with ⁵⁷Fe but also with ¹³C and D in the methylene groups of the ADT ligand. We accomplished this by preparing a [2Fe]_H precursor, the ⁵⁷Fe-labelled salt (Et₄N)₂[⁵⁷Fe₂[(SCH₂)₂NH](CN)₂(CO)₄] (**1**) as well as its variant also labelled with ¹³C and D on the two methylene groups of the ADT ligand (¹³CD-**1**).³² We then used these samples to reconstitute an apo form of CrHydA1 containing the [4Fe-4S]_H cluster but lacking the [2Fe]_H subsite.³³

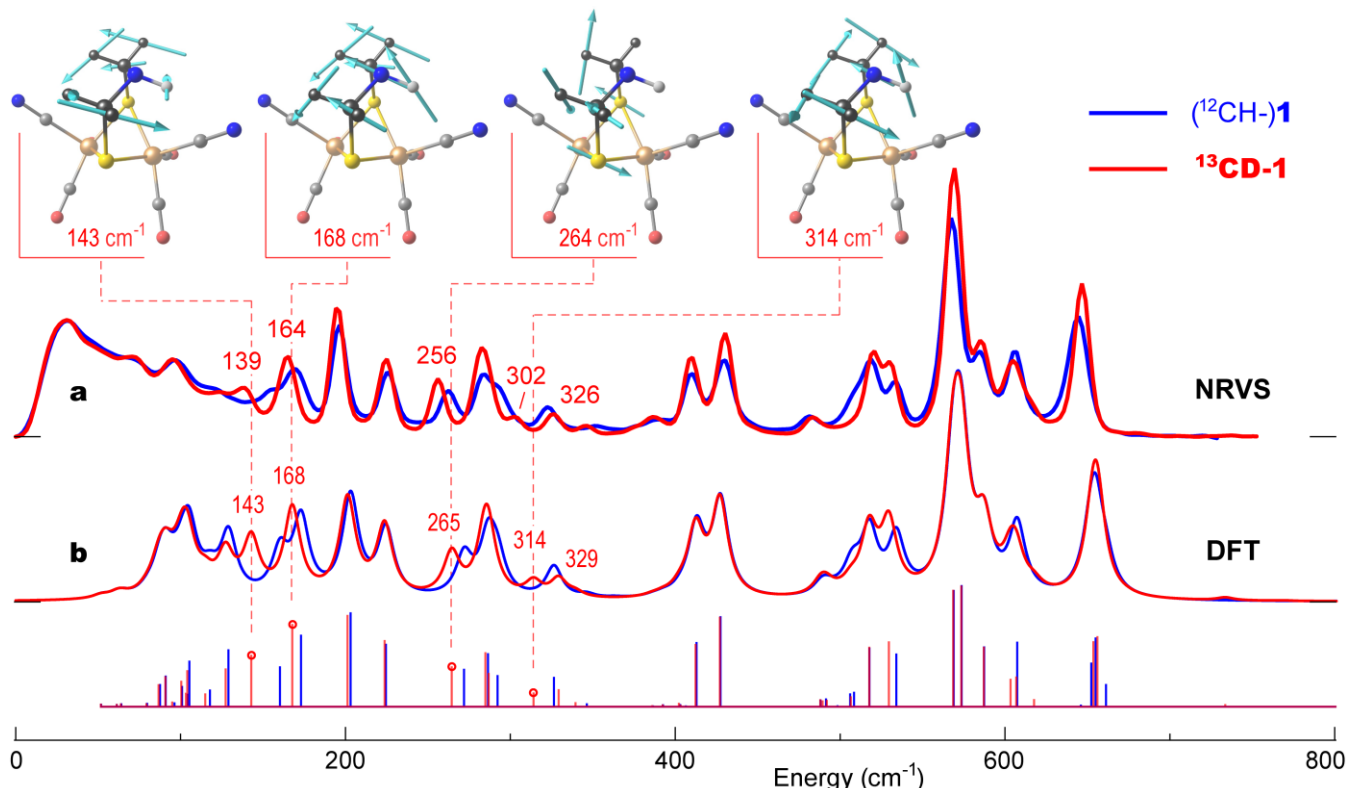
We first examine NRVS spectra for the precursor isotopologues **1** vs. ¹³CD-**1** in **Figure 2a**. Close inspection reveals a number of subtle changes to band positions and intensities in the broad ~100–700 cm⁻¹ range, most of them well reproduced by the DFT simulation shown in **Figure 2b**. We note that this is the first demonstration of NRVS isotope shifts from labeling in the 2nd and 3rd coordination spheres of ⁵⁷Fe, although such shifts have been seen before in resonance Raman spectra.^{34–35} In the following, when referring to the bands observed (or vibrational frequencies calculated) for the two isotopologues, we use a nomenclature x→y (cm⁻¹) where x and y represent **1** and ¹³CD-**1**, respectively.

