Protonation/reduction dynamics at the [4Fe–4S] cluster of the hydrogen-forming cofactor in [FeFe]-hydrogenases

Moritz Senger, Stefan Mebs, Jifu Duan, Olga Shulenina, Konstantin Laun, Leonie Kertess, Florian Wittkamp, Ulf-Peter Apfel, Martin Winkler, Michael Haumann and Sven T. Stripp

The [FeFe]-hydrogenases of bacteria and algae are the most efficient hydrogen conversion catalysts in nature. Their active-site cofactor (H-cluster) comprises a [4Fe–4S] cluster linked to a unique diiron site that binds three carbon monoxide (CO) and two cyanide (CN–) ligands. Understanding microbial hydrogen conversion requires elucidation of the interplay of proton and electron transfer events at the H-cluster. We performed real-time spectroscopy on [FeFe]-hydrogenase protein films under controlled variation of atmospheric gas composition, sample pH, and reductant concentration. Attenuated total reflection Fourier-transform infrared spectroscopy was used to monitor shifts of the CO/CN– vibrational bands in response to redox and protonation changes. Three different [FeFe]-hydrogenases and several protein and cofactor variants were compared, including element and isotopic exchange studies. A protonated equivalent (HoxH) of the oxidized state (Hox) was found, which preferentially accumulated at acidic pH and under reducing conditions. We show that the one-electron reduced state Hred represents an intrinsically protonated species. Interestingly, the formation of HoxH and Hred was independent of the established proton pathway to the diiron site. Quantum chemical calculations of the respective CO/CN– infrared band patterns favored a cysteine ligand of the [4Fe–4S] cluster as the protonation site in HoxH and Hred. We propose that proton-coupled electron transfer facilitates reduction of the [4Fe–4S] cluster and prevents premature formation of a hydride at the catalytic diiron site. Our findings imply that protonation events both at the [4Fe–4S] cluster and at the diiron site of the H-cluster are important in the hydrogen conversion reaction of [FeFe]-hydrogenases.

Introduction

Hydrogenases are enzymes that catalyze hydrogen conversion in archaea, bacteria, fungi, and algae. Among the three types of hydrogenases only [FeFe]-hydrogenases are found in eukaryotes, for example HYDA1 of the unicellular green alga Chlamydomonas reinhardtii. HYDA1 represents the ‘minimal unit’ of biological hydrogen conversion as it exclusively binds the catalytic cofactor. Prokaryotic [FeFe]-hydrogenases carry additional iron–sulfur clusters and typically stem from obligate anaerobic bacteria such as Clostridium pasteurianum (CPI) or Desulfovibrio desulfiticans (DDH). [FeFe]-hydrogenases are bidirectional enzymes that catalyze H2 oxidation and H2 release from proton reduction at negligible electrochemical overpotential. Hydrogen conversion in [FeFe]-hydrogenases was proposed to be limited by the diffusion velocity of reactants and understanding the molecular principles of hydrogen conversion in [FeFe]-hydrogenases will be an important step towards the design of bio-inspired catalysts with comparable H2 release activity. The active site cofactor of [FeFe]-hydrogenases is referred to as the H-cluster (Fig. 1). It comprises a [4Fe–4S] cluster covalently linked to a unique diiron site with a ‘proximal’ and ‘distal’ iron ion (FeP and FeD, respectively). An azadithiolate ligand (adt) bridges the two iron ions and introduces a
Hred$_2$ + 1 \text{I/II} \rightarrow 32 and 33 differ in the location of the surplus electron at the H-cluster. 29–31

CN$_2$Hox — + 2 \text{I/II} — 46–48

Hred — + 2 \text{I/II} — 25–28

Hox-CO that Fe d exhibits a vacant, apical coordination site in EPR-active.

EPR-active.

