Published in final edited form as:

J Am Chem Soc. 2017 November 22; 139(46): 16894–16902. doi:10.1021/jacs.7b09751.

Reaction Coordinate Leading to H₂ Production in [FeFe]-Hydrogenase Identified by NRVs and DFT

Vladimir Pelmeshnikov^{1,†}, James A. Birrell^{2,†}, Cindy C. Pham^{3,†}, Nakul Mishra³, Hongxin Wang³, Constanze Sommer², Edward Reijerse², Casseday P. Richers⁴, Kenji Tamasaku⁵, Yoshitaka Yoda⁵, Thomas B. Rauchfuss⁴, Wolfgang Lubitz², and Stephen P. Cramer³

¹Institut für Chemie, Technische Universität Berlin, Strasse des 17 Juni 135, 10623 Berlin, Germany

²Max-Planck-Institut für Chemische Energiekonversion, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany

³Department of Chemistry, University of California, Davis, One Shields Ave, Davis, CA 95616, USA

⁴School of Chemical Sciences, University of Illinois, 600 S. Mathews Ave, Urbana, IL 61801, USA

⁵JASRI, 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 ⁵⁷Fe 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, with 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 H₂ formation. The observed vibrational spectrum, therefore, provides mechanistic insight into the reaction coordinate for H₂ bond formation by [FeFe]-hydrogenases.

Graphical abstract

Correspondence to: Stephen P. Cramer.

[†]These three authors contributed equally to this work.

Supporting Information: Supplementary FTIR, NRVs, and DFT figures and tables; further details on the DFT models; coordinates of the DFT models as XYZ files; animated vibrational normal modes as GIF files. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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 ($>10,000 \text{ s}^{-1}$ 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 $[2\text{Fe}]_{\text{H}}$ and iron-sulfur $[4\text{Fe}-4\text{S}]_{\text{H}}$ subclusters (Figure 1). The Fe_{p} and Fe_{d} sites of $[2\text{Fe}]_{\text{H}}$, respectively proximal and distal to $[4\text{Fe}-4\text{S}]_{\text{H}}$, are 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.⁴⁻⁶ Fe_{d} , the site at which hydrogen binds, is the Lewis acid.^{7,8} Many previous experimental and theoretical studies⁹⁻¹² implicate a $\text{Fe}_{\text{d}}\text{-H}_{\text{h}}\cdots\text{H}_{\text{A}}\text{-N}_{\text{ADT}}$ species as the key intermediate leading to H-H bond formation (Figure 1A). Despite its mechanistic importance, however, experimental characterization of such HLH 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* (*CHydA1*) and *Desulfovibrio desulfuricans* (*DdHydAB*) both exhibit species with these key $\text{Fe}_{\text{d}}\text{-H}_{\text{h}}\cdots\text{H}_{\text{A}}\text{-N}_{\text{ADT}}$ interactions. These turn-over 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 *CHydA1/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 $[4\text{Fe}-4\text{S}]_{\text{H}}$, vary significantly between organisms. For example, *CHydA1* lacks accessory clusters altogether, while *DdHydAB* contains two additional $[4\text{Fe}-4\text{S}]$ clusters, and the bidirectional hydrogenase from *Clostridium pasteurianum* (*CpHydA1*) contains one $[2\text{Fe}-2\text{S}]$ and three $[4\text{Fe}-4\text{S}]$ 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 *CHydA1*,^{7,17} whereas C298D of *CaHydA* retains significant activity.¹⁸ Modification of the proton transfer pathway by

artificial maturation with complexes that substitute the –NH– ADT bridgehead with –O– (ODT) or –CH₂– (PDT) also causes nearly complete loss of activity.^{1,19}

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 H_{hyd}.^{7,17} The H_{hyd} state is thought to feature a reduced [4Fe-4S]_H⁺ subcluster and a homovalent Fe_p(II)Fe_d(II) binuclear [2Fe]_H site, with a hydride H_h bound in the apical position on Fe_d.²⁰

In previous work, we used Nuclear Resonance Vibrational Spectroscopy (NRVS) to examine the [2⁵⁷Fe-ODT]_H-C_HHydA1 H_{hyd} 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 of [FeFe]-hydrogenase with [2⁵⁷Fe]_H-labeling makes the technique completely selective for the normal modes of the subcluster.²⁵⁻²⁷ For the [2⁵⁷Fe-ODT]_H-C_HHydA1 H_{hyd} 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 terminal hydride Fe_d-H_h bending modes. The corresponding red-shifted Fe_d-D_h modes were associated with the perturbed Fe_{d/p}-CO bands at 625 and 564 cm⁻¹ under D₂/D₂O conditions.²¹ The Fe_d-H_h vibrations at 670 and 727 cm⁻¹ were assigned, respectively, to H⁻ motion perpendicular to and within the approximate mirror symmetry plane of [2Fe]_H, which passes through Fe_p, Fe_d, and H_h (Fe_pFe_dH_h plane). Here we focus on the NRVS spectra for the H_{hyd} species from C_HHydA1 and D_dHydAB artificially matured 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 Fe_d-H_h center. These remote effects include hydrogen bonding between Fe_d-H_h and ADT and between ADT and Cys169/178, the very sites that were modified in previous studies that demonstrated the existence of iron hydrides.^{21,28} 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

H_{hyd} State Accumulation

The H_{hyd} states of the catalytically active [2⁵⁷Fe]_H-C_HHydA1 and [2⁵⁷Fe]_H-D_dHydAB enzymes were stabilized at low pH values (pH 6 and pH 4, respectively) and with high concentrations of a low potential reductant (sodium dithionite).²⁹ These turn-over conditions give high steady state levels of H_{hyd}, 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 H_{sred}H⁺ state is rate limiting. Protonation at low pH, of the amino acids on the proton transfer pathway, increases the rate of proton transfer from the nitrogen base to the open coordination site on Fe_d forming the H_{hyd} 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 Fe_d in the oxidized state reforming the H_{hyd} state.