Since the bands from 400 to 660 cm⁻¹ are dominated by Fe-CN and Fe-CO motion, we focus instead on differences in the region from 100 to 350 cm⁻¹, which contains delocalized bending and torsional modes as well as Fe-S stretching. In the ¹³CD-**1** spectra, several bands exhibit clear downshifts from the (¹²CH-)**1** data, for example at 150→139, 168→164, and 260→256 cm⁻¹ (**Figure 2a**). This pattern is echoed in the ¹³CD-**1** DFT simulations, with downshifted bands at 161→143, 173→168, and 273→265 cm⁻¹ (**Figure 2b**). The normal mode analysis also reveals isotope-dependent redistribution of the intensities underlying the DFT bands at 326→329/314 cm⁻¹, mapping onto the NRVS features at 322→326/302 cm⁻¹.

Having identified the most significant isotope shifts in the precursor spectra, we now illustrate the atomic motions deduced from the DFT calculations. As displayed in **Figure 2** for ¹³CD-**1**, the normal mode calculated at 143 cm⁻¹ is mostly out-of-phase rotation of the two ADT -μSCH₂- groups around their S-C axes, combined with some motion of the μS pivot points due to Fe-S-Fe bending (see animated representations of the calculated vibrational modes as part of the **Supporting Information**, and their characterization in **Table S2**). The large amount of methylene motion explains the significant isotope shift. In contrast, the 168 cm⁻¹ mode involves rocking of the entire -H₂C-NH-CH₂- assembly in one direction while the underlying Fe₂S₂ cluster (and associated ligands) rotate in the opposite direction. At higher frequencies, the 264 cm⁻¹ mode involves out-of-phase displacements of the -μSCH₂- fragments with substantial Fe-S stretching character, while the 314 cm⁻¹ mode is an in-phase -CH₂- methylene group motion, accompanied by wagging of the -NH- bridgehead in the opposite direction.

Our key observations from these precursor studies are that (i) the ¹³CD substitution in the Fe-bridging ADT ligand induces measurable isotope shifts in the ⁵⁷Fe NRVS spectra, on the order of the 8 cm⁻¹ instrumental resolution, (ii) the DFT calculations are sufficiently accurate to reproduce these shifts, allowing confidence in the motions assigned to these modes, and (iii) the calculations predict a variety of ADT flexing modes with significant motion of the -NH- bridgehead.

Precursors **1** and ¹³CD-**1** were used for maturation of the apo CrHydA1 containing natural abundance Fe in the [4Fe-4S]_H subcluster. This yielded holo CrHydA1 labeled with ⁵⁷Fe in the [2Fe]_H subcluster, and with either a natural abundance ADT ligand (**1**-