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Reactant gases (12CO, 13CO, H2 from Linde, Germany) were added to the N2 stream via separate flow controllers. A 200 mbar check valve separates the dry gas section (A) from the wet gas section (B). In order to create a reactive aerosol, the gas stream was passed through a wash bottle containing 150 mL mixed buffer (50 mM Tris, MES, PIPPS, pH 3–9) and sodium dithionite (DT) and/or methyl viologen (MV). In Fig. S1 (ESI†), we demonstrate how water-soluble ingredients are transported with the aerosol stream, i.e. to reduce MV via DT enrichment. Section (C) shows the spectroscopic set-up. The aerosol was fed into a gas-tight polychlorotrifluoroethylene (PCTFE) compartment, attached on top of the ATR crystal plate and equipped with six optional gas inlets, a manometer for pressure control, and a transparent glass window for UV/vis irradiation.\(^\text{28}\) The FTIR spectrometer (Tensor27, Bruker Optik, Germany) was equipped with a triple-reflection ZnSe/Si crystal ATR cell (Smith Detection, USA) and placed in an anaerobic chamber (Coy Laboratories, USA). Infrared spectra were recorded with 80 kHz scanning velocity at a spectral resolution of 1 cm\(^{-1}\) from 3800 to 1200 cm\(^{-1}\). Under these conditions, the time-resolution of data acquisition is in the range of 1 s (one interferometer scan in the forward/backward direction). In order to determine the timescale of equilibration in the aerosol set-up, we probed a fast process, i.e. CO inhibition.\(^\text{28}\) Fig. S2 (ESI†) shows the accumulation of Hox\(^{\text{CO}}\) upon exchange of N2 to CO gas. Here, the Hox \(\rightarrow\) Hox\(^{\text{CO}}\) conversion rate determined by ATR FTIR largely exceeds the value suggested by flash-photolysis IR spectroscopy \((t_{1/2} \approx 3\) s and 15 ms, respectively).\(^\text{58–60}\) The former represents the timescale of equilibration and defines the temporal resolution of the aerosol set-up. Note that all analyzed titrations proceeded by far slowly and were therefore not limited by the experimental time resolution. ATR FTIR measurements were performed at 25 °C air-conditioning and on hydrogenase films derived by controlled dehydration and rehydration of 1 \(\mu\)L protein sample as reported earlier (500 \(\mu\)M hydrogenase in 100 mM Tris/HCl pH 8, 100 mM NaCl, and 2 mM DT if not stated otherwise). Samples of anaerobically purified and activated [FeFe]-hydrogenase typically contained the H-cluster \(1\) from 3800 to 1200 cm\(^{-1}\), was enriched in the film under a constant stream of N2 aerosol for 30 minutes prior to the pH or redox titrations.

**Infrared data evaluation**

A quantitative analysis of infrared spectra was performed using in-house software tools (Fig. S2, ESI†). Spectra in the 1750–2150 cm\(^{-1}\) frequency range were corrected for background contributions (liquid H\(_2\)O) by subtraction of polynomial spline functions. The resulting spectra were normalized to unity area in the CO/CN\(^{-1}\) spectral region and the CO/CN\(^{-1}\) bands were fitted by Voigt functions (50% Gaussian and Lorentzian characters) for frequency, peak width, and relative intensity determination. A global analysis approach was used (simultaneous fitting of time-series of spectra with a common set of frequencies and relative intensities for each H-cluster species). The relative population of a specific H-cluster species at a given time point was derived as the fraction of its summed CO/CN\(^{-1}\) band intensities of the total (normalized) spectral intensity. We measured an experimental
uncertainty of <25% for the determination of rate constants in pH and redox titrations (Fig. S2, ESI†).