FTIR Spectroscopy

The FTIR spectra for the H_{hyd} state in both [2⁵⁷Fe]_H-C_HHydA1 and [2⁵⁷Fe]_H-D_dHydAB under H₂/H₂O and D₂/D₂O (Figure 2) are very similar to those observed for [2⁵⁷Fe-ODT]_H-C_HHydA1.²¹ 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 Fe_d-H_h vibration.²¹ 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.^{14,30,31} The H_{hyd} state in [2⁵⁷Fe]_H-C_HHydA1 was formed in high stoichiometric yield (~90%) and appeared to be very stable (Figure S1A). In D_dHydAB, however, lower stability of the H_{hyd} state (Figure S1B) due to the much higher activity of this enzyme (~10000 s⁻¹ compared with ~1000 s⁻¹ for C_HHydA1) gave slightly lower yields (~50%) of the H_{hyd} state.^{32,33} The major impurities were the H_{ox} state and a protonated form of the H_{ox} state (termed H_{ox}-blue).^{29,34} The Mössbauer (Figure S2A) and EPR (Figure S2B) spectra of C_HHydA1 in the H_{hyd} state under H₂/H₂O are very similar to those previously published for both wild type C_HHydA1 and the C169S mutant.²⁸

NRVS Spectroscopy

NRVS spectra for the two [FeFe]-hydrogenases under H₂/H₂O and D₂/D₂O conditions are illustrated in Figure 3A. Features in the 150–400 cm⁻¹ range are primarily due to Fe-S 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 are modes mostly of 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.²¹

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 H_{hyd} state. However, to our initial surprise, the two main hydride bands for the enzyme spectra are upshifted vs those observed for [2⁵⁷Fe-ODT]_H-C_HHydA1 (Figure S3).²¹ For both proteins, the [2⁵⁷Fe-ODT]_H 670 cm⁻¹ band is upshifted by 5 cm⁻¹ to 675 cm⁻¹, while the [2⁵⁷Fe-ODT]_H 727 cm⁻¹ band upshifts by 17/20 cm⁻¹ to 744/747 cm⁻¹ for [2⁵⁷Fe]_H-C_HHydA1/D_dHydAB. 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_d, 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,^{14,30,31} structural studies are lacking for the enzyme with a bound hydride. Four schematic alternatives on H_{hyd} based on earlier proposals²¹ are shown in Figure 5F. Two of them, designated $H_{\text{hyd-A}}$ and $H_{\text{hyd-E}}$, assume an uncharged bridgehead amino group, in either ‘axial’ $-N_{\text{ADT}}H_A-$ (H_A pointing toward Fe_d) or ‘equatorial’ $-N_{\text{ADT}}H_E-$ (H_E pointing away from Fe_d) conformation. The other two alternatives $H_{\text{hyd}}^*H^+$ and $H_{\text{hyd}}H^+$ assumed a protonated $-N_{\text{ADT}}H_2^+-$ form wherein both $H_{A/E}$ locations are occupied, and the $[4Fe-4S]_H$ - $[2Fe]_H$ subclusters in either $[4Fe-4S]^+-Fe_p(II)Fe_d(II)$ or $[4Fe-4S]^{2+}-Fe_p(I)Fe_d(II)$ oxidation levels.

DFT modeling of the H_{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 $[2Fe]_H$ subcluster and the Cys421/382 side chain that coordinates Fe_p (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 $[2^{57}\text{Fe-ODT}]_H\text{-CHydA1}$.²¹ As shown in Figure S10, the *S* modeling of the four H_{hyd} states produced a significantly variable distribution of the $Fe_d\text{-}H_h$ bands. Only *S*- $H_{\text{hyd-A}}$ with its $Fe_d\text{-}H_h$ modes at 668 and 764 cm^{-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 $[4Fe-4S]_H$ subcluster and the protein environment surrounding the H-cluster play crucial roles in the enzyme's properties,¹⁶ and H-bonding between ADT protons and the neighboring Cys169/178 residue are also important. We, therefore, proceeded to explore the four H_{hyd} alternatives using a ‘*Large*’ modeling level *L* that, beyond *S*, integrated protein side chains surrounding the $[2Fe]_H$ subcluster (see Figures 4A and B, S4 and S6). A second large model, labelled *L'* and additionally included the $[4Fe-4S]_H$ subcluster, was evaluated (see Figure S7).