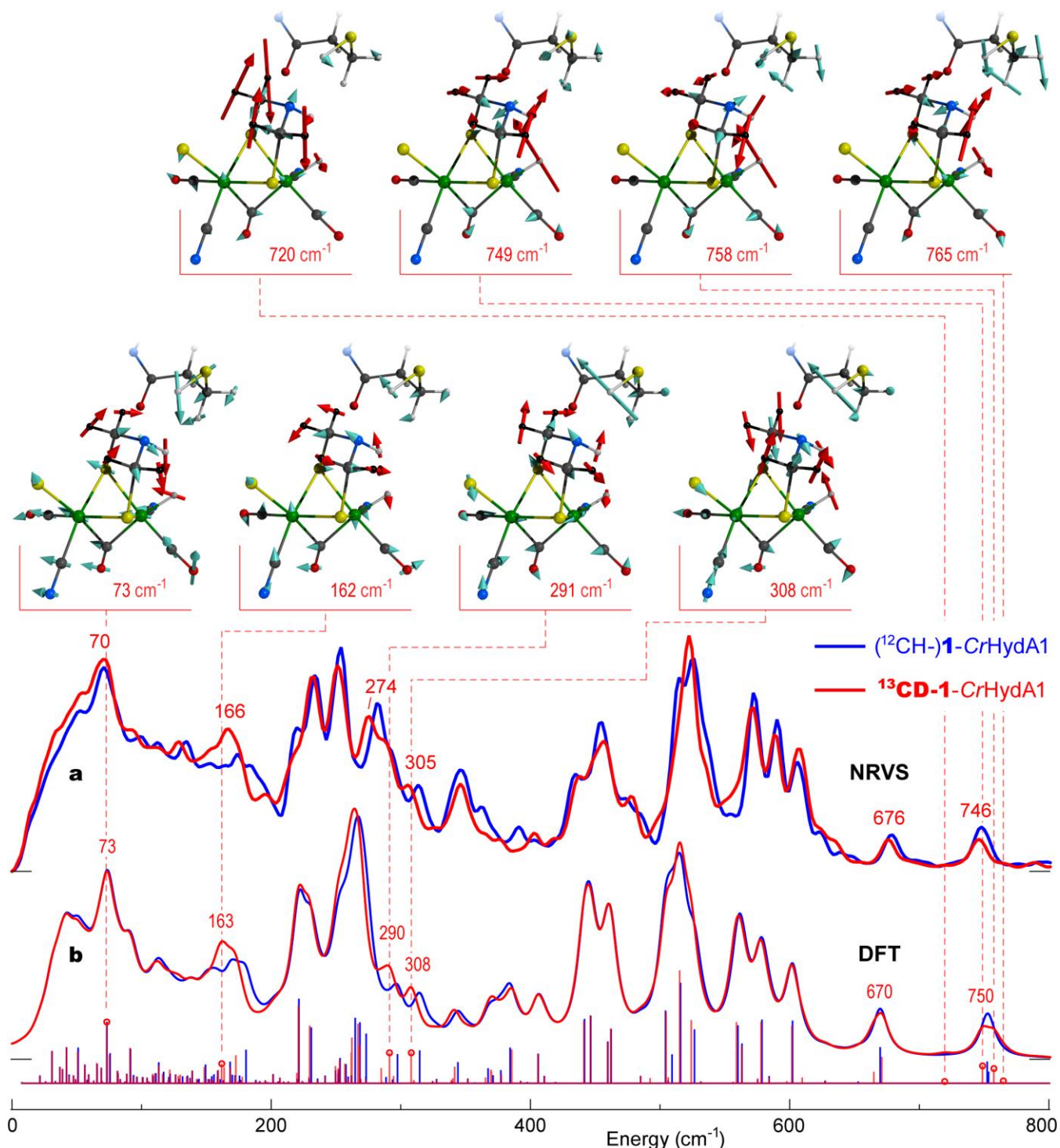
CrHydA1) or $^{-13}\text{CD-}$ in the methylene portions of ADT ($^{13}\text{CD-1-CrHydA1}$). These samples were poised in the H_{hyd} state by reduction with 100 mM sodium dithionite at pH 6. As shown by infrared (IR) spectra in **Figure S1**, both samples exhibited the standard H_{hyd} IR signature, with minimal contribution from other redox states.

Figure 2. ^{57}Fe -PVDOS for the $[2\text{Fe}]_{\text{H}}$ precursor isotopologues **1** (blue) vs. $^{13}\text{CD-1}$ (red) from (a) NRVS experiment and (b) DFT calculations. Sticks correspond to individual DFT normal mode energies and intensities before broadening. For $^{13}\text{CD-1}$, important band positions are labeled and atomic motions in selected normal modes are shown.