**Density functional theory calculations**

DFT calculations on model structures of the H-cluster were performed at the TPSSH functional and TZVP basis-set level of theory using Gaussian09. Starting models comprised the complete H-cluster and were constructed on the basis of the crystal structure (PDB entry 4XDC) of CPI [FeFe]-hydrogenase as described previously. In some cases, adjacent amino acids and water molecules were included (see below). Anti-ferromagnetic coupling was assigned to the H-cluster prior to geometry optimization by proper definition of molecular fragments (broken symmetry approach). Calculations involved the COSMO solvation model (ε = 4). H-cluster structures with varying protonation sites were calculated. During structural relaxation, the positions of the four carbon atoms of the S-CH₃ groups (representing the truncated terminal cysteine ligands of the H-cluster) were restraint at their crystallographic positions. A maximum deviation of ~3 cm⁻¹ was observed for the CO and CN⁻ band frequencies between calculations on constraint or unconstraint model structures (average deviation of ~1.5 cm⁻¹). Vibrational frequencies for the relaxed molecular structures were derived from normal mode analysis as implemented in Gaussian09. Summed band intensities of CO/CN⁻ ligands from the calculations were normalized to unity for comparison with experimental data. For quantification of the correlation between experimental (exp) and calculated (cal) frequencies (F) and relative band intensities (I), the root-mean-square-deviation (rmsd) was calculated (eqn (1), n = number of CO/CN⁻ bands). For band intensities, rmsdₐ values were derived using the uncorrected calculated intensities (an almost ideally linear correlation was observed for Hox \( \Delta \omega_{ox} = 0.2\% + 0.99F_{ox}^{cal} \)). For band frequencies, rmsdᵦ values were calculated after correction of the calculated frequencies for a systematic deviation from ideal correlation due to the DFT theory level. Calculated frequencies for Hox were plotted versus the experimental Hox frequencies, a linear fit was performed yielding slope \( (s_{ox} = 1.15) \) and offset \( (o_{ox} = -243 \text{ cm}^{-1}) \) values for Hox (eqn (2)). The corrected (cor) frequencies for all H-cluster species (i) were calculated using eqn (3) \( (n = \text{number of bands, i.e. } 2 \text{ CN}^{-} + 3 \text{ CO} = 5 \text{ bands for, e.g., Hox}) \) and the \( s_{ox} \) and \( o_{ox} \) values (meaning that rmsdᵦ values for all species were directly comparable to the rmsdᵦ for Hox).

\[
\text{rmsd} = \sqrt{\frac{1}{n-2} \sum_{i=1}^{n} (F_i^{cal} - F_i^{cor})^2} \\
F_i^{cal} = o_{ox} + s_{ox}F_i^{exp} \\
F_i^{cor} = (F_i^{cal} - o_{ox})/s_{ox}
\]

**Results and discussion**

**IR band shifts upon acidification of [FeFe]-hydrogenase**

Fully active [FeFe]-hydrogenase HYDA1 as reconstituted with the native aden cofactor was used to form a protein film on the ATR silicon crystal. The active-ready, oxidized state Hox was enriched to purity under a stream of humidified N₂ at pH 8 and 2 mM sodium dithionite (DT).²⁸ In contrast to earlier studies, no build-up of reduced H-cluster species was observed, most likely due to release of H₂ from the protein film. It can be assumed that the decay rate of reduced species exceeds their formation rate and the removal of H₂ via the N₂ carrier gas precludes a re-reduction of enzyme. When the film was exposed to an acidified aerosol (pH 4, 2 mM DT), the IR band pattern of Hox was lost in favor of a novel spectrum (Fig. 3A and B). We note that the indicated pH values refer to the pH of the respective buffer used in the experiments. Based on calibration experiments, a difference of approximately ~0.25 pH units between the buffer and the sample film was estimated (Fig. S3, ESI†). The novel pattern showed an up-shift of the CO/CN⁻ bands by 4-6 cm⁻¹ with similar relative intensities as in the Hox spectrum. Furthermore, the pattern comprised the characteristic low-frequency band of the μCO ligand, up-shifted by 10 cm⁻¹. The conversion was found to be fully reversible when changing the pH in the aerosol back to alkaline values (Fig. S4 and S5, ESI†). Although the HYDA1 H₂ release activity was increasingly inhibited from pH 7 to 5, no structural changes were observed in the amide region when titrating the film from pH 8 to 4 (Fig. S6, ESI†). Note that extreme conditions (pH 3, 500 mM DT) let to irreversible loss of cofactor bands (Fig. S4, ESI†). Frequency shifts by 4-10 cm⁻¹ are not compatible with redox changes at the diiron site and the shift to higher frequencies excludes a reduction of the H-cluster, e.g. by DT. Dithionite is commonly used as a reductant to promote H₂ evolution from [FeFe]-hydrogenases in solution.⁶⁵ Accordingly, we attribute the observed ‘blue shift’ to a protonation event and denote the novel species HoxH (Table 2). It is important to note that the Hox → HoxH conversion was not observed in the absence of DT (Fig. S4, ESI†). This result suggests that reducing conditions are required to induce the protonation event and the shift of cofactor bands is unrelated to protonation of amino acid residues.