The 600-900 cm^{-1} ‘active window’ is uniquely sensitive to Fe-H/D bending motions. As shown in Figure 5A-E, model *L*- $H_{\text{hyd-A}}$ is the only simulation that reasonably replicates the positions and intensities of the two observed Fe-H bands. The other *L*- H_{hyd} models predict either too many bands (*L*- $H_{\text{hyd-E}}$), only a single band (*L*- $H_{\text{hyd}}^*H^+$), or hardly any bands (*L*- $H_{\text{hyd}}H^+$), with intensity levels above the experimental noise. Interestingly, the total intensity concentrated in the calculated $Fe_d\text{-}H_h$ bands decreases in the sequence

$H_{\text{hyd-E}} > H_{\text{hyd-A}} > H_{\text{hyd}}^*H^+ > H_{\text{hyd}}H^+$, which correlates with the strength of the ADT-hydride interaction, as reflected by the $H_h\cdots H_A$ distances collected in Table S3. Only for the $H_{\text{hyd-E}}$ alternative that lacks the H_A proton, modeling *S* and *L* predict essentially the same set of $Fe_d\text{-}H_h$ bands (Figure S10C). For other H_{hyd} alternatives the $H_h\cdots H_A$ 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*- $H_{\text{hyd-A}}$ model leads to an improvement in the higher

frequency $\text{Fe}_d\text{-H}_h$ mode position by 15 cm^{-1} (764 in $S\text{-H}_{\text{hyd-A}}$ and 749 in $L\text{-H}_{\text{hyd-A}}$, vs $744/749\text{ cm}^{-1}$ observed, see Figure S10B). We note also that our preference for $\text{H}_{\text{hyd-A}}$ is supported by its $\approx 6\text{-}9\text{ kcal/mol}$ lower energy vs $\text{H}_{\text{hyd-E}}$ (Table S5). Simulations for the entire spectral range reported in Figures S9 and S11-S14 provide additional support for $\text{H}_{\text{hyd-A}}$, and unequivocally indicate the benefits of the large DFT models for reproducing NRVs bands $<400\text{ cm}^{-1}$.

The motion of the various nuclei that is the source of the calculated intensities for the $L\text{-H}_{\text{hyd-A}}$ bands (Figure 5B) 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_h motion normal to the $\text{Fe}_p\text{Fe}_d\text{H}_h$ plane. In contrast, the three higher frequency modes are in-plane H_h 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: $[\text{H}_h\downarrow, \text{H}_A\downarrow, \text{H}_C\downarrow]$ at 749 , $[\text{H}_h\uparrow\text{H}_A\downarrow, \text{H}_C\downarrow]$ at 766 , and $[\text{H}_h\uparrow\text{H}_A\downarrow, \text{H}_C\uparrow]$ at 788 cm^{-1} . Among the in-plane modes, only the one at 749 cm^{-1} has sufficient ^{57}Fe motion to be detected in the NRVs experiment and associated with the H_{hyd} band observed at $744/747\text{ cm}^{-1}$. Transition from the S to L modeling for the $\text{H}_{\text{hyd-A}}$ spectral simulations reveals that the *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 $[\text{}^{257}\text{Fe-ODT}]_H$ vs $[\text{}^{257}\text{Fe}]_H$ protein systems on the two Fe-H band shifts (see the NRVs section above); only in the $[\text{}^{257}\text{Fe}]_H$ systems the $\text{H}_h\cdots\text{H}_A$ interaction is present, and the higher energy $\text{Fe}_d\text{-H}_h$ mode becomes delocalized.

Mechanistic Proposal

The details of the catalytic mechanism of $[\text{FeFe}]$ -hydrogenases have been debated for decades.^{1,8,11,12,35} Past proposals for the catalytic mechanism can be divided into one set involving a bridging hydride between Fe_p and Fe_d ³⁶ (Mechanism I as described by Trohalaki and Pachter¹¹) and another set involving a terminal hydride on Fe_d (Mechanism II).³⁵ We see no evidence that H_{hyd} involves a bridging hydride. Notably, in contrast to the $[\text{}^{257}\text{Fe}]_H\text{-CtHydA1/DdHydAB}$ samples and the best-fit $\text{H}_{\text{hyd-A}}$ state producing *two* (Fe-H bending) bands in the NRVs ‘active window’ (Figure 5A and B), bridging $\text{Ni(II)}(\mu\text{-H})^{57}\text{Fe(II)}$ hydrides in $[\text{NiFe}]$ -hydrogenase and a model complex produced only a *single* (Ni-H-Fe wagging) band.³⁷ A qualitatively similar result displaying strong deviations to the observed H_{hyd} spectra is obtained from a $^{57}\text{Fe}_p(\text{II})(\mu\text{-H})^{57}\text{Fe}_d(\text{II})$ DFT candidate called $L\text{-H}_{\mu\text{-hyd-A}}$ (see Figures S8 and S15, and Supplementary Discussion in Supporting Information). Other reports also disfavor bridging hydride-based mechanisms on a variety of grounds.^{12,38} 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_d hydride is the key step in H_2 formation.