NRVS data for **1-CrHydA1** and $^{13}\text{CD-1-CrHydA1}$ are shown in **Figure 3a**, with the corresponding DFT simulations in **Figure 3b**. The calculated spectra were generated using a DFT model of H_{hyd} including the entire H-cluster and its immediate protein environment,^{21-22, 36} see DFT methods in the **Supporting Information** for further details. Again, we focus first on differences in the low-energy region, where we see the most obvious isotope effects. These include NRVS downshifts at $281 \rightarrow 274$ and $313 \rightarrow 305$ cm^{-1} , with the DFT simulations yielding corresponding modifications at $296 \rightarrow 290$ and $314 \rightarrow 308$ cm^{-1} . The 150–200 cm^{-1} isotope-dependent NRVS region ($\sim 174 \rightarrow 166$ cm^{-1}) of H_{hyd} essentially repeats in the DFT spectra ($\sim 171 \rightarrow 163$ cm^{-1}), indicating overlapping contributions from different modes. Complementary DFT simulation for the $[4\text{Fe-4S}]_{\text{H}}^{2+}\text{-Fe}_p(\text{II})\text{Fe}_d(\text{I})$ redox state of the H-cluster, H_{ox} , reveals comparable $^{12}\text{CH} \rightarrow ^{13}\text{CD}$ spectral shifts in the broader $\sim 150\text{--}330$ cm^{-1} region (**Figure S2**); this indicates that the ADT labeling effects observed in NRVS are stable against potential impurities from additional redox states of the H-cluster.

The atomic motions deduced from the DFT calculations are displayed in **Figure 3**. The 162 cm^{-1} band of $^{13}\text{CD-1-CrHydA1}$ contains $[2\text{Fe}]_{\text{H}}$ modes heavily mixed with the protein environment, but an important feature here is rocking of the $-\text{N}_{\text{ADT}}\text{H}_{\text{ADT}}-$ bridgehead toward the distal iron hydride $\text{Fe}_d\text{-H}_h$, along with out-of-phase rotation of the ADT $-\text{CH}_2-$ groups; this character matches the $^{13}\text{CD-1}$ precursor mode at 143 cm^{-1} . At higher energies, the 291 cm^{-1} mode exhibits a breathing motion of the Fe_2S_2 moiety, which leads to changing the distance between Fe_p and the $[4\text{Fe-4S}]_{\text{H}}$ subcluster; in this case there is an in-phase motion of the ADT methylene groups in the opposite direction of the amine bridgehead, similar to the $^{13}\text{CD-1}$ mode at 314 cm^{-1} described above. The 308 cm^{-1} mode exhibits an entire ADT fragment wagging/rotation relative to Fe_p and Fe_d , equivalent to the $^{13}\text{CD-1}$ mode at 287 cm^{-1} . We also illustrate the 73 cm^{-1} mode, which is highly delocalized with torsional motions of the entire H-cluster.

We now turn to the higher-energy side of the H_{hyd} spectra, which contains two distinct $\text{Fe}_d\text{-H}_h$ bending mode peaks observed at 679/676 and 748/746 cm^{-1} , and calculated at 670/670 and 753/750 cm^{-1} (**Figure 3**). These were the focus of previous studies because they characterize the terminal iron hydride bonding and its interactions with surroundings.²⁰⁻²² The two main features arise from relatively pure H_h hydride bending motion perpendicular to and parallel to the plane defined by the $\text{Fe}_p\text{---Fe}_d$ axis and the $\text{Fe}_d\text{-H}_h$ bond, respectively. Although the isotope-dependent shifts in these bands are small and nearly unmeasurable, fine structure of the underlying normal modes displays a difference. In the current DFT analysis there are