Exposure of bacterial [FeFe]-hydrogenases CPIadt and native DDH to pH 4 and 2 mM DT caused a similar ‘blue shift’ of the Hox band pattern (Fig. 3C). Formation of HoxH was not affected by the accessary iron-sulfur clusters in the prakroykine enzymes or amino acid differences near the H-cluster. We note that HYDA1 and CPI apo-enzymes were artificially maturated whereas DDH was isolated from *D. desulfuricans* in the native form.⁵⁷ The formation of HoxH hence is unrelated to the maturation procedure. Besides HYDA1adt, we investigated cofactor variants with a less basic (odt, sdt) or hydrophobic (pdt, edt) bridgehead group at the diiron site. For all modified enzymes, a similar Hox → HoxH conversion at pH 4 and 2 mM DT was observed (Fig. 3D). This clearly proves that the bridgehead atom does not correspond to the protonation site. However, the cofactor variant HYDA1pdt showed an approximately 20-fold slower formation of HoxH in comparison to HYDA1adt (Fig. S5, ESI†), presumably related to the much smaller H₂ release activity of the pdt variant.⁶⁶ Protein variants with amino acid exchanges in the proton pathway towards the diiron site were studied (C169D and C169A). Although typical IR spectra and quantitative formation of HoxH were
HoxH functions as the protonation site in the C169D variant (Fig. S5, ESI†). In the C169A variant, H\textsubscript{2} release activity significantly decreased compared to wild-type HYDA1 adt or HYDA1 C169D. Table S1 (ESI†) lists the vibrational frequencies for all analyzed variants.

Fig. 3 FTIR difference spectra for the Hox \rightarrow HoxH conversion (‘blue shift’). The CO/CN\textsuperscript{−} spectral region is shown. (A) Absolute spectra for the Hox \rightarrow HoxH conversion in native HYDA1\textsuperscript{adt} obtained at pH 8 (black trace) and pH 4 in the presence of 2 mM DT (blue trace). Assignment of bands to vibrational modes was derived from DFT calculations (CN\textsuperscript{−} and CO/\mu CO marked in red). (B) Difference spectra obtained during Hox (negative bands) conversion to HoxH (positive bands) conversion in HYDA1\textsuperscript{adt} from the same data set as in (A). (C) Difference spectra for bacterial [FeFe]-hydrogenases CPI\textsuperscript{adt} and DDH\textsuperscript{adt}. (D) Difference spectra for HYDA1 cofactor variants odt, sdt, pdt, and edt. (E) Difference spectra for HYDA1\textsuperscript{adt} amino acid variants C169A and C169D. Table S1 (ESI†) lists the vibrational frequencies for all analyzed variants.

Table 2: IR band frequencies of CO and CN\textsuperscript{−} ligands in HYDA1\textsuperscript{adt}.

<table>
<thead>
<tr>
<th>H-Cluster species</th>
<th>IR frequency/cm\textsuperscript{-1}</th>
<th>Additional reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CN\textsuperscript{−} bands</td>
<td>CO bands</td>
</tr>
<tr>
<td>HoxH</td>
<td>2088 2070 1964 1940 1802</td>
<td>21, 22 and 28</td>
</tr>
<tr>
<td>HoxH\textsuperscript{H}</td>
<td>2092 2074 1970 1946 1812</td>
<td>This study</td>
</tr>
<tr>
<td>Hred\textsuperscript{a}</td>
<td>2084 2066 1962 1933 1792</td>
<td>29, 31 and 49</td>
</tr>
<tr>
<td>Hred\textsuperscript{H}</td>
<td>2086 2068 1966 1938 1800</td>
<td>This study</td>
</tr>
<tr>
<td>Hyd</td>
<td>2088 2076 1980 1960 1860</td>
<td>40</td>
</tr>
<tr>
<td>Hred \textsuperscript{a}</td>
<td>2070 2033 1963\textsuperscript{a} 1915 1891</td>
<td>21, 22 and 31</td>
</tr>
<tr>
<td>Hsred</td>
<td>2068 2026 1953\textsuperscript{a} 1918 1882</td>
<td>32</td>
</tr>
<tr>
<td>Hox-CO</td>
<td>2091 2081 1968 1962\textsuperscript{b} 1808</td>
<td>25–28</td>
</tr>
<tr>
<td>HoxH-CO</td>
<td>2094 2086 1972 1966\textsuperscript{b} 1816</td>
<td>This study</td>
</tr>
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\textsuperscript{a} The bridging carbonyl (\mu CO) is moved to a terminal position at Fe\textsubscript{a} in Hred and Hsred. \textsuperscript{b} In the CO-inhibited states, the coupled pCO/dCO stretching vibration gives rise to an additional IR band (Hox-CO: 2012 cm\textsuperscript{-1}, HoxH-CO: 2006 cm\textsuperscript{-1}). IR frequencies were determined from the spectra shown in Fig. 3, 6 and Fig. S7, S10 (ESI).