We note that model $L\text{-H}_{\text{hyd-A}}$ predicts a $\text{H}_h\cdots\text{H}_A$ distance of $2.01\text{ \AA}$ (Table S3), a similar Fe-H $\cdots$ H-N distance of $1.88\text{ \AA}$ observed in the $[(\text{H})\text{Fe}_2(\text{adt-NH}_2)(\text{CO})_2(\text{dppv})_2](\text{BF}_4)_2$ complex

by X-ray crystallography.⁹ This distance is still quite a bit longer than the ≈ 1.5 Å H $\cdots$ H distance reported by Bullock and coworkers for a strong Fe-H $\cdots$ H-N dihydrogen interaction.¹⁰ The long Fe-H $\cdots$ H-N distance in the complex is however a consequence of hydrogen bonding to the BF₄⁻ 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 H₂ formation. H₂ production from state H_{hyd-A} requires transfer of the amino proton to subsequently yield an Fe_d-H₂ complex (see Figure 6B). This process requires assistance from the proton transport chain, either to first produce -NH₂⁺- (or to transfer H⁺ in a concerted mechanism). DFT modeling *L*' including both [2Fe]_H and [4Fe-4S]_H subclusters indicates that protonation of H_{hyd-A} yields an [4Fe-4S]²⁺-Fe_p(I)Fe_d(II) species H_{hyd}H⁺, and the [4Fe-4S]⁺-Fe_p(II)Fe_d(II) alternative *H*_{hyd}^{*}H⁺ is disfavored (see Figure 5F, Table S4, and Supplementary Discussion in Supporting Information).

For the next step, Bullock and coworkers have proposed that -NH₂⁺-/Fe-H and -NH-/Fe-H₂ species exist in rapid equilibrium, both in their model complex and in [FeFe]-hydrogenases.¹⁰ Our calculations for model *L*-H_{hyd}H⁺, which posits an Fe_p(I)Fe_d(II) redox level and -NH₂⁺- / Fe-H coordination, finds an H_h $\cdots$ H_A distance of 1.38 Å (Table S3), even shorter than in the Bullock complex, and it is thus reasonable that a similar -NH₂⁺- / Fe-H $\rightleftharpoons$ -NH- / Fe-H₂ equilibrium would occur. We propose that protonation of H_{hyd-A}, in concert with electron transfer from [4Fe-4S]_H to [2Fe]_H, yields H_{hyd}H⁺, which can directly yield an Fe-H₂ species. Capturing this dihydrogen complex experimentally is an excellent target for future studies.

Conclusions

The key hydride intermediate in [FeFe]-hydrogenase catalysis has been characterized for the first time in an unperturbed [FeFe]-hydrogenase. The NRVS vibrational data are consistent with a terminal hydride at the distal iron of the [2Fe]_H subsite interacting with the amine proton of the azadithiolate cofactor. As indicated by the diversity of the DFT-simulated spectra, the Fe_d-H_h center in the [FeFe]-hydrogenases is exquisitely sensitive to its environment. The protonation states of the amine and its interaction with the cysteine residue at the end of the proton supply chain, as well as the redox levels of the [2Fe]_H and [4Fe-4S]_H subclusters are shown to strongly influence the Fe_d-H_h bending vibrations. Rationalized by theory, distinctive ⁵⁷Fe-H/D spectroscopic signatures from NRVS are therefore anticipated to serve diagnostic in other Fe-containing enzymes and molecular systems. Although iron hydrides have been detected previously in variants of the [FeFe]-hydrogenases, the present work gives the first structure for the enzyme-substrate complex in a catalytically competent state. Furthermore, the structure of this hydride intermediate paints a coherent picture for the sequence of events leading to evolution of H₂.

Methods

Sample Preparation

[²⁵⁷Fe]_H-CHydA1 and [²⁵⁷Fe]_H-DdHdAB were prepared as described previously,^{27,40} but using an ⁵⁷Fe-labelled azadithiolate cofactor.²⁵ All sample preparation steps were performed in an anaerobic glovebox (Coy) under 2% H₂ (or D₂)/98% N₂. Samples of [²⁵⁷Fe]_H-

CHydA1 (60 μ L of 3 - 5 mM) in pH 6 buffer (50 mM MES, 50 mM HEPES, 150 mM NaCl, either in H₂O or D₂O) and [⁵⁷Fe]_H-*DdHydAB* (60 μ L of 2 - 4 mM) in pH 4 buffer (100 mM sodium acetate, 150 mM NaCl, either in H₂O or D₂O) were reduced with 100 mM sodium dithionite, 50 μ L were transferred to each NRVS cell and frozen in liquid nitrogen, and the remaining 10 μ L were loaded into an FTIR cell and measured within 5 min.

Fourier-Transform Infrared Spectroscopy

FTIR spectra were collected using a Bruker IFS 66v/S FT-IR spectrometer equipped with a liquid nitrogen cooled Bruker mercury cadmium telluride (MCT) detector. Measurements were performed at room temperature in the double-sided, forward-backward mode with a resolution of 2 cm⁻¹, an aperture setting of 1.5 mm and a scan velocity of 20 Hz. Data were processed using home-written routines in the MATLAB™ platform.

Nuclear Resonance Vibrational Spectroscopy

NRVS spectra for [⁵⁷Fe]_H-*CHydA1* and [⁵⁷Fe]_H-*DdHydAB* were recorded at SPring-8 BL09XU⁴¹ and BL19LXU.⁴² BL09XU uses a Si(111) double crystal in a high heat load monochromator (HLM) to produce 14.414 keV radiation with ≈ 1.0 eV resolution, followed by a high energy resolution monochromator (HRM) [Ge(422) $\times$ 2Si(975)] to increase the resolution to 0.8 meV. The beam flux was $\sim 2.5 \times 10^9$ photons/s and the beam size was about 0.6 (height) $\times$ 1 (width) mm². A 2 $\times$ 2 avalanche photodiode (APD) detector array was used to collect the delayed nuclear fluorescence and the K α fluorescence following nuclear excitation. The temperature at the base of the sample was maintained at 10 K with a LHe cryostat. The Stoke/anti-Stoke imbalance derived real sample temperatures were 40-70 K.⁴³