1-¹³CD-1-CrHydA1 ‘perpendicular’ modes at 670/665,671 cm^{-1} , and ‘parallel’ modes at 752,754/749,758 cm^{-1} respectively. The ¹³CD-labeling introduces $\text{N}_{\text{ADT}}\text{-H}_{\text{ADT}}$ bending admixtures to the $\text{Fe}_d\text{-H}_h$ modes, where the H_{ADT} and H_h nuclei displace either in- or out-of-phase. E.g. the 758 cm^{-1} ‘parallel’ mode (**Figure 3**) brings H_h and H_{ADT} closer during half of each excursion cycle. The calculations suggest an increased involvement of the heavier ¹³CD-ADT fragment in the $\text{Fe}_d\text{-H}_h$ bends, with rotations of the two – ¹³CD₂– methylene groups contributing at least 16% to the vibrational kinetic energy. Similar modes are calculated in the ADT-labeled ¹³CD-1 precursor in the ~670–770 cm^{-1} region, while the unlabeled (¹²CH-)1 variant

Figure 3. ⁵⁷Fe-PVDOS for the H_{hyd} state isotopologues 1-CrHydA1 (blue) vs. ¹³CD-1-CrHydA1 (red) from (a) NRVS experiment and (b) DFT calculations. Sticks correspond to individual DFT normal mode energies and intensities before broadening. For ¹³CD-1-CrHydA1, important band positions are labeled and atomic motions in selected normal modes are shown. Only the $[\text{2Fe}]_h$ and C169 fragments of the DFT model are shown with the methylene, H_h , and H_{ADT} hydrogen nuclei displacements indicated by red arrows.

produces their counterparts at frequencies only above 800 cm^{-1} (**Table S2**).

The DFT analysis therefore indicates that some modes in the Fe_d–H_h bending region involve mixing with motions inherent to the ¹³CD-labeled ADT ligand. A search for such ‘satellite’ modes is what initially prompted our isotopic labelling investigation. The experimental data might show weak ‘satellite’ features on either side of the main Fe_d–H_h bending peaks (**Figure 3**). Yet, despite prolonged data collection in this region to improve the signal-to-noise (S/N) ratio, firm assignment of the small differences to ‘satellites’ is not yet possible. The exact calculated energies of the ‘satellites’ should also be taken with caution, because they are governed by motion of a very light H_h nucleus that mediates interaction between ⁵⁷Fe_d and the ADT bridgehead. Further experimental insight into these modes will require significantly higher NRVs photon flux, which may be available in the next generation of synchrotron sources, e.g. PETRA-IV.³⁷

The accuracy of the DFT calculations at reproducing the experimental NRVs spectra of the unlabeled and isotopically labelled precursor and [2Fe]_H, here and in our previous work,^{20–22, 36} gives us confidence that it is valuable to consider the predicted ‘satellite’ modes in H_{hyd}, whether or not they can be conclusively detected by NRVs. Illustrations of these ‘satellite’ modes at **720** and **765** cm^{–1} are included in **Figure 3**. The latter two modes involve ‘parallel’ Fe_d–H_h bending, similar to the **749** and **758** cm^{–1} modes. Interestingly, some of these modes involve motion of the nearby cysteine at the end of the proton transfer channel leading to the ADT ligand. These vibrational modes appear to represent a pathway for coupled proton transfer from (C169)S_C–H_C to N_{ADT} and from N_{ADT}–H_{ADT} to Fe_d–H_h.

Are any other modes relevant to H₂ production catalysis? We inspected the DFT calculations for changes in H_{ADT}···H_h and N_{ADT}···H_C distances that occur during normal mode displacements, see **Figure S3**. The results for the modes with the greatest distance changes are summarized in **Table S1**. The equilibrium 2.06 Å H_{ADT}···H_h distance is already firmly in the 1.7–2.2 Å range for a ‘dihydrogen bond’,³⁸ and it is similar to the 2.02 Å value seen as the shortest H···H distance in solid BH₃NH₃.³⁹ We found that a few modes contribute a disproportionate amount of motion involving the H_{ADT}···H_h distance as well as the N_{ADT}···H_C distance. In particular, the ‘parallel’ Fe_d–H_h bending modes at 752/758 cm^{–1} yield the record ~0.14/0.15 Å contractions in the H_{ADT}···H_h distance across the entire vibrational spectra. For the N_{ADT}···H_C distance, the largest vibrational contraction of ~0.11 Å is achieved in the S_C–H_C stretching mode calculated at 2449/2449 cm^{–1}.