observed in both variants (Fig. 3E), the rate of HoxH formation in the C169A variant was smaller than in wild-type HYDA1\textsuperscript{adt} or in the C169D variant (Fig. S5, ESI†), ruling out that cysteine 169 functions as the protonation site in HoxH. The amino acid variant HYDA1 C169D showed H\textsubscript{2} release activity significantly similar to native enzyme (up to 80%) while HYDA1 C169A did not evolve H\textsubscript{2} in the presence of DT and MV. We therefore assume that H\textsubscript{2} release activity is correlated with the formation rate of HoxH.

Proton and reductant concentration effects on HoxH formation

Protein concentration, hydration level, and temperature affected the Hox \rightarrow HoxH conversion rate (Fig. S6, ESI†). Accordingly, a quantitative comparison demanded tight control of all experimental parameters. We determined an experimental uncertainty in the determination of conversion rates of less than 25\% (Fig. S2, ESI†) and observed negligible differences in the hydration level (Fig. S6, ESI†). Accordingly, it was possible to investigate the influence of proton and DT concentration on the yield and formation rate of HoxH. Fig. 4A shows the Hox \rightarrow HoxH conversion with individual protein films after a change of the pH. The sigmoidal time course of the Hox \rightarrow HoxH transition suggested that at least two consecutive processes are involved, i.e. proton transfer and electron transfer. In contrast, back conversion to Hox showed exponential behavior and the rate was found to be virtually independent of pH and DT (Fig. S5, ESI†). These observations might suggest that the divergent velocity of the Hox \rightarrow HoxH conversion is related to protein structural rearrangements in the ‘blue shift’ reaction. However, the formation of HoxH was not associated with changes in the protein structure or denaturation (Fig. S6, ESI†). Despite the high concentrations of DT used in some experiments, no enrichment of reduced H-cluster species was observed.

The formation rate of HoxH increased linearly with proton concentration in a pH range of 4–7 at 10 mM DT (Fig. 5A). A pH titration with a 50-fold increase of DT (500 mM) showed an overall faster Hox \rightarrow HoxH conversion and linear regression
from pH 3 to 7 (Fig. S4, ESI†). In contrast, saturation behavior of the HoxH formation rate was observed at excess DT concentrations (Fig. 5B). Accelerated formation of HoxH was also observed in the presence of the redox mediator MV (Fig. S5, ESI†) which is often used to optimize electron transfer from DT to hydrogenase enzymes.65 Fig. 5 compares the HoxH yield for pH titrations at 10 mM or 500 mM DT, respectively. Significant formation of HoxH was observed even at alkaline pH, and the apparent pK shifted from ~5.5 to ~7.3 for higher DT concentrations. The titration behavior of a protonatable group should be independent of reductant concentration. The variable, apparent pK value thus excludes a specific amino acid residue as the protonation site in HoxH.