BL19LXU⁴² provides the fundamental beam at 7.2-18 keV energy region, covering ⁵⁷Fe nuclear resonance at 14.414 keV. Its 25-m undulator produces about 5 $\times$ higher beam flux at HLM than that at BL09XU. Therefore, it is a good choice for NRVS measurements on weak vibrational features. BL19LXU is not a dedicated nuclear scattering beamline, and a similar HMR and associated NRVS measurement system was integrated into the BL19LXU's experimental hutch during the first 36-48 h of each beamtime. Therefore, BL19LXU provided 14.414 keV radiation with 0.8 meV resolution, with an average photo flux of $\sim 5.4 \times 10^9$ photons/s. The beam size was also 0.6 $\times$ 1 mm². The delayed signal was also measured with a 2 $\times$ 2 APD array. The real sample temperatures were also 40-70 K.

NRVS spectral analysis was performed using the PHOENIX software package⁴⁴ executed through spectraltools.⁴⁵ The energy scales were calibrated with a standard sample of [NEt₄][⁵⁷FeCl₄] with a prominent peak at 380 cm⁻¹.

Since the relative strength of NRVS transitions varies dramatically and since it is necessary to emphasize one region of interest (*e.g.* for searching for Fe-H-Fe or X-Fe-H), the scans were divided into segments with different data collection times (second per point, or s/p). In general, 1 s/p was used for the range from -240 to 400 cm⁻¹ (covering the Fe-S region), then 5-10 s/p. for the Fe-CN and Fe-CO region from 400-620 cm⁻¹. A longer scanning time (16-23 s) was used for the Fe-H or X-Fe-H region (*e.g.* at 640-800 cm⁻¹). The scan ranges are all relative about the resonance energy.

DFT Calculations

The density functional theory (DFT) methodology presently applied to the [FeFe]-hydrogenase H-cluster modeling is mostly equivalent to that employed earlier.²¹ The structural optimizations and subsequent normal mode calculations were performed using GAUSSIAN 09 Revision D.01⁴⁶ based on the densities exported from single point calculations performed by JAGUAR 9.4²⁶ that provided high-quality initial guess. The BP86^{47,48} functional and the LACV3P** basis set as implemented in JAGUAR were employed, unless otherwise mentioned. For the first- and second-row elements, LACV3P** implies 6-311G** triple- ζ basis sets including polarization functions. For the Fe atoms, LACV3P** consists of a triple- ζ 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 (IEF-PCM)⁴⁹ as implemented in GAUSSIAN 09, with the static dielectric constant set to $\epsilon = 4.0$ 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 non-hybrid PBE,⁵⁰ hybrid PBE0,⁵¹ and hybrid B3LYP⁵²⁻⁵⁵ 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,⁵⁶ as well as (*iii*) D3 reformulated with Becke-Johnson damping.⁵⁷ Optimal results for reproducing the observed NRVS spectra were found from using scheme (*i*) for modeling levels *S* and *L* > 400 cm⁻¹, and from using scheme (*ii*) for modeling level *L*' < 400 cm⁻¹; the H-cluster modeling levels *S/L/L*' are explained in section 'Model Setup' of the Supporting Information and Table S1, and shown in Figures 4A and S4.

DFT Spectra Simulation

DFT-based vibrational data (Figures 5B-E, S9-S15) were generated using selective [⁵⁷Fe]_H subcluster labeling as employed in the NRVS experiment. Simulation of the deuterated samples, referred in the text as 'D-variants', introduced additional H-to-D substitutions applied to hydrogen nuclei labeled as H_B/H_A/H_E/H_C throughout the manuscript. The ⁵⁷Fe-PVDOS and hydride-only H⁻/D⁻-PVDOS intensities were extracted from normal mode outputs using an in-house Q-SPECTOR program, successfully applied earlier.^{21,37,58-62} To account for the resolution of the present NRVS experiment, computed ⁵⁷Fe-PVDOS intensities were broadened by convolution with a full width at half maximum (FWHM) = 14 cm⁻¹ Lorentzian. Homogeneous empirical scaling of the calculated frequencies by 96 % was applied to modeling levels *S* and *L*, and no frequency scaling has been applied (100 %) to modeling level *L*'. Based on the approach described in Supplementary Discussion of the Supporting Information, the representative large model full-range spectra (Figures S9, S11-S14) combined ⁵⁷Fe-PVDOS from modeling levels *L* > 400 cm⁻¹ and *L*' < 400 cm⁻¹ and referred to as '*L*-modeling'. This combined approach provided essentially unambiguous

mapping between the ^{57}Fe -PVDOS features observed for the protein samples and calculated for the best-fit state $\text{H}_{\text{hyd-A}}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH GM-65440 (S.P.C.), NIH GM-GM-61153 (T.B.R.), the Cluster of Excellence “Unifying Concepts in Catalysis” initiative of DFG (V.P.), and the Max Planck Society. NRVS data collection was supported by the following proposals to SPring-8: [2015B1134], [2016B1347], [2016A1154], long term proposals at BLXU09: [2015A0103], [2015B0103], [2016A0103], and long-term proposals at BLXU19: [20140033], [20150048], [20160063].

References

1. Lubitz W, Ogata H, Rüdiger O, Reijerse E. Chem Rev. 2014; 114:4081. [PubMed: 24655035]
2. Peters JW, Schut GJ, Boyd ES, Mulder DW, Shepard EM, Broderick JB, King PW, Adams MWW. Biochim Biophys Acta. 2015; 1853:1350. [PubMed: 25461840]
3. Hatchikian EC, Magro V, Forget N, Nicolet Y, Fontecilla-Camps JC. J Bact. 1999; 181:2947. [PubMed: 10217791]
4. Silakov A, Wenk B, Reijerse E, Lubitz W. Phys Chem Chem Phys. 2009; 11:6592. [PubMed: 19639134]
5. Carr SB, Evans RM, Brooke EJ, Wehlin SAM, Nomerotskaia E, Sargent F, Armstrong FA, Phillips SEV. Biochem Soc Trans. 2016; 44:863. [PubMed: 27284053]
6. Kalz KF, Brinkmeier A, Dechert S, Mata RA, Meyer F. J Am Chem Soc. 2014; 136:16626. [PubMed: 25353322]
7. Mulder DW, Ratzloff MW, Bruschi M, Greco C, Koonce E, Peters JW, King PW. J Am Chem Soc. 2014; 136:15394. [PubMed: 25286239]
8. Schilter D, Camara JM, Huynh MT, Hammes-Schiffer S, Rauchfuss TB. Chem Rev. 2016; 116:8693. [PubMed: 27353631]
9. Carroll ME, Barton BE, Rauchfuss TB, Carroll PJ. J Am Chem Soc. 2012; 134:18843. [PubMed: 23126330]
10. Liu TB, Wang XP, Hoffmann C, DuBois DL, Bullock RM. Angew Chem Int Ed. 2014; 53:5300.
11. Trohalaki S, Pachter R. Int J Hyd En. 2010; 35:5318.
12. Finkelmann AR, Stiebritz MT, Reiher M. Chem Sci. 2014; 5:215.
13. Yang Z, Lasker K, Schneidman-Duhovny D, Webb B, Huang CC, Pettersen EF, Goddard TD, Meng EC, Sali A, Ferrin TE. J Struct Biol. 2012; 179:269. [PubMed: 21963794]
14. Nicolet Y, Piras C, Legrand P, Hatchikian CE, Fontecilla-Camps JC. Structure. 1999; 7:13. [PubMed: 10368269]
15. Hong G, Cornish AJ, Hegg EL, Pachter R. BBA-Bioenergetics. 2011; 1807:510. [PubMed: 21296047]
16. Knörzer P, Silakov A, Foster CE, Armstrong FA, Lubitz W, Happe T. J Biol Chem. 2012; 287:1489. [PubMed: 22110126]
17. Das R, Neese F, van Gastel M. Phys Chem Chem Phys. 2016; 18:24681. [PubMed: 27545687]
18. Morra S, Giraudo A, Di Nardo G, King PW, Gilardi G, Valetti F. PLOS One. 2012; 7
19. Siebel JF, Adamska-Venkatesh A, Weber K, Rumpel S, Reijerse E, Lubitz W. Biochemistry. 2015; 54:1474. [PubMed: 25633077]
20. Tal S, Chun TW, Gavini N, Burgess BK. J Biol Chem. 1991; 266:10654. [PubMed: 2037604]
21. Reijerse EJ, Pham CC, Pelmenschikov V, Gilbert-Wilson R, Adamska-Venkatesh A, Siebel JF, Gee LB, Yoda Y, Tamasaku K, Lubitz W, Rauchfuss TB, Cramer SP. J Am Chem Soc. 2017; 139:4306. [PubMed: 28291336]