From time-resolved photochemical IR studies, Sanchez *et al.* have shown that the decay of H_{hyd} is kinetically competent as a near-final step in the [FeFe] hydrogenase catalytic cycle.⁴⁰ However, since the pK_a for a neutral secondary amine such as the ADT bridgehead nitrogen is extremely high, an intervening protonated ADT –NH₂⁺– intermediate, H_{hyd}H⁺, has often been included in the catalytic cycle.^{17, 24, 41–44} Our results, which document the role of ADT flexibility in normal modes that bring H_{ADT} and H_h closer together, offer the possibility of a mechanism update.

In this speculative scenario, high-frequency modes such as at 752/758 and 2449/2449 cm^{–1}, combined with low-frequency modes such as at 73 cm^{–1}, would involve coordinated motion of H_{ADT} toward H_h, while H_C moves toward N_{ADT}. This might precipitate a ‘deep tunneling’ transfer of H_{ADT} to H_h, while S_C–H_C transfer replenishes the N_{ADT}–H_A, and with the S_C–H_C proton reloaded from the H₂O in the proton transfer chain. Champion and coworkers have shown that deep tunneling can allow high pK_a residues to participate in proton transfer chains, as invoked for a serine residue in green fluorescent protein.⁴⁵ If the transfer reaction for H_{hyd} were facilitated by electron transfer from the [4Fe–4S]_H⁺ to the [Fe^{II}Fe^{II}]_H subsite, the overall PCET reaction would yield an H_{ox} electronic state with bound H₂. This scenario agrees with calculations on the reverse reaction of H₂ activation by Greco *et al.*⁴⁶

In summary, we have investigated vibrations of the [FeFe] hydrogenase active site in the H_{hyd} state through ⁵⁷Fe, ¹³C, and D isotopic labeling, combined with ⁵⁷Fe NRVs measurements and DFT calculations. This represents the first observation of 2nd and 3rd coordination sphere isotope effects using NRVs. We identified normal modes involving the flexing of the bridging ADT ligand that point to its unique properties as an active site ligand. The combined motions of the Fe_d–H_h, N_{ADT}–H_{ADT}, and (C169)S_C–H_C protons are presumably coupled to the remainder of the proton transfer chain as well as electron transfer. These effects may be important for catalysis and will be investigated in future studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental and computational procedures, supplementary figures and tables (PDF) Animated vibrational modes of the precursor and H_{hyd} DFT models as GIF files (ZIP) Coordinates of the precursor, H_{hyd}, and H_{ox} DFT models as XYZ files (ZIP)

AUTHOR INFORMATION

Corresponding Authors

James A. Birrell – orcid.org/0000-0002-0939-0573

Email: james.birrell@cec.mpg.de

Thomas B. Rauchfuss – orcid.org/0000-0003-2547-5128

Email: rauchfuz@illinois.edu

Stephen P. Cramer – orcid.org/0000-0002-3751-7623

Email: scramer@seti.org

Authors

Vladimir Pelmeshnikov – orcid.org/0000-0002-0523-4418

Leland B. Gee – orcid.org/0000-0002-5817-3997
 Casseday P. Richers – orcid.org/0000-0002-1324-0447
 Edward J. Reijerse – orcid.org/0000-0001-9605-4510
 Hongxin Wang – orcid.org/0000-0002-7325-0161
 Simon Arragain – orcid.org/0000-0002-6500-0066
 Nakul Mishra – orcid.org/0000-0003-4382-933X
 Yoshitaka Yoda – orcid.org/0000-0003-3062-8651
 Hiroaki Matsuura – orcid.org/0000-0002-8659-9952
 Lei Li – orcid.org/0000-0001-7909-6487
 Kenji Tamasaku – orcid.org/0000-0003-1440-0518
 Wolfgang Lubitz – orcid.org/0000-0001-7059-5327

Notes

The authors declare no competing financial interest.

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