Ligand exchange and isotope editing at the H-cluster

Further information on the formation of HoxH was obtained from CO-inhibition and isotopic exchange studies. HYDA1adt was quantitatively inhibited with 1% CO gas, as indicated by the typical IR bands of Hox-CO (Table 2).28 Acidification in the presence of 2 mM DT under 1% CO induced an overall similar ‘blue shift’ of the CO/CN/C0 bands as observed for Hox (Fig. S7, ESI†). We attribute the shifted spectrum to a protonated, CO-inhibited H-cluster species, HoxH-CO. Formation of HoxH-CO implies that protonation cannot occur at the distal iron ion because its ligand sphere was completed by the additional CO ligand already.24–28 Similar to the Hox → HoxH conversion, DT was required for the formation of HoxH-CO. Exchange of the bridging sulfur (µS) against selenium (µSe) in the [4Fe–4S] cluster of the catalytic cofactor has been shown to barely affect catalytic activity.56 Selenium-substituted HYDA1adt showed unchanged Hox and HoxH IR-band patterns. Although the pK values of thiols and selenols can differ by up to three units69–71...
and a protonation event at μSe of the [4Fe–4Se] cluster should influence the formation rate of HoxH, the latter was not found to be affected. For a distinct assignment of IR band formation of the concomitant decrease of bands at 1933 cm⁻¹ a direct denaturation from pH 8 to pH 6 (Fig. S9, ESI†). For diiron model complexes, protonation at the bridging thiolate sulfur atoms has been considered. The frequency down-shift of the S–H vibration upon deuteration from ~2550 to ~1850 cm⁻¹ could help in identifying protonation of the dithiolate ligand at the diiron site due to vibrational coupling with the CO/·CN⁻ ligands. However, formation of HoxH in the presence of 0.5% D₂SO₄ and 2 mM DT did not result in altered Hox and HoxH IR-band patterns (Fig. S9, ESI†), even when HYDA1 was isolated and activated in the presence of D₂O before film formation and acidification. A direct detection of SH/SD bands in the sample is unrelated to vibrational coupling with the cofactor ligands and was not attempted. These results render protonation of the thiolate sulfur atoms at the diiron site in HoxH less likely.

Reduction of HYDA1 with H₂

The H₂ uptake activity of HYDA1 adt was exploited to enrich reduced H-cluster species in the protein film. In the presence of 10% H₂ the IR signature of HYDA1 pdt was lost in favor of reduced species mainly attributable to reduced species (Fig. 6 and Table 2). In the H₂–N₂ difference spectrum at pH 8, prominent CO bands at 1933 cm⁻¹ and 1792 cm⁻¹ resembled a pattern previously observed in reduced HYDA1 pdt enzyme. These bands have been assigned to Hred in HYDA1 adt recently. Interestingly, we found that Hred was favored over Hred when progressing from acidic to alkaline pH, and vice versa (Fig. S10, ESI†). This can be explained by the PCET reactivity in the formation of Hred and destabilization of the reduced diiron site at alkaline pH. For a distinct assignment of IR band patterns, we exchanged the bridging and distal CO ligands in HYDA1 adt to ¹³CO, following a previously established protocol. The isotope-edited enzyme with H₂ facilitated direct assignment of the bands at 1891 cm⁻¹ and 1933 cm⁻¹ to dCO of Hred adt and Hred adt, respectively (Fig. 6). Whether the μCO band at 1792 cm⁻¹ belonged to Hred or Hred still remains unclear. To refine the assignment, HYDA1 adt was reduced under 10% H₂ and titrated from pH 8 to pH 6 (Fig. 6 and Fig. S10, ESI†). Although the H₂ concentration was kept constant, acidification caused pronounced changes in the relative population of redox species and Hsred and Hred were lost in favor of Hred and Hox. Due to the concomitant decrease of bands at 1933 cm⁻¹ (dCO) and 1793 cm⁻¹ (μCO), the experiment facilitated a clear assignment of these bands to Hred (Table 2). The deviating pH-dependence of bands at 1933 cm⁻¹ and 1891 cm⁻¹ further distinguishes Hred from Hred (Fig. S10, ESI†). In agreement with former observations, an increase of Hhyd was observed when decreasing the pH from 8 to 6 (Fig. 6). We observed no ‘blue shift’ of the marker bands of Hred (1891 cm⁻¹) and Hsred (1882 cm⁻¹) at acidic pH.

To investigate Hred in greater detail, we focused on HYDA1 cofactor variant pdt. This variant does not form Hred and Hsred and therefore represents an excellent model to study the relation between Hox and Hred. Although HYDA1 pdt has been reported to show only minimal H₂ uptake activity, we observed quantitative Hox → Hred conversion under 100% H₂ (Fig. 7). At pH 8, both states showed similar IR band patterns as in HYDA1 adt with relative frequency shifts of less than 5 cm⁻¹. When HYDA1 pdt was purged with N₂ at pH 4 and in the presence of 2 mM DT, an IR spectrum similar to the one of
HoxH was observed (compare Fig. 3). Exposure to 100% H₂ under acidic conditions resulted in a minor ‘blue shift’ of all CO/CN/C0 bands with respect to Hred₀ (Fig. 7). We denote the species responsible for this shifted spectrum as Hred₀H (Table 2). Redox titrations indicated the presence of Hred₀ in HYDA₁ adt as well, although this state was more difficult to detect in native enzyme due to competing population of Hred/Hred and Hhyd (Fig. S10, ESI†).40 When the hydrogenase concentration in the film was decreased by dilution with bovine serum albumin protein (BSA), the Hox ↔ Hred conversion rate drastically dropped for relative concentrations of HYDA₁pd below 25% and no formation of reduced states was observed with 5% HYDA₁ (Fig. S11, ESI†). This behavior suggests that the inter-molecular electron transfer between [FeFe]-hydrogenase molecules (disproportionation) was involved in the formation of one-electron reduced Hred from oxidized Hox via two-electron reduced species transiently formed upon reduction with H₂. Inefficient inter-molecular electron transfer may explain the low rates of H₂ uptake that have been reported for HYDA₁pd in solution.46 In contrast, the formation rate of HoxH was only slightly affected by dilution of HYDA₁pd with BSA (Fig. S11, ESI†). This observation rules out inter-molecular electron transfer as an explanation for the apparent absence of reduced species in the presence of high DT concentrations.

Density functional theory on H-cluster protonation

DFT calculations on H-cluster model structures (Fig. S12, ESI†) were used to explore conceivable protonation sites. Respective positions included N(adt), the μS atoms in the diiron site (S1, S2) and in the [4Fe–4S] cluster (S3–S6), as well as the sulfur atoms of the three terminal cysteine ligands (S8–S10). Good agreement between the experimental and calculated CO/CN band patterns of Hox was obtained, as judged on the basis of the small root-mean-square-deviations (rmsd) derived from experimental and calculated band frequencies (f) and intensities (I), i.e. rmsdf ∼ 8 cm⁻¹ and rmsdi < 3% (Fig. 8). We note that relative band positions and intensities of all investigated H-cluster species were largely independent of the size of the model structure and geometry-optimization restraints that were
used in the calculations (Fig. S13, ESI†). Protonation at N(adt) or S1/S2 at the diiron site caused frequency up-shifts three to four fold larger than experimentally observed for HoxH (compare Table 1). The large rmsd values (15–25 cm⁻¹) suggested that these groups are unlikely protonation sites (Fig. S14, ESI†). Protonation at the μS atoms of the [4Fe–4S] cluster caused band shifts in better agreement with the experiment (rmsdμ 8–11 cm⁻¹). However, protonation of S3 or S5 produced a downshift of the pCO band and intensity changes less compatible with the experimental observations. Furthermore, protonation at S4 or S6 caused considerable distortion of the [4Fe–4S] cluster (e.g. >0.5 Å elongation of the S4/S6-Fe bonds), which rendered μS atom protonation at the [4Fe–4S] cluster less likely. This interpretation is in line with previous theoretical results on [4Fe–4S] clusters.⁷⁶ Protonation at S10 of a terminal cysteine ligand yielded a frequency down-shift of the pCO band not observed in the experiment. In contrast, good agreement was found for protonation at S8 or S9 (rmsdμ ~ 7 cm⁻¹). Best overall agreement with the HoxH band pattern was observed for protonation at S9 (Fig. 8). Protonation at water molecules in the vicinity of the [4Fe–4S] cluster or at the bridging cysteine S7 caused significantly larger IR band shifts than protonation at S9 (Fig. S14, ESI†). Notably, deuteration at S9 caused negligible CO/CN⁻ band shifts