22. Wang H, Alp EE, Yoda Y, Cramer SP. *Methods Mol Biol.* 2014; 1122:125. [PubMed: 24639257]
23. Scheidt WR, Durbin SM, Sage JT. *J Inorg Biochem.* 2005; 99:60. [PubMed: 15598492]
24. Seto M, Yoda Y, Kikuta S, Zhang XW, Ando M. *Phys Rev Lett.* 1995; 74:3828. [PubMed: 10058307]
25. Gilbert-Wilson R, Siebel JF, Pham CC, Adamska-Venkatesh A, Reijerse E, Wang H, Cramer SP, Lubitz W, Rauchfuss TB. *J Am Chem Soc.* 2015; 137:8998. [PubMed: 26091969]
26. Berggren G, Adamska A, Lambert C, Simmons TR, Esselborn J, Atta M, Gambarelli S, Mouesca JM, Reijerse E, Lubitz W, Happe T, Artero V, Fontecave M. *Nature.* 2013; 499:66. [PubMed: 23803769]
27. Esselborn J, Lambert C, Adamska-Venkatesh A, Simmons T, Berggren G, Nothl J, Siebel J, Hemschemeier A, Artero V, Reijerse E, Fontecave M, Lubitz W, Happe T. *Nature Chem Biol.* 2013; 9:607. [PubMed: 23934246]
28. Mulder DW, Guo Y, Ratzloff MW, King PW. *J Am Chem Soc.* 2017; 139:83. [PubMed: 27973768]
29. Winkler MSM, Duan J, Esselborn J, Wittkamp F, Hofmann E, Apfel UP, Stripp ST, Happe T. *Nat Commun.* 2017; 8:16115. [PubMed: 28722011]
30. Esselborn J, Muraki N, Klein K, Engelbrecht V, Metzler-Nolte N, Apfel UP, Hofmann E, Kurisu G, Happe T. *Chem Sci.* 2016; 7:959.
31. Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC. *Science.* 1998; 282:1853. [PubMed: 9836629]
32. Glick BR, Martin WG, Martin SM. *Can J Microbiol.* 1980; 26:1214. [PubMed: 7006765]
33. Happe T, Naber JD. *Eur J Biochem.* 1993; 214:475. [PubMed: 8513797]
34. Senger M, Mebs S, Duan J, Shulenina O, Laun K, Kertess L, Wittkamp F, Apfel UP, Happe T, Winkler M, Haumann M, Stripp ST. *Phys Chem Chem Phys.* 2018; Advance Article. doi: 10.1039/C7CP04757F
35. Siegbahn PEM, Tye JW, Hall MB. *Chem Rev.* 2007; 107:4414–4435. [PubMed: 17927160]
36. Zhou TJ, Mo YR, Liu AM, Zhou ZH, Tsai KR. *Inorg Chem.* 2004; 43:923. [PubMed: 14753812]
37. Ogata H, Kraemer T, Wang H, Schilter D, Pelmenschikov V, van Gastel M, Neese F, Rauchfuss TB, Gee LB, Scott AD, Yoda Y, Tanaka Y, Lubitz W, Cramer SP. *Nat Commun.* 2015; 6
38. Mebs S, Senger M, Duan J, Wittkamp F, Apfel UP, Happe T, Winkler M, Stripp ST, Haumann M. *J Am Chem Soc.* 2017; 139:12157. [PubMed: 28825810]
39. Huynh MT, Wang W, Rauchfuss TB, Hammes-Schiffer S. *Inorg Chem.* 2014; 53:10301. [PubMed: 25207842]
40. Birrell JA, Wrede K, Pawlak K, Rodriguez-Maciá P, Rüdiger O, Reijerse EJ, Lubitz W. *Isr J Chem.* 2016; 56:852.
41. Yoda Y, Yabashi M, Izumi K, Zhang XW, Kishimoto S, Kitao S, Seto M, Mitsui T, Harami T, Imai Y, Kikuta S. *Nucl Inst Meth A.* 2001; 467:715.
42. Yabashi M, Tamasaku K, Kikuta S, Ishikawa T. *Rev Sci Instrum.* 2001; 72:4080.
43. Wang HX, Yoda Y, Kamali S, Zhou ZH, Cramer SP. *J Synchrotron Rad.* 2012; 19:257.
44. Sturhahn W, Toellner TS, Alp EE, Zhang X, Ando M, Yoda Y, Kikuta S, Seto M, Kimball CW, Dabrowski B. *Phys Rev Lett.* 1995; 74:3832. [PubMed: 10058308]
45. Crouthers DJ, Ding SD, Denny JA, Bethel RD, Hsieh CH, Hall MB, Darensbourg MY. *Angew Chem Int Ed.* 2015; 54:11102.
46. Frisch, MJ., Trucks, GW., Schlegel, HB., Scuseria, GE., Robb, MA., Cheeseman, JR., Scalmani, G., Barone, V., Mennucci, B., Petersson, GA., Nakatsuji, H., Caricato, M., Li, X., Hratchian, HP., Izmaylov, AF., Bloino, J., Zheng, G., Sonnenberg, JL., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery, JA., Jr, Peralta, JE., Ogliaro, F., Bearpark, M., Heyd, JJ., Brothers, E., Kudin, KN., Staroverov, VN., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, JC., Iyengar, SS., Tomasi, J., Cossi, M., Rega, N., Millam, JM., Klene, M., Knox, JE., Cross, JB., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, RE., Yazyev, O., Austin, AJ., Cammi, R., Pomelli, C., Ochterski, JW., Martin, RL., Morokuma, K., Zakrzewski, VG., Voth, GA., Salvador, P., Dannenberg, JJ., Dapprich, S., Daniels, AD., Farkas, Ö., Foresman, JB., Ortiz, JV., Cioslowski, J., Fox, DJ. *Gaussian 09, Revision D.01.* Gaussian Inc.; Wallingford CT: 2009.
47. Becke AD. *Phys Rev A.* 1988; 38:3098.

48. Perdew JP. Phys Rev B. 1986; 33:8822.
49. Tomasi J, Mennucci B, Cammi R. Chem Rev. 2005; 105:2999. [PubMed: 16092826]
50. Perdew JP, Burke K, Ernzerhof M. Phys Rev Lett. 1996; 77:3865. [PubMed: 10062328]
51. Adamo C, Barone V. J Chem Phys. 1999; 110:6158.
52. Becke AD. J Chem Phys. 1993; 98:5648.
53. Lee CT, Yang WT, Parr RG. Phys Rev B. 1988; 37:785.
54. Vosko SH, Wilk L, Nusair M. Can J Phys. 1980; 58:1200.
55. Stephens PJ, Devlin FJ, Chabalowski CF, Frisch MJ. J Phys Chem-Us. 1994; 98:11623.
56. Grimme S, Antony J, Ehrlich S, Krieg H. J Chem Phys. 2010; 132:154104. [PubMed: 20423165]
57. Grimme S, Ehrlich S, Goerigk L. J Comput Chem. 2011; 32:1456. [PubMed: 21370243]
58. Schilter D, Pelmenschikov V, Wang H, Meier F, Gee LB, Yoda Y, Kaupp M, Rauchfuss TB, Cramer SP. Chem Commun. 2014; 50:13469.
59. Mitra D, George SJ, Guo YS, Kamali S, Keable S, Peters JW, Pelmenschikov V, Case DA, Cramer SP. J Am Chem Soc. 2013; 135:2530. [PubMed: 23282058]
60. Yan LF, Pelmenschikov V, Dapper CH, Scott AD, Newton WE, Cramer SP. Chem-Eur J. 2012; 18:16349. [PubMed: 23136072]
61. Pelmenschikov V, Guo YS, Wang HX, Cramer SP, Case DA. Faraday Discuss. 2011; 148:409. [PubMed: 21322496]
62. Mitra D, Pelmenschikov V, Guo YS, Case DA, Wang HX, Dong WB, Tan ML, Ichiye T, Jenney FE, Adams MWW, Yoda Y, Zhao JY, Cramer SP. Biochemistry. 2011; 50:5220. [PubMed: 21500788]

Figure 1.

(A) Schematic illustration of the [FeFe]-hydrogenase H-cluster proposed for the H_{hyd} state. The hydride H_H at the iron site of the [2Fe]_H subcluster Fe_d, distal to [4Fe-4S]_H, interacts with the ‘Axial’ proton H_A of the ADT bridgehead nitrogen N_{ADT}. The cysteine, shown here as –S_C–H_C, terminates the proton supply chain and is in contact with N_{ADT}. (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 grey arrows. Red and blue numbers correspond to the amino acid residues in *CtHydA1* and *DdHydAB*, respectively.

Figure 2.

FT-IR spectra of the H_{hyd} state. 10 μ L samples of 3 – 5 mM $[^{257}\text{Fe}]_H\text{-CrHydA1}$ at pH 6 (A) and 2 – 4 mM $[^{257}\text{Fe}]_H\text{-DdHydAB}$ at pH 4 (B) were reduced with 100 mM sodium dithionite and transferred to the FT-IR cells. The samples were prepared either in H_2O under a 2% H_2 atmosphere (upper spectra) or in D_2O under a 2% D_2 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: H_{ox} (purple), H_{ox}^{blue} (blue), H_{hyd} (pink), $H_{red}H^+$ (green), $H_{sred}H^+$ (orange), H_{ox-CO} (grey).

Figure 3.

NRVS spectra of [FeFe]-hydrogenases. (A) The overall NRVs for samples of $[2^{57}\text{Fe}]_{\text{H}}\text{-CrHydA1}$ (top) and $[2^{57}\text{Fe}]_{\text{H}}\text{-DdHydAB}$ (bottom) prepared under $\text{H}_2/\text{H}_2\text{O}$ (blue) and $\text{D}_2/\text{D}_2\text{O}$ (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 $\text{H}_2/\text{H}_2\text{O}$ (blue) and $\text{D}_2/\text{D}_2\text{O}$ (red) samples.

Figure 4.

DFT modeling of the H_{hyd} state alternatives. (A) The best-fit H_{hyd-A} state optimized at three different modeling levels S (purple tubes), L (green tubes) and L' (tubes in element colors) (see Supporting Information), overlaid with the X-ray crystal structure³⁰ of the *Cpl* [FeFe]-hydrogenase (grey wire frame). All protons except the mechanistically central protons (H_H , H_A and H_C) are omitted for clarity. The same structures with all protons included are shown in Figure S4. (B) Close view of the alternative H_{hyd} configurations H_{hyd-A} (green), H_{hyd-E} (blue), $H_{hyd-H^+}^*$ (yellow) and H_{hyd-H^+} (red) from L modeling, overlaid with the X-ray crystal structure (grey). Dashed lines indicate interatomic interactions $H_H \cdots H_A$, $NADT \cdots H_C$, and $S_C \cdots H_E$ within 3 Å as detailed in Table S3. For the complete L models view, see Figure S6.

Figure 5.

NRVS spectra in the 600-900 cm⁻¹ 'active window' region (A) for [²⁵⁷Fe]_H-CrHydA1/*DdHydAB* compared with spectra for the states (B) H_{hyd}-A, (C) H_{hyd}-E, (D) H_{hyd}H⁺ and (E) H_{hyd}H⁺ simulated using *L* modeling. Blue spectra are for the H-variants and red spectra are for the D-variants. Stick-style peaks in (B-E) are H_H/D_H-PVDOS (partial vibrational density of states) spectra that quantify the hydride-only contribution to the computed vibrational modes. The vertical dotted lines indicate the main peak positions in the observed NRVs spectra. The right panel (F) shows a schematic view of the H_{hyd} variants, placed into the context of the [FeFe]-hydrogenase mechanism.

Figure 6.

DFT-calculated normal modes and possible mechanism of H₂ formation in [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 5B). Contributions from the H_h and ⁵⁷Fe nuclei to the vibrational energies are given. (B) Proposed sequence of events leading to H₂ formation: H_{hyd} becomes protonated leading to H_{hyd}H⁺, and subsequently bond formation occurs giving the H₂ product complex. H₂ release generates the H_{ox} state of the enzyme, which can be reduced and protonated to form H_{hyd} again.