How [FeFe]-Hydrogenase Facilitates Bidirectional Proton Transfer

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Hydrogenases are metalloenzymes that catalyse the interconversion of protons and molecular hydrogen, H2. [FeFe]-hydrogenases show particularly high rates of hydrogen turnover and have inspired numerous compounds for biomimetic H2 production. Two decades of research on the active site cofactor of [FeFe]-hydrogenases have put forward multiple models of the catalytic proceedings. In comparison, understanding of the catalytic proton transfer is poor. We were able to identify the amino acid residues forming a proton transfer pathway between active site cofactor and bulk solvent; however, the exact mechanism of catalytic proton transfer remained inconclusive. Here, we employ in situ IR difference spectroscopy on the [FeFe]-hydrogenase from Chlamydomonas reinhardtii evaluating dynamic changes in the hydrogen-bonding network upon catalytic proton transfer. Our analysis allows for a direct, molecular unique assignment to individual amino acid residues. We found that transient protonation changes of arginine and glutamic acid residues facilitate bidirectional proton transfer in [FeFe]-hydrogenases.
How [FeFe]-Hydrogenase Facilitates Bidirectional Proton Transfer

Moritz Senger¹, Viktor Eichmann¹, Konstantin Laun¹, Jifu Duan², Florian Wittkamp³, Günther Knör⁴, Ulf-Peter Apfel³,⁵, Thomas Happe², Martin Winkler², Joachim Heberle¹, Sven Timo Stripp¹*

¹ Experimental Molecular Biophysics, Department of Physics, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany

² Photobiotechnology, Faculty of Biology and Biotechnology, Ruhr-Universität Bochum, Universitätsstraße 150, 44801 Bochum, Germany

³ Activation of Small Molecules, Faculty of Chemistry and Biochemistry, Ruhr-Universität Bochum, Universitätsstraße 150, 44801 Bochum, Germany

⁴ Institute of Inorganic Chemistry, Johannes Kepler Universität Linz, Altenberger Straße 69, 4040 Linz, Austria

⁵ Fraunhofer UMSICHT, 46047 Oberhausen, Germany

*E-mail: sven.stripp@fu-berlin.de
ABSTRACT

Hydrogenases are metalloenzymes that catalyse the interconversion of protons and molecular hydrogen, H₂. [FeFe]-hydrogenases show particularly high rates of hydrogen turnover and have inspired numerous compounds for biomimetic H₂ production. Two decades of research on the active site cofactor of [FeFe]-hydrogenases have put forward multiple models of the catalytic proceedings. In comparison, understanding of the catalytic proton transfer is poor. We were able to identify the amino acid residues forming a proton transfer pathway between active site cofactor and bulk solvent; however, the exact mechanism of catalytic proton transfer remained inconclusive. Here, we employ in situ IR difference spectroscopy on the [FeFe]-hydrogenase from Chlamydomonas reinhardtii evaluating dynamic changes in the hydrogen-bonding network upon catalytic proton transfer. Our analysis allows for a direct, molecular unique assignment to individual amino acid residues. We found that transient protonation changes of arginine and glutamic acid residues facilitate bidirectional proton transfer in [FeFe]-hydrogenases.
Hydrogenases are gas-processing iron-sulphur enzymes that catalyse the reversible reduction of protons to molecular hydrogen in all kingdoms of life.\textsuperscript{1,2} Most hydrogenases are biased towards $\text{H}_2$ oxidation, for example in the context of energy metabolism and $\text{H}_2$ sensing.\textsuperscript{3–5} The [FeFe]-hydrogenases from bacteria and algae, in contrast, are truly bidirectional and catalyse $\text{H}_2$ oxidation and $\text{H}_2$ evolution with similar efficiency.\textsuperscript{6–8} Combining high turnover frequencies ($U > 10,000 \text{ H}_2 \text{ s}^{-1}$) and low electrochemical overpotential (E $\approx 420$ mV vs. SHE),\textsuperscript{9–11} the active site cofactor of [FeFe]-hydrogenases (“H-cluster”) inspired the design of numerous biomimetic complexes for $\text{H}_2$ production.\textsuperscript{12–14}

Figure 1
The H-cluster comprises a conventional [4Fe-4S] centre linked to a bimetallic iron-sulphur complex of formal similarity with [2Fe-2S] clusters (Fig. 1a).\textsuperscript{15–17} The diiron site carries two terminal carbonyl and cyanide ligands (CO, CN\textsuperscript{–}) as well as a single carbonyl ligand in Fe-Fe bridging position (\(\mu\text{CO}\)).\textsuperscript{18–20} An aminodithiolate group (ADT) connects the proximal and distal iron ion (Fe\textsubscript{p} and Fe\textsubscript{d}, relative to the [4Fe-4S] centre).\textsuperscript{21} The ADT group functions as proton relay between cofactor and protein environment.

[FeFe]-hydrogenases exchange protons with bulk solvent \textit{via} a trajectory of conserved, polar amino acid residues that connect catalytic cofactor and protein surface (Fig. 1a).\textsuperscript{22–24} Making use of protein crystallography and infrared spectroscopy, we were able to identify the residues that render catalytic proton transfer possible.\textsuperscript{25} In the [FeFe]-hydrogenase from \textit{Chlamydomonas reinhardtii}, this includes C169, E141, S189, E144, R148, and water cluster W1. A second, mostly aqueous proton transfer pathway facilitates protonation of the [4Fe-4S] centre.\textsuperscript{26} These protons are not consumed in the H\textsubscript{2} release reaction but stabilize the active-ready geometry and compensate for the drop in redox potential after a first reduction step.\textsuperscript{27,28} The dynamics of catalytic proton transfer have been addressed by molecular dynamics simulations before\textsuperscript{29–31}; however, no experimental data exists on the changes in the hydrogen-bonding network, e.g. when switching from H\textsubscript{2} evolution to H\textsubscript{2} oxidation. Such data is key to understanding the bidirectional catalysis of [FeFe]-hydrogenases.

Fourier-transformed infrared (FTIR) difference spectroscopy is an exquisite method to trace proton transfer steps and is routinely used for the analysis of light-induced reactions in chromophoric proteins.\textsuperscript{32–34} Redox dyes provide an opportunity to characterize hydrogen-bonding networks in enzymes that lack a natural chromophore.\textsuperscript{35} Here, we explore the dynamics of proton transfer in [FeFe]-hydrogenases by \textit{in situ} attenuated total reflection (ATR) FTIR difference spectroscopy.
spectroscopy. Photoreduction of the H-cluster by 5'-carboxy eosin Y (5CE)\textsuperscript{36} and ethylenediaminetetraacetic acid (EDTA) as sacrificial electron donor was utilized to enrich the one-electron reduced state $\text{Hred}$ over the oxidized resting state, $\text{Hox}$ (Fig. 1b). Photoreduction resulted in a fast ($t_{1/2} \sim 20$ s), near complete (>90%), and highly selective redox conversion (< 5% other species) that was not achieved by \textit{in situ} gas treatments\textsuperscript{26,37} or electrochemical methods.\textsuperscript{28,38} We analysed the [FeFe]-hydrogenase from \textit{C. reinhardtii}, HYDA1. Proton uptake upon formation of $\text{Hred}$ over $\text{Hox}$ induced spectral differences in the IR regime from 1750 – 1650 cm\textsuperscript{-1}. Exploiting \textit{in situ} H/D exchange and site-directed mutagenesis, these differences are assigned to the C=O stretching vibrations of carboxylic acid side chains (COOH) and the coupled vibrational mode of an protonated arginine side chain (C(NH\textsubscript{2})\textsuperscript{3+}). Infrared spectroscopy provides evidence for changes in hydrogen bonding involving glutamic acid E141 and serine S189 close to the active site as well as glutamic acid E144 and arginine R148 near the protein surface. This work presents the first direct, experimental characterization of the hydrogen-bonding network that facilitates catalytic proton transfer in [FeFe]-hydrogenases.

\textbf{RESULTS}

Figure 2a shows an overlay of absorbance spectra in the range of 3900 – 1300 cm\textsuperscript{-1} in the dark (black) and light (magenta). The ratio of \~1.3 for amide I (1635 cm\textsuperscript{-1}) to amide II (1545 cm\textsuperscript{-1}) absorbance indicates a well hydrated protein film.\textsuperscript{26} From 2700 – 1800 cm\textsuperscript{-1} neither liquid water (H\textsubscript{2}O) nor protein solution show strong IR intensities, which allows analysing the CO/CN\textsuperscript{-} bands of the H-cluster in absolute spectra. The inset highlights the IR signature of the H-cluster from 2150 – 1750 cm\textsuperscript{-1}. In the dark, the hydrogenase adopted $\text{Hox}$ while $\text{Hred}$ clearly dominated under illumination conditions (main bands 1940 cm\textsuperscript{-1} and 1891 cm\textsuperscript{-1}, respectively).\textsuperscript{38,39} The comparison of absolute spectra indicates a redox conversion larger than 90%.
In contrast to the CO/CN$^-$ bands, the intense absorbance of liquid water (HOH bending) and protein backbone (amide I, amide II) overlaps with signals in the COOH regime from 1750 – 1650 cm$^{-1}$ and precludes any meaningful analysis in absolute spectra. Figure 2b shows a “dark – light” difference spectrum of the single channel spectra that generated the absorbance spectra in Fig. 2a. The cofactor bands clearly dominate the Hred – Hox difference spectrum (see Fig. S1 for a closer examination). Efficient photoreduction prevents an accumulation of unspecific changes in the film (i.e. hydration level, protein concentration) and allows analysing the full spectrum. This includes the OH, SH, CO$_2$, and COOH regime as well as frequencies <1600 cm$^{-1}$ comprising vibrational marker bands of the photosensitizer 5CE (Fig. S2).
All difference bands in the COOH regime are specific for functional HYDA1. The inset in Fig. 2b shows that no such changes were observed when HYDA1 apo-protein was probed (apo-HYDA1 lacks the diiron site and is catalytically unreactive\(^{40-42}\)). Moreover, difference spectra of HYDA1 recorded upon exposure to CO (Hox-CO over Hox) or in the presence of zinc porphyrin as an alternative redox dye\(^{43}\) (Hred’ over Hox) confirmed that all bands in the COOH regime are specific for the conversion of Hred over Hox (Fig. S2). Please find a brief discussion of the different redox states of [FeFe]-hydrogenases in Supporting Information or elsewhere.\(^{44}\)

It is important to point out that our results do not suggest protonation or hydrogen bonding differences involving OH or SH groups (Fig. S3). The former would give rise to sharp absorbance bands around 3650 cm\(^{-1}\) indicative of “dangling”, weakly hydrogen-bonded water.\(^{45,46}\) The SH group absorbs around 2550 cm\(^{-1}\) and is very sensitive to changes in hydrophilicity.\(^{46,47}\) Typically, this frequency regime is addressed to analyse hydrogen-bonding changes involving the sidechain of a cysteine, e.g. C169. While the signal-to-noise ratio of the Hred – Hox difference spectrum is sufficiently good to pick up changes in the OH and SH regime (Fig. S3), the lack thereof suggests an invariable hydrogen-bonding network between water cluster W1, C169, and the ADT head group of the H-cluster (Fig. 1a). In the following, we will concentrate on the assignment of changes in the C=O stretching frequencies of carboxylic side chains (COOH).

**Band Fitting and Tentative Assignments.** The Hred – Hox spectrum in the COOH regime was best described by a fit routine including 7 + 2 Gaussians with a fixed half-max width of 6 – 8 cm\(^{-1}\) and third-order polynomial baseline correction (Fig. 3a). The temporal evolution of these bands is in excellent agreement with those of the H-cluster (Fig. S1). Vibrations at frequencies > 1700 cm\(^{-1}\) are typically assigned to the C=O stretches of the COOH side chain from aspartic acid or
glutamic acid residues (E, D).\textsuperscript{47,48} The C=O stretching frequency is inversely proportional to the hydrogen-bonding strength and can vary between none to multiple hydrogen-bonding partners from 1750 – 1700 cm\textsuperscript{-1}. Besides clearly discriminable bands at 1721, 1715, and 1700 cm\textsuperscript{-1} our fit routine suggested additional contributions centred at 1710, 1696, 1690, and 1681 (Fig. 3a). The negative band at 1681 cm\textsuperscript{-1} may be attributed to the asymmetric C(NH\textsubscript{2})\textsuperscript{3+} vibration of the protonated arginine side chain\textsuperscript{49–51} while the broader features at ~1670 cm\textsuperscript{-1} and 1655 cm\textsuperscript{-1} may be assigned to changes in amide I absorbance.\textsuperscript{52} The later likely reflects minor changes in secondary structure induced upon reduction of the H-cluster.

\textbf{Figure 3}
H/D Exchange. In order to achieve an experimental band assignment in the COOH regime, we
performed photoreduction on hydrated and deuterated hydrogenases films. Bands indicative of
hydrogen bonding or protonation changes involving the carboxylic side chains are supposed to
shift to lower frequencies in deuterated sample.\textsuperscript{52} Absorbance spectra of the HYDA1:5CE:EDTA
reaction mixture show a complete exchange of solvent in the presence of either H$_2$O or D$_2$O and
Hred – Hox difference spectra prove that deuteration did not affect the H-clusters’ CO/CN$^-$ band
position (Fig. S4). However, in the COOH regime, the spectra show significant changes (Fig. 3b).
The prominent H/D shift of 1715 and 1700 cm$^{-1}$ to 1709 and 1694 cm$^{-1}$ immediately supports an
assignment of this motif to a titratable group, e.g. and aspartic or glutamic acid side chain. Bands
at 1696 and 1690 cm$^{-1}$ were affected by the H/D shift as well. While a dissection of components
is not immediately possible here, the mean frequency downshift by 12 ± 2 cm$^{-1}$ suggests an
assignment to strongly hydrogen-bonded aspartic or glutamic acid side chains.\textsuperscript{47,48} The positive
band at 1721 cm$^{-1}$ is insensitive to H/D exchange.

To achieve an unambiguous experimental band assignment, we analysed three different amino
acid variants of the proton transfer pathway. The enrichment of Hred over Hox depends on
functional proton transfer.\textsuperscript{38,39,53} In particular, amino acid residues C169 and E141 close to the
H-cluster were found to be susceptible to variations of the hydrogen-bonding network, slowly
accumulating the hydride state Hhyd over Hox rather than Hred (Fig. S5). This impedes a direct
comparison, and only a limited number of variants allowed screening the hydrogen-bonding
changes associated with catalytic proton transfer. An invariable hydrogen-bonding network
between H-cluster and E141 is in striking agreement with the aforementioned lack of hydrogen-
bonding changes around W1 and C169 (Fig. S3). Relative to the H-cluster, we will refer to C169,
W1, and E141 as “inner core” of the proton transfer pathway.
By contrast, Fig. 4 shows \textbf{Hred} – \textbf{Hox} difference spectra of HYDA1 variants that constitute the “outer core” of the proton transfer pathway, namely R148, E144, and S189. Site-directed mutagenesis at these position included an exchange against alanine (A). No accumulation of \textbf{Hred} was observed upon photoreduction of E144A (Fig. S5) so that the conservative variation of glutamic to aspartic acid was analysed instead. Variants R148A, E144D, and S189A adopted \textbf{Hred} upon illumination but showed only 15 – 25% of the native conversion efficiency (Fig. S6).

For comparison with native HYDA1, difference spectra were normalized to the amplitude of the band pair at 1715 and 1700 cm$^{-1}$ that was found to be prominently conserved in all spectra. The resulting scaling factors were in good agreement with the amplitudes observed for the CO difference bands of the conversion of \textbf{Hred} over \textbf{Hox}. Figure S6 depicts the spectral transitions over time for each variant including an evaluation of signal-to-noise in the COOH regime.
**Amino Acid Variant R148A.** The H$_2$ evolution activity of ~50% for HYDA1 variant R148A indicates that glutamic acid E144 can partially replace R148 as proton loading site. Figure 4a shows an overlay of H$_{red}$–H$_{ox}$ difference spectra in the COOH regime for R148A and native HYDA1 (left panel). The right panel depicts an overlay of the respective crystal structures.\textsuperscript{25} For the sake of convenience, we will use HYDA1 numbering here. Site-directed mutagenesis resulted in spectra with missing features at 1721, 1696, or 1681 cm$^{-1}$ (marked “X”) while the shift from 1715 to 1700 cm$^{-1}$ and a negative band at 1690 cm$^{-1}$ was conserved among variant and native HYDA1.

Above, we tentatively assigned the negative feature at 1681 cm$^{-1}$ to the asymmetric C(NH$_2$)$_3^+$
vibration of an arginine residue.\textsuperscript{40-51} The evident lack of this band in amino acid variant R148A supports this assignment. Moreover, the band at 1681 cm\textsuperscript{-1} shifted to 1607 cm\textsuperscript{-1} in deuterated sample which is in excellent agreement to guanidine hydrochloride reference spectra (Fig. S4). Therefore, we conclude deprotonation of \textit{R148}\textsuperscript{+} upon formation of \textit{Hred} in native HYDA1.

Poisson-Boltzmann calculations predicted a pK\textsubscript{a} of ~3.5 for E144 in native HYDA1 (Tab. S1). Although our experiments were conducted at pH 6, we suggest hydrogen bonding of E144 to R148 (\textsim 2.8 Å) and S189 (\textsim 3.1 Å) stabilizing the carboxylic over the carboxylate form of E144. However, the carboxylate form likely prevails in the absence of the arginine side chain. The lack of spectral features at 1721 and 1696 cm\textsuperscript{-1} in the R148A difference spectrum ("X") therefore facilitates the assignment to hydrogen-bonding changes involving E144 in native HYDA1.

**Glutamic Acid Variant E144D.** As observed for the arginine variant, the interaction between R148 and E144 (E144D) is not strictly essential for catalytic activity. Amino acid variant E144D is reported with \textsim 50\% H\textsubscript{2} evolution activity.\textsuperscript{25} Figure 4b shows an overlay of \textit{Hred} – \textit{Hox} difference spectra in the COOH regime for R144D and native HYDA1 (left panel). The right panel depicts an overlay of the respective CPI crystal structures.\textsuperscript{25} Site-directed mutagenesis resulted in spectra with a pronounced band upshift from 1721 to 1742 cm\textsuperscript{-1} and an intensity inversion of the band at around 1690 cm\textsuperscript{-1} (negative in native HYDA1, slightly shifted and positive in E144D). The band pair at 1715 and 1700 cm\textsuperscript{-1} and negative bands at 1694 and 1681 cm\textsuperscript{-1} are conserved among variant and native HYDA1.

Shortening of the alkyl side chain at position 144 causes a different hydrogen-bonding situation (Fig. 4b, right panel). Instead of forming a hydrogen bond with R148 (\textsim 5.0 Å) rotation of the aspartic acid side chain forces D144 into a weak complex with S189 in \textit{Hox} (3.0 and 3.7 Å) that reflects in a pK\textsubscript{a} increase of nearly three units compared to native HYDA1 (Tab. S1). The upshift of the E144 band
from 1721 to 1742 cm\(^{-1}\) suggests significantly weaker hydrogen-bonding in reduced enzyme.

Furthermore, variant E144D allows differentiating the 1696/1690 cm\(^{-1}\) peak doublet. The latter band appears positive in the spectrum (at 1688 cm\(^{-1}\)) thus only the 1696 cm\(^{-1}\) band is assigned to E144 in native HYDA1.

**Serine Variant S189A.** Figure 4c shows an overlay of Hred – Hox difference spectra in the COOH regime for S189A and native HYDA1 (left panel). The right panel depicts an overlay of the respective CPI crystal structures.\(^{25}\) Despite the relatively low H\(_2\) evolution activity of \(~10\%\), site-directed mutagenesis resulted in spectra indicative of only minor differences to native HYDA1. The crystal structure of the S189A variant revealed an additional water molecule (W*) between E144 and E141 (Fig. 4c, right panel). This arrangement was proposed to compensate the lack of the serine side chain.\(^{25}\) E144 is in fair hydrogen-bonding distance to W* (~2.4 Å) and R148 (~2.9 Å), which largely restores the spectral phenotype of native HYDA1. However, the distance of 5.8 Å between E141 and W* is clearly out of range for hydrogen bonding or proton transfer.

**Glutamic Acid E141.** The band pair at 1715 and 1700 cm\(^{-1}\) is prominently conserved in all protein samples that accumulate Hred over Hox. The H/D specific band shift hints at a carboxylic group and suggest efficient proton exchange; however, the motif could not be assigned to E144.

Residing in a hydrophobic pocket at the interface of inner and outer core of the proton transfer pathway, glutamic acid E141 has been calculated to adopt the carboxylic acid form for pH < 8.\(^{25}\) Accordingly, changes associated with E141 will be visible in the COOH regime.\(^{47}\) Any variation of E141 abolished catalytic activity and the formation of Hred (Fig. S5) hinting at the central role of E141 in proton transfer. Based on this line of evidence, we assign the band pair at 1715 and 1700 cm\(^{-1}\) to E141. Figure S7 provides a conclusive overview on the observed frequencies and experiment band assignment.
The C=O stretching frequencies of glutamic acid E141 indicate strong hydrogen-bonding contacts, irrespective of redox state.\textsuperscript{47,48} To this end, the crystal structure of oxidized enzyme supports a trans complex between E141 and W1 (distances 2.4 and 3.4 Å).\textsuperscript{54–56} The 15 cm\textsuperscript{-1} frequency downshift upon reduction may reflect a release of the E141/W1 complex in favour of hydrogen bonding with S189 (or W* in serine variant S189A). This demands a certain level of structural flexibility as the distance between E141 and S189 accounts to ~3.8 Å in oxidized enzyme. Molecular dynamics simulations showed that E141 and S189 change between hydrogen-bonding donor and acceptor when switching from proton uptake to proton release.\textsuperscript{29,31}

Apparently, smaller structural changes at the interface of inner and outer core are well within the thermodynamic range of functional [FeFe]-hydrogenases. The large distance between E141 and W* (5.8 Å) reduces the probability of proton transfer in serine variant S189A to approximately 10% H\textsubscript{2} release activity.\textsuperscript{25}

**DISCUSSION**

Figure 5a depicts the progression of amino acid residues involved in catalytic proton transfer as identified in the crystal structure of oxidized [FeFe]-hydrogenase (H\textit{ox}).\textsuperscript{25} This arrangement favours proton uptake and H\textsubscript{2} evolution. Arginine R148\textsuperscript{+} donates a hydrogen bond to glutamic acid E144 (3.1 Å), the latter forming a hydrogen bond with serine S189 (2.8 Å).\textsuperscript{29–31} Based on p\textsubscript{K}a calculations, we previously favoured an ionic bond between R148\textsuperscript{+} and the carboxylate of E141\textsuperscript{25}; however, such stabilization fails to explain the FTIR band changes. Trapped between R148 and S189, E144 likely persist in protonated, carboxylic acid form, even at pH values well above the predicted p\textsubscript{K}a of 3.5 (Tab. S1).
Serine S189 is located at the interface of inner and outer core of the proton transfer pathway. The distance of ~3.8 Å between S189 and E141 does reflect discontinued hydrogen bonding and the probability of proton transfer appears insufficient to justify turnover frequencies > 10,000 H\textsubscript{2} s\textsuperscript{-1}. Furthermore, E141 and water molecule W1 form a trans complex that represents the most stable configuration of COOH groups in aqueous solution.\textsuperscript{54-56} This arrangement interrupts the catalytic hydrogen-bonding network as indicated by the yellow boxes in Fig. 5. The E141/W1 complex is weakened by the comparatively long donor distance (\(\alpha = 3.4\) Å), which may alleviate changing from W1 to S189 as hydrogen-bond acceptor, e.g. upon reduction of the cofactor.

Figure 5b illustrates how the catalytic proton transfer pathway between cofactor and solvent is significantly more continuous in the reduced enzyme (H\textsubscript{red}). The downshift of the E141 band
may reflect dissolution of the E141/W1 complex and hydrogen bonding to S189 with $\alpha > \beta$.

Serine S189 donates a single bond to E144; however, no second hydrogen bond is formed by E144 due to de-protonation of R148. This induces the pronounced upshift of the E144 band. We assume that a re-protonation of R148 is precluded by the unfavourable local electrostatics between the OH group of E144 and R148$^+$. Based on energetic considerations, the stability of de-protonated arginine residues in proteins has been questioned. Yet, our spectroscopic investigation on hydrated and deuterated [FeFe]-hydrogenase unambiguously resolved the de-protonation of R148$^+$ upon reduction of the enzyme. This converted arginine functions as proton donor to the active site cofactor, fine-tuning proton transfer efficiency and catalytic bias.

The mechanism of discontinuous proton transfer conceptualised above likely includes a transient step we can speculate about now (Fig. S8). Reduction of the H-cluster by one electron leads to an increase in basicity and the formation of $\text{H}_{\text{red}}$ upon protonation via the catalytic proton transfer pathway. Based on the lack of difference signals in the SH and OH regime, we consider a rigid donor/acceptor conformation between the H-cluster, C169, and W1. Protonation of the ADT head group in $\text{H}_{\text{red}}$ was discussed and computed earlier but the present data support protonation changes neither at the ADT head group, cysteine thiolate, nor water cluster. Transiently, however, formation of $\text{H}_{\text{red}}$ may trigger de-protonation of E141 (Fig. S8). In a second step, the high basicity of E141$^-$ would induce steady-state de-protonation of R148$^+$, proton transfer via E144 and S189, and re-protonation of E141, now hydrogen-bonded to S189 instead of W1 (Fig. 5b).

Discontinuous proton transfer is common in nature, e.g. in retinal proteins, photosystem II, cytochrome c oxidase, and other systems, but has not yet been considered in hydrogenases. Our model rationalises how ions transcend the gap between inner and outer core of the proton.
transfer pathway. Furthermore, it provides a reasonable explanation for the catalytic
bidirectionality of [FeFe]-hydrogenases.\textsuperscript{6–8} Glutamic acid E141 switches as hydrogen-bonding
donor between W1 (proton uptake) and S189 (proton release). Deviations in distance $< 0.5$ Å
suggest a flexible hydrogen-bonding network that facilitates both H\textsubscript{2} evolution (proton uptake)
and H\textsubscript{2} oxidation (proton release).

**CONCLUSIONS**

In this work, we demonstrate how \textit{in situ} infrared spectroscopy was applied to analyse the
hydrogen-bonding network of the catalytic proton transfer pathway in [FeFe]-hydrogenases.
Discontinuous proton transfer was triggered by the enhanced basicity of the active site cofactor
(H-cluster) upon photoreduction of a highly active iron-sulphur enzyme lacking a natural
chromophore. Infrared spectroscopy provides a direct read-out for changes in hydrogen bonding
perfectly complementary to X-ray crystallography. Thereby, the first experimental description of
the dynamic hydrogen-bonding changes in the catalytic proton transfer pathway of [FeFe]-
hydrogenases was accomplished.

Reduction of the H-cluster induces a transient de-protonation of glutamic acid residue E141 right
in the middle of the proton transfer pathway. Re-protonation of E141 by arginine residue R148
restores the conductivity of the proton transfer pathway and enables a second proton transfer
event that leads to H\textsubscript{2} evolution eventually. Our data unambiguously allow concluding on de-
protonation of an arginine residue, which has never been shown in an enzyme before. In
summary, we found that the flexible hydrogen-bonding network around E141 allows [FeFe]-
hydrogenases catalysing H\textsubscript{2} evolution and H\textsubscript{2} uptake with similar efficiency.
METHODS

The [FeFe]-hydrogenase from *C. reinhardtii* HYDA1 was expressed and synthesized in *E. coli*, purified by strep-tactin affinity chromatography, and activated *in vitro* with ADT cofactor under anaerobic conditions. After removal of excess cofactor, the protein concentration was adjusted to approximately 3 mM (~150 g/L). 5'-carboxy eosin Y (5CE) and EDTA were prepared in aqueous stock solutions of 6 mM and 90 mM, respectively. One part of each component was mixed to yield a HYDA1/5CE/EDTA ratio of 1:2:30.

All spectroscopic experiments were performed under anaerobic conditions, at room temperature, ambient pressure, and on hydrated protein films of physiological pH values. First, 1 – 2 µL of the reaction mix was pipetted onto the silicon crystal of the ATR unit (DuraDisc SampIR-2, Smiths Detection) in the FTIR spectrometer (Tensor 27, Bruker). Spectra from 3900 – 1300 cm\(^{-1}\) were recorded with a narrow-band MCT detector with a spectral resolution of 2 cm\(^{-1}\) and 25 interferometer scans each. The solution was protected from stray light, dried under N\(_2\), and rehydrated via the gas phase with 10 mM MES buffer (pH 6). Traces of reduced and CO-inhibited species were lost in favour of Hox after 1 – 3 h auto-oxidation, as reported earlier. The experiment was initiated upon continuous illumination of the film at 505 nm and followed by FTIR spectroscopy with a time resolution of 5 s (Fig. S1). After ~20 s, half of the Hox population converted into Hred, with an overall redox efficiency of > 90%. Importantly, no other reduced species than Hred were observed although H\(_2\) released upon reduction of HYDA1 may re-react with the enzyme forming Hred', Hsred, and Hhyd. Therefore, the continuous exchange of gas in our setup was found to be of particular importance as it precludes a build-up of multiple reduced species. No photoreduction was observed under off-resonant conditions (590 nm) whereas illumination at 455 nm induced notable cofactor corruption (Fig. S9), as noted earlier.
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Figure 1 – The proton transfer pathway of [FeFe]-hydrogenases and experimental strategy.

(a) Hydrogen turnover is catalysed at the H-cluster that is formed by a diiron site attached to the protein via a [4Fe-4S] centre. The diiron site binds 5 – 6 CO and CN⁻ ligands, one of which that can be found in Fe-Fe bridging position (µCO). The ADT group functions as proton relay between C169 and the distal iron ion of the H-cluster, Fe₆. Residues involved in proton transfer are identified. Cartoon model of the oxidized [FeFe]-hydrogenase from CPI according to pdb entry 4XDC, numbering refers to HYDA1. (b) Continuous illumination at 505 nm of 5’-carboxy eosin Y (5CE, photosensitizer) with ethylenediaminetetraacetic acid (EDTA) as sacrificial electron donor was exploited to enrich Hred over Hox in [FeFe]-hydrogenase HYDA1. Increasing the basicity upon reduction induces a protonation of the H-cluster. The associated changes in the hydrogen-bonding network of the catalytic proton transfer pathway are followed by in situ ATR FTIR difference spectroscopy.

Figure 2 – Absorbance and light-induced difference spectra of [FeFe]-hydrogenase. (a) ATR FTIR absorbance spectra of the hydrated reaction mixture (HYDA1:5CE:EDTA) in the dark (black) and upon illumination at 505 nm (magenta). Inset: magnification of the cofactor regime. The changes show reduction of HYDA1 from Hox (black) to Hred (magenta). (b) Subtraction of single channel spectra from the same data set as in (a). The “dark – light” difference spectrum is dominated by the CO/CN⁻ bands of the H-cluster. Additionally, it allows analysing the OH, SH, and COOH regime as well as frequencies < 1500 cm⁻¹ comprising vibrational marker bands of photosensitizer 5CE. Inset: magnification of the COOH regime. The band changes are specific for functional HYDA1 (black) and not observed in HYDA1 apo-protein (red). * 2337 cm⁻¹, assigned to CO₂.
**Figure 3 – Band fit and H/D exchange.** (a) The ATR FTIR difference spectrum of the Hred – Hox conversion (solid line) was fitted with a minimum of $7 + 2$ Gaussians in the COOH regime (blue traces). All frequencies are given in the legend. The red dotted line depicts the resulting envelope. (b) Comparison of ATR FTIR difference spectra on hydrated and deuterated film (black and red, respectively). The data indicates a downshifts of $6 – 14$ cm$^{-1}$ for bands associated with Hox (negative intensities) and similar frequency difference for bands accumulating upon reduction (positive intensities). No significant shift was noted for the positive bands at 1721 cm$^{-1}$ and ~1670 cm$^{-1}$. See Fig. S4 for further details.

**Figure 4 – Photoreduction of [FeFe]-hydrogenase variants.** In situ ATR FTIR difference spectra in the COOH regime for three different amino acid residues compared to native HYDA1 (dotted traces, scaled to the 1715/1700 difference signal that was observed in all experiments). The crystallographic comparison includes pdb coordinates 4XDC, 6GM2, 6GM3, and 6GM4. (a) Arginine variant R148A lacks the positive 1721 cm$^{-1}$ feature and shows significantly diminished contributions at 1696 and 1681 cm$^{-1}$ (“X”). (b) Glutamic acid variant E144D exhibits a ~21 cm$^{-1}$ upshift of the native 1721 cm$^{-1}$ band to 1742 cm$^{-1}$. Negative bands at 1692 and 1682 cm$^{-1}$ suggest similarities with native enzyme whereas the band intensity at 1688 cm$^{-1}$ is inverted. (c) The difference spectrum of serine variant S189A indicates a largely native phenotype with only smaller differences in band intensity. See Fig. S6 for further details.

**Figure 5 – The hydrogen-bonding network of the catalytic proton transport pathway.** (a) Progression of amino acid residues as observed in the crystal structure of oxidized [FeFe]-hydrogenase CPI (Hox). All distances refer to pdb coordinates 4XDC (HYDA1 numbering). Our data suggest hydrogen-bonding contacts between S189, E144, and R148$^{+}$ of the catalytic proton transport pathway. Furthermore, invariable hydrogen-bonding between ADT, C169, and W1 was
observed. The W1/E141 complex establishes the difference between inner and outer core of the catalytic proton transport pathway. (b) In reduced [FeFe]-hydrogenase (Hred), the W1/141 complex is terminated in favour of hydrogen bonding between E141, S189, and E144, which facilitates continuous proton transfer (see main text for details). Our data indicate deprotonation of R148$^+$ upon formation of Hred.
How [FeFe]-Hydrogenase Facilitates Bidirectional Proton Transfer

Moritz Senger¹, Viktor Eichmann¹, Konstantin Laun¹, Jifu Duan², Florian Wittkamp³, Günther Knör⁴, Ulf-Peter Apfel³, Thomas Happe², Martin Winkler², Joachim Heberle¹, Sven Timo Stripp¹*

¹ Experimental Molecular Biophysics, Department of Physics, Freie Universität Berlin,
Arnimallee 14, 14195 Berlin, Germany
² Photobiotechnology, Faculty of Biology and Biotechnology, Ruhr-Universität Bochum,
Universitätsstraße 150, 44801 Bochum, Germany
³ Activation of Small Molecules, Faculty of Chemistry and Biochemistry, Ruhr-Universität Bochum,
Universitätsstraße 150, 44801 Bochum, Germany
⁴ Institute of Inorganic Chemistry, Johannes Kepler Universität Linz, Altenberger Straße 69, 4040 Linz, Austria
⁵ Fraunhofer UMSICHT, 46047 Oberhausen, Germany

*E-mail: sven.stripp@fu-berlin.de
ABSTRACT

Hydrogenases are metalloenzymes that catalyse the interconversion of protons and molecular hydrogen, H₂. [FeFe]-hydrogenases show particularly high rates of hydrogen turnover and have inspired numerous compounds for biomimetic H₂ production. Two decades of research on the active site cofactor of [FeFe]-hydrogenases have put forward multiple models of the catalytic proceedings. In comparison, understanding of the catalytic proton transfer is poor. We were able to identify the amino acid residues forming a proton transfer pathway between active site cofactor and bulk solvent; however, the exact mechanism of catalytic proton transfer remained inconclusive. Here, we employ in situ IR difference spectroscopy on the [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* evaluating dynamic changes in the hydrogen-bonding network upon catalytic proton transfer. Our analysis allows for a direct, molecular unique assignment to individual amino acid residues. We found that transient protonation changes of arginine and glutamic acid residues facilitate bidirectional proton transfer in [FeFe]-hydrogenases.
Hydrogenases are gas-processing iron-sulphur enzymes that catalyse the reversible reduction of protons to molecular hydrogen in all kingdoms of life. Most hydrogenases are biased towards H2 oxidation, for example in the context of energy metabolism and H2 sensing. The [FeFe]-hydrogenases from bacteria and algae, in contrast, are truly bidirectional and catalyse H2 oxidation and H2 evolution with similar efficiency. Combining high turnover frequencies (U > 10,000 H2 s-1) and low electrochemical overpotential (E ~420 mV vs. SHE), the active site cofactor of [FeFe]-hydrogenases (“H-cluster”) inspired the design of numerous biomimetic complexes for H2 production.
The H-cluster comprises a conventional [4Fe-4S] centre linked to a bimetallic iron-sulphur complex of formal similarity with [2Fe-2S] clusters (Fig. 1a).\textsuperscript{15-17} The diiron site carries two terminal carbonyl and cyanide ligands (CO, CN\textsuperscript{–}) as well as a single carbonyl ligand in Fe-Fe bridging position (\(\mu\)CO).\textsuperscript{18-20} An aminodithiolate group (ADT) connects the proximal and distal iron ion (Fep and Fed, relative to the [4Fe-4S] centre).\textsuperscript{21} The ADT group functions as proton relay between cofactor and protein environment.

[FeFe]-hydrogenases exchange protons with bulk solvent via a trajectory of conserved, polar amino acid residues that connect catalytic cofactor and protein surface (Fig. 1a).\textsuperscript{22-24} Making use of protein crystallography and infrared spectroscopy, we were able to identify the residues that render catalytic proton transfer possible.\textsuperscript{25} In the [FeFe]-hydrogenase from \textit{Chlamydomonas reinhardtii}, this includes C169, E141, S189, E144, R148, and water cluster W1. A second, mostly aqueous proton transfer pathway facilitates protonation of the [4Fe-4S] centre.\textsuperscript{26} These protons are not consumed in the H\textsubscript{2} release reaction but stabilize the active-ready geometry and compensate for the drop in redox potential after a first reduction step.\textsuperscript{27,28} The dynamics of catalytic proton transfer have been addressed by molecular dynamics simulations before\textsuperscript{29-31}, however, no experimental data exists on the changes in the hydrogen-bonding network, e.g. when switching from H\textsubscript{2} evolution to H\textsubscript{2} oxidation. Such data is key to understanding the bidirectional catalysis of [FeFe]-hydrogenases.

Fourier-transformed infrared (FTIR) difference spectroscopy is an exquisite method to trace proton transfer steps and is routinely used for the analysis of light-induced reactions in chromophoric proteins.\textsuperscript{32-34} Redox dyes provide an opportunity to characterize hydrogen-bonding networks in enzymes that lack a natural chromophore.\textsuperscript{35} Here, we explore the dynamics of proton
transfer in [FeFe]-hydrogenases by in situ attenuated total reflection (ATR) FTIR difference spectroscopy. Photoreduction of the H-cluster by 5’-carboxy eosin Y (5CE) and ethylenediaminetetraacetic acid (EDTA) as sacrificial electron donor was utilized to enrich the one-electron reduced state \( \text{Hred} \) over the oxidized resting state, \( \text{Hox} \) (Fig. 1b). Photoreduction resulted in a fast \( (t_{1/2} \sim 20 \text{ s}) \), near complete (>90%), and highly selective redox conversion (< 5% other species) that was not achieved by in situ gas treatments or electrochemical methods.

We analysed the [FeFe]-hydrogenase from \( \text{C. reinhardtii} \), HYDA1. Proton uptake upon formation of \( \text{Hred} \) over \( \text{Hox} \) induced spectral differences in the IR regime from 1750 – 1650 cm\(^{-1}\). Exploiting in situ H/D exchange and site-directed mutagenesis, these differences are assigned to the \( \text{C}=\text{O} \) stretching vibrations of carboxylic acid side chains (COOH) and the coupled vibrational mode of an protonated arginine side chain (\( \text{C(NH}_2\text{)}^+ \)). Infrared spectroscopy provides evidence for changes in hydrogen bonding involving glutamic acid E141 and serine S189 close to the active site as well as glutamic acid E144 and arginine R148 near the protein surface. This work presents the first direct, experimental characterization of the hydrogen-bonding network that facilitates catalytic proton transfer in [FeFe]-hydrogenases.

**RESULTS**

Figure 2a shows an overlay of absorbance spectra in the range of 3900 – 1300 cm\(^{-1}\) in the dark (black) and light (magenta). The ratio of \( \sim 1.3 \) for amide I (1635 cm\(^{-1}\)) to amide II (1545 cm\(^{-1}\)) absorbance indicates a well hydrated protein film. From 2700 – 1800 cm\(^{-1}\) neither liquid water \( \text{H}_2\text{O} \) nor protein solution show strong IR intensities, which allows analysing the CO/CN\(^{-}\) bands of the H-cluster in absolute spectra. The inset highlights the IR signature of the H-cluster from 2150 – 1750 cm\(^{-1}\). In the dark, the hydrogenase adopted \( \text{Hox} \) while \( \text{Hred} \) clearly dominated under
Illumination conditions (main bands 1940 cm\(^{-1}\) and 1891 cm\(^{-1}\), respectively).\(^{38,39}\) The comparison of absolute spectra indicates a redox conversion larger than 90%.

Figure 2

In contrast to the CO/CN\(^{-}\) bands, the intense absorbance of liquid water (HOH bending) and protein backbone (amide I, amide II) overlaps with signals in the COOH regime from 1750 – 1650 cm\(^{-1}\) and precludes any meaningful analysis in absolute spectra. Figure 2b shows a “dark – light” difference spectrum of the single channel spectra that generated the absorbance spectra in Fig. 2a. The cofactor bands clearly dominate the H\(_{\text{red}}\) – H\(_{\text{ox}}\) difference spectrum (see Fig. S1 for a closer examination). Efficient photoreduction prevents an accumulation of unspecific changes in the film (i.e. hydration level, protein concentration) and allows analysing the full spectrum.
This includes the OH, SH, CO₂, and COOH regime as well as frequencies <1600 cm⁻¹ comprising vibrational marker bands of the photosensitizer 5CE (Fig. S2).

All difference bands in the COOH regime are specific for functional HYDA1. The inset in Fig. 2b shows that no such changes were observed when HYDA1 apo-protein was probed (apo-HYDA1 lacks the diiron site and is catalytically unreactive⁴⁰–⁴²). Moreover, difference spectra of HYDA1 recorded upon exposure to CO (Hox-CO over Hox) or in the presence of zinc porphyrin as an alternative redox dye⁴³ (Hred over Hox) confirmed that all bands in the COOH regime are specific for the conversion of Hred over Hox (Fig. S2). Please find a brief discussion of the different redox states of [FeFe]-hydrogenases in Supporting Information or elsewhere.⁴⁴

It is important to point out that our results do not suggest protonation or hydrogen bonding differences involving OH or SH groups (Fig. S3). The former would give rise to sharp absorbance bands around 3650 cm⁻¹ indicative of “dangling”, weakly hydrogen-bonded water.⁴⁵,⁴⁶ The SH group absorbs around 2550 cm⁻¹ and is very sensitive to changes in hydrophilicity.⁴⁶,⁴⁷ Typically, this frequency regime is addressed to analyse hydrogen-bonding changes involving the sidechain of a cysteine, e.g. C169. While the signal-to-noise ratio of the Hred – Hox difference spectrum is sufficiently good to pick up changes in the OH and SH regime (Fig. S3), the lack thereof suggests an invariable hydrogen-bonding network between water cluster W1, C169, and the ADT head group of the H-cluster (Fig. 1a). In the following, we will concentrate on the assignment of changes in the C=O stretching frequencies of carboxylic side chains (COOH).

Band Fitting and Tentative Assignments. The Hred – Hox spectrum in the COOH regime was best described by a fit routine including 7 + 2 Gaussians with a fixed half-max width of 6 – 8 cm⁻¹.
and third-order polynomial baseline correction (Fig. 3a). The temporal evolution of these bands is in excellent agreement with those of the H-cluster (Fig. S1). Vibrations at frequencies > 1700 cm\(^{-1}\) are typically assigned to the C=O stretches of the COOH side chain from aspartic acid or glutamic acid residues (E, D).\(^{47,48}\) The C=O stretching frequency is inversely proportional to the hydrogen-bonding strength and can vary between none to multiple hydrogen-bonding partners from 1750 – 1700 cm\(^{-1}\). Besides clearly discriminable bands at 1721, 1715, and 1700 cm\(^{-1}\) our fit routine suggested additional contributions centred at 1710, 1696, 1690, and 1681 cm\(^{-1}\) (Fig. 3a). The negative band at 1681 cm\(^{-1}\) may be attributed to the asymmetric C(NH\(_2\))\(^+\) vibration of the protonated arginine side chain\(^{49-51}\) while the broader features at \(~1670\) cm\(^{-1}\) and 1655 cm\(^{-1}\) may be assigned to changes in amide I absorbance.\(^{52}\) The later likely reflects minor changes in secondary structure induced upon reduction of the H-cluster.
131H/D Exchange. In order to achieve an experimental band assignment in the COOH regime, we performed photoreduction on hydrated and deuterated hydrogenases films. Bands indicative of hydrogen bonding or protonation changes involving the carboxylic side chains are supposed to shift to lower frequencies in deuterated sample. Absorbance spectra of the HYDA1:5CE:EDTA reaction mixture show a complete exchange of solvent in the presence of either H$_2$O or D$_2$O and Hred – Hox difference spectra prove that deuteration did not affect the H-clusters’ CO/CN$^-$ band position (Fig. S4). However, in the COOH regime, the spectra show significant changes (Fig. 3b). The prominent H/D shift of 1715 and 1700 cm$^{-1}$ to 1709 and 1694 cm$^{-1}$ immediately supports an
Assignment of this motif to a titratable group, e.g. and aspartic or glutamic acid side chain. Bands at 1696 and 1690 cm\(^{-1}\) were affected by the H/D shift as well. While a dissection of components is not immediately possible here, the mean frequency downshift by 12 ± 2 cm\(^{-1}\) suggests an assignment to strongly hydrogen-bonded aspartic or glutamic acid side chains.\(^{47,48}\) The positive band at 1721 cm\(^{-1}\) is insensitive to H/D exchange.

To achieve an unambiguous experimental band assignment, we analysed three different amino acid variants of the proton transfer pathway. The enrichment of H\(_{\text{red}}\) over H\(_{\text{ox}}\) depends on functional proton transfer.\(^{38,39,53}\) In particular, amino acid residues C169 and E141 close to the H-cluster were found to be susceptible to variations of the hydrogen-bonding network, slowly accumulating the hydride state H\(_{\text{hyd}}\) over H\(_{\text{ox}}\) rather than H\(_{\text{red}}\) (Fig. S5). This impedes a direct comparison, and only a limited number of variants allowed screening the hydrogen-bonding changes associated with catalytic proton transfer. An invariable hydrogen-bonding network between H-cluster and E141 is in striking agreement with the aforementioned lack of hydrogen-bonding changes around W1 and C169 (Fig. S3). Relative to the H-cluster, we will refer to C169, W1, and E141 as “inner core” of the proton transfer pathway.

By contrast, Fig. 4 shows H\(_{\text{red}}\) – H\(_{\text{ox}}\) difference spectra of HYDA1 variants that constitute the “outer core” of the proton transfer pathway, namely R148, E144, and S189. Site-directed mutagenesis at these position included an exchange against alanine (A). No accumulation of H\(_{\text{red}}\) was observed upon photoreduction of E144A (Fig. S5) so that the conservative variation of glutamic to aspartic acid was analysed instead. Variants R148A, E144D, and S189A adopted H\(_{\text{red}}\) upon illumination but showed only 15 – 25% of the native conversion efficiency (Fig. S6). For comparison with native HYDA1, difference spectra were normalized to the amplitude of the band pair at 1715 and 1700 cm\(^{-1}\) that was found to be prominently conserved in all spectra. The
resulting scaling factors were in good agreement with the amplitudes observed for the CO difference bands of the conversion of Hred over Hox. Figure S6 depicts the spectral transitions over time for each variant including an evaluation of signal-to-noise in the COOH regime.

Figure 4

**Amino Acid Variant R148A.** The H₂ evolution activity of ~50% for HYDA1 variant R148A indicates that glutamic acid E144 can partially replace R148 as proton loading site. Figure 4a shows an overlay of Hred – Hox difference spectra in the COOH regime for R148A and native HYDA1 (left panel). The right panel depicts an overlay of the respective crystal structures. For the sake of convenience, we will use HYDA1 numbering here. Site-directed mutagenesis resulted in
spectra with missing features at 1721, 1696, or 1681 cm\(^{-1}\) (marked “X”) while the shift from 1715 to 1731 cm\(^{-1}\) and a negative band at 1690 cm\(^{-1}\) was conserved among variant and native HYDA1.

Above, we tentatively assigned the negative feature at 1681 cm\(^{-1}\) to the asymmetric C(NH\(_2\))\(_3^+\) vibration of an arginine residue.\(^{49-51}\) The evident lack of this band in amino acid variant R148A supports this assignment. Moreover, the band at 1681 cm\(^{-1}\) shifted to 1607 cm\(^{-1}\) in deuterated sample which is in excellent agreement to guanidine hydrochloride reference spectra (Fig. S4). Therefore, we conclude deprotonation of R148\(^+\) upon formation of H\(_{red}\) in native HYDA1.

Poisson-Boltzmann calculations predicted a \(pK_a\) of \(~3.5\) for E144 in native HYDA1 (Tab. S1). Although our experiments were conducted at pH 6, we suggest hydrogen bonding of E144 to R148 (~2.8 Å) and S189 (~3.1 Å) stabilizing the carboxylic over the carboxylate form of E144. However, the carboxylate form likely prevails in the absence of the arginine side chain. The lack of spectral features at 1721 and 1696 cm\(^{-1}\) in the R148A difference spectrum (“X”) therefore facilitates the assignment to hydrogen-bonding changes involving E144 in native HYDA1.

Glutamic Acid Variant E144D. As observed for the arginine variant, the interaction between R148 and E144 (E144D) is not strictly essential for catalytic activity. Amino acid variant E144D is reported with \(~50\%\) H\(_2\) evolution activity.\(^{25}\) Figure 4b shows an overlay of H\(_{red}\) – H\(_{ox}\) difference spectra in the COOH regime for R144D and native HYDA1 (left panel). The right panel depicts an overlay of the respective CPI crystal structures.\(^{25}\) Site-directed mutagenesis resulted in spectra with a pronounced band upshift from 1721 to 1742 cm\(^{-1}\) and an intensity inversion of the band at around 1690 cm\(^{-1}\) (negative in native HYDA1, slightly shifted and positive in E144D). The band pair at 1715 and 1921 cm\(^{-1}\) and negative bands at 1694 and 1681 cm\(^{-1}\) are conserved among variant and native HYDA1.
Shortening of the alkyl side chain at position 144 causes a different hydrogen-bonding situation (Fig. 4b, right panel). Instead of forming a hydrogen bond with R148 (~5.0 Å) rotation of the aspartic acid side chain forces D144 into a weak complex with S189 in Hox (3.0 and 3.7 Å) that reflects in a pKᵢ increase of nearly three units compared to native HYDA1 (Tab. S1). The upshift of the E144 band from 1721 to 1742 cm⁻¹ suggests significantly weaker hydrogen-bonding in reduced enzyme.

Furthermore, variant E144D allows differentiating the 1696/1690 cm⁻¹ peak doublet. The latter band appears positive in the spectrum (at 1688 cm⁻¹) thus only the 1696 cm⁻¹ band is assigned to E144 in native HYDA1.

Serine Variant S189A. Figure 4c shows an overlay of Hred – Hox difference spectra in the COOH regime for S189A and native HYDA1 (left panel). The right panel depicts an overlay of the respective CPI crystal structures. Despite the relatively low H2 evolution activity of ~10%, site-directed mutagenesis resulted in spectra indicative of only minor differences to native HYDA1. The crystal structure of the S189A variant revealed an additional water molecule (W*) between E144 and E141 (Fig. 4c, right panel). This arrangement was proposed to compensate the lack of the serine side chain. E144 is in fair hydrogen-bonding distance to W* (~2.4 Å) and R148 (~2.9 Å), which largely restores the spectral phenotype of native HYDA1. However, the distance of 5.8 Å between E141 and W* is clearly out of range for hydrogen bonding or proton transfer.

Glutamic Acid E141. The band pair at 1715 and 1700 cm⁻¹ is prominently conserved in all protein samples that accumulate Hred over Hox. The H/D specific band shift hints at a carboxylic group and suggest efficient proton exchange; however, the motif could not be assigned to E144. Residing in a hydrophobic pocket at the interface of inner and outer core of the proton transfer pathway, glutamic acid E141 has been calculated to adopt the carboxylic acid form for pH < 8. Accordingly, changes associated with E141 will be visible in the COOH regime.
of E141 abolished catalytic activity and the formation of \textbf{Hred} (Fig. S5) hinting at the central role of E141 in proton transfer. Based on this line of evidence, we assign the band pair at 1715 and 1791 cm$^{-1}$ to E141. Figure S7 provides a conclusive overview on the observed frequencies and experiment band assignment.

The C=O stretching frequencies of glutamic acid E141 indicate strong hydrogen-bonding contacts, irrespective of redox state.$^{47,48}$ To this end, the crystal structure of oxidized enzyme supports a \textit{trans} complex between E141 and W1 (distances 2.4 and 3.4 Å).$^{54-56}$ The 15 cm$^{-1}$ frequency downshift upon reduction may reflect a release of the E141/W1 complex in favour of hydrogen bonding with S189 (or W* in serine variant S189A). This demands a certain level of structural flexibility as the distance between E141 and S189 accounts to ~3.8 Å in oxidized enzyme. Molecular dynamics simulations showed that E141 and S189 change between hydrogen-bonding donor and acceptor when switching from proton uptake to proton release.$^{29,31}$ Apparently, smaller structural changes at the interface of inner and outer core are well within the thermodynamic range of functional [FeFe]-hydrogenases. The large distance between E141 and W* (5.8 Å) reduces the probability of proton transfer in serine variant S189A to approximately 10% \textsubscript{H$_2$} release activity.$^{25}$

\textbf{DISCUSSION}

Figure 5a depicts the progression of amino acid residues involved in catalytic proton transfer as identified in the crystal structure of oxidized [FeFe]-hydrogenase (\textbf{Hox}).$^{25}$ This arrangement favours proton uptake and \textsubscript{H$_2$} evolution. Arginine R148$^+$ donates a hydrogen bond to glutamic acid E144 (3.1 Å), the latter forming a hydrogen bond with serine S189 (2.8 Å).$^{29-31}$ Based on pK$_a$ calculations, we previously favoured an ionic bond between R148$^+$ and the carboxylate of E141$^-$; however, such stabilization fails to explain the FTIR band changes. Trapped between R148 and
S189, E144 likely persist in protonated, carboxylic acid form, even at pH values well above the predicted pKₐ of 3.5 (Tab. S1).

Figure 5

Serine S189 is located at the interface of inner and outer core of the proton transfer pathway. The distance of ~3.8 Å between S189 and E141 does reflect discontinued hydrogen bonding and the probability of proton transfer appears insufficient to justify turnover frequencies > 10,000 H₂ s⁻¹. Furthermore, E141 and water molecule W1 form a trans complex that represents the most stable configuration of COOH groups in aqueous solution. This arrangement interrupts the catalytic hydrogen-bonding network as indicated by the yellow boxes in Fig. 5. The E141/W1 complex is weakened by the comparatively long donor distance (α = 3.4 Å), which may alleviate changing from W1 to S189 as hydrogen-bond acceptor, e.g. upon reduction of the cofactor.
Figure 5b illustrates how the catalytic proton transfer pathway between cofactor and solvent is significantly more continuous in the reduced enzyme ($\text{H}_{\text{red}}$). The downshift of the E141 band may reflect dissolution of the E141/W1 complex and hydrogen bonding to S189 with $\alpha > \beta$. Serine S189 donates a single bond to E144; however, no second hydrogen bond is formed by E144 due to de-protonation of R148. This induces the pronounced upshift of the E144 band. We assume that a re-protonation of R148 is precluded by the unfavourable local electrostatics between the OH group of E144 and R148$.^{57}$ Yet, our spectroscopic investigation on hydrated and deuterated [FeFe]-hydrogenase unambiguously resolved the de-protonation of R148$^+$ upon reduction of the enzyme. This conversed arginine functions as proton donor to the active site cofactor, fine-tuning proton transfer efficiency and catalytic bias.

The mechanism of discontinuous proton transfer conceptualised above likely includes a transient step we can speculate about now (Fig. S8). Reduction of the H-cluster by one electron leads to an increase in basicity and the formation of $\text{H}_{\text{red}}$ upon protonation via the catalytic proton transfer pathway. Based on the lack of difference signals in the SH and OH regime, we consider a rigid donor/acceptor conformation between the H-cluster, C169, and W1. Protonation of the ADT head group in $\text{H}_{\text{red}}$ was discussed$^{38,39,53}$ and computed earlier$^{29–31}$ but the present data support protonation changes neither at the ADT head group, cysteine thiolate, nor water cluster.

Transiently, however, formation of $\text{H}_{\text{red}}$ may trigger de-protonation of E141 (Fig. S8). In a second step, the high basicity of E141$^-$ would induce steady-state de-protonation of R148$^+$, proton transfer via E144 and S189, and re-protonation of E141, now hydrogen-bonded to S189 instead of W1 (Fig. 5b).
Discontinuous proton transfer is common in nature, e.g. in retinal proteins\textsuperscript{58,59}, photosystem II\textsuperscript{60}, cytochrome c oxidase\textsuperscript{61}, and other systems,\textsuperscript{34,62} but has not yet been considered in hydrogenases. Our model rationalises how ions transcend the gap between inner and outer core of the proton transfer pathway. Furthermore, it provides a reasonable explanation for the catalytic bidirectionality of [FeFe]-hydrogenases.\textsuperscript{6–8} Glutamic acid E141 switches as hydrogen-bonding donor between W1 (proton uptake) and S189 (proton release). Deviations in distance < 0.5 Å suggest a flexible hydrogen-bonding network that facilitates both H\textsubscript{2} evolution (proton uptake) and H\textsubscript{2} oxidation (proton release).

CONCLUSIONS

In this work, we demonstrate how in situ infrared spectroscopy was applied to analyse the hydrogen-bonding network of the catalytic proton transfer pathway in [FeFe]-hydrogenases. Discontinuous proton transfer was triggered by the enhanced basicity of the active site cofactor (H-cluster) upon photoreduction of a highly active iron-sulphur enzyme lacking a natural chromophore. Infrared spectroscopy provides a direct read-out for changes in hydrogen bonding perfectly complementary to X-ray crystallography. Thereby, the first experimental description of the dynamic hydrogen-bonding changes in the catalytic proton transfer pathway of [FeFe]-hydrogenases was accomplished.

Reduction of the H-cluster induces a transient de-protonation of glutamic acid residue E141 right in the middle of the proton transfer pathway. Re-protonation of E141 by arginine residue R148 restores the conductivity of the proton transfer pathway and enables a second proton transfer event that leads to H2 evolution eventually. Our data unambiguously allow concluding on de-protonation of an arginine residue, which has never been shown in an enzyme before. In
summary, we found that the flexible hydrogen-bonding network around E141 allows [FeFe]-
hydrogenases catalysing H2 evolution and H2 uptake with similar efficiency.

METHODS

The [FeFe]-hydrogenase from C. reinhardtii HYDA1 was expressed and synthesized in E. coli,
purified by strep-tactin affinity chromatography, and activated in vitro with ADT cofactor under
anaerobic conditions. After removal of excess cofactor, the protein concentration was adjusted
to approximately 3 mM (~150 g/L). 5`-carboxy eosin Y (5CE) and EDTA were prepared in
aqueous stock solutions of 6 mM and 90 mM, respectively. One part of each component was
mixed to yield a HYDA1/5CE/EDTA ratio of 1:2:30.

All spectroscopic experiments were performed under anaerobic conditions, at room temperature,
ambient pressure, and on hydrated protein films of physiological pH values. First, 1 – 2 µL of the
reaction mix was pipetted onto the silicon crystal of the ATR unit (DuraDisc SamplIR-2, Smiths
Detection) in the FTIR spectrometer (Tensor 27, Bruker). Spectra from 3900 – 1300 cm⁻¹ were
recorded with a narrow-band MCT detector with a spectral resolution of 2 cm⁻¹ and 25
interferometer scans each. The solution was protected from stray light, dried under N₂, and
rehydrated via the gas phase with 10 mM MES buffer (pH 6). Traces of reduced and CO-
inhibited species were lost in favour of Hox after 1 – 3 h auto-oxidation, as reported earlier. The
experiment was initiated upon continuous illumination of the film at 505 nm and followed by
FTIR spectroscopy with a time resolution of 5 s (Fig. S1). After ~20 s, half of the Hox population
converted into Hred, with an overall redox efficiency of > 90%. Importantly, no other reduced
species than Hred were observed although H₂ released upon reduction of HYDA1 may re-react.
with the enzyme forming **Hred’, Hsred**, and **Hhyd**. Therefore, the continuous exchange of gas in our setup was found to be of particular importance as it precludes a build-up of multiple reduced species. No photoreduction was observed under off-resonant conditions (590 nm) whereas illumination at 455 nm induced notable cofactor corruption (Fig. S9), as noted earlier.

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LEGENDS

**Figure 1 – The proton transfer pathway of [FeFe]-hydrogenases and experimental strategy.**

(a) Hydrogen turnover is catalysed at the H-cluster that is formed by a diiron site attached to the protein via a [4Fe-4S] centre. The diiron site binds 5 – 6 CO and CN⁻ ligands, one of which that can be found in Fe-Fe bridging position (µCO). The ADT group functions as proton relay between C169 and the distal iron ion of the H-cluster, Feₐ. Residues involved in proton transfer are identified. Cartoon model of the oxidized [FeFe]-hydrogenase from CPI according to pdb entry 4XDC, numbering refers to HYDA1. (b) Continuous illumination at 505 nm of 5’-carboxy eosin Y (5CE, photosensitizer) with ethylenediaminetetraacetic acid (EDTA) as sacrificial electron donor was exploited to enrich H_red over H_ox in [FeFe]-hydrogenase HYDA1. Increasing the basicity upon reduction induces a protonation of the H-cluster. The associated changes in the hydrogen-bonding network of the catalytic proton transfer pathway are followed by *in situ* ATR FTIR difference spectroscopy.
Figure 2 – Absorbance and light-induced difference spectra of [FeFe]-hydrogenase. (a) ATR FTIR absorbance spectra of the hydrated reaction mixture (HYDA1:5CE:EDTA) in the dark and upon illumination at 505 nm (magenta). Inset: magnification of the cofactor regime. The changes show reduction of HYDA1 from Hox (black) to Hred (magenta). (b) Subtraction of single channel spectra from the same data set as in (a). The “dark – light” difference spectrum is dominated by the CO/CN bands of the H-cluster. Additionally, it allows analysing the OH, SH, and COOH regime as well as frequencies < 1500 cm$^{-1}$ comprising vibrational marker bands of photosensitizer 5CE. Inset: magnification of the COOH regime. The band changes are specific for functional HYDA1 (black) and not observed in HYDA1 apo-protein (red). * 2337 cm$^{-1}$, assigned to CO$_2$.

Figure 3 – Band fit and H/D exchange. (a) The ATR FTIR difference spectrum of the Hred – Hox conversion (solid line) was fitted with a minimum of 7 + 2 Gaussians in the COOH regime (blue traces). All frequencies are given in the legend. The red dotted line depicts the resulting envelope. (b) Comparison of ATR FTIR difference spectra on hydrated and deuterated film (black and red, respectively). The data indicates a downshifts of 6 – 14 cm$^{-1}$ for bands associated with Hox (negative intensities) and similar frequency difference for bands accumulating upon reduction (positive intensities). No significant shift was noted for the positive bands at 1721 cm$^{-1}$ and ~1670 cm$^{-1}$. See Fig. S4 for further details.

Figure 4 – Photoreduction of [FeFe]-hydrogenase variants. In situ ATR FTIR difference spectra in the COOH regime for three different amino acid residues compared to native HYDA1 (dotted traces, scaled to the 1715/1700 difference signal that was observed in all experiments). The crystallographic comparison includes pdb coordinates 4XDC, 6GM2, 6GM3, and 6GM4. (a) Arginine variant R148A lacks the positive 1721 cm$^{-1}$ feature and shows significantly diminished
contributions at 1696 and 1681 cm\(^{-1}\) ("X"). (b) Glutamic acid variant E144D exhibits a ~21 cm\(^{-1}\) upshift of the native 1721 cm\(^{-1}\) band to 1742 cm\(^{-1}\). Negative bands at 1692 and 1682 cm\(^{-1}\) suggest similarities with native enzyme whereas the band intensity at 1688 cm\(^{-1}\) is inverted. (c) The difference spectrum of serine variant S189A indicates a largely native phenotype with only smaller differences in band intensity. See Fig. S6 for further details.

**Figure 5 – The hydrogen-bonding network of the catalytic proton transport pathway.** (a) Progression of amino acid residues as observed in the crystal structure of oxidized [FeFe]-hydrogenase CPI (Hox). All distances refer to pdb coordinates 4XDC (HYDA1 numbering). Our data suggest hydrogen-bonding contacts between S189, E144, and R148\(^{+}\) of the catalytic proton transport pathway. Furthermore, invariable hydrogen-bonding between ADT, C169, and W1 was observed. The W1/E141 complex establishes the difference between inner and outer core of the catalytic proton transport pathway. (b) In reduced [FeFe]-hydrogenase (Hred), the W1/141 complex is terminated in favour of hydrogen bonding between E141, S189, and E144, which facilitates continuous proton transfer (see main text for details). Our data indicate deprotonation of R148\(^{+}\) upon formation of Hred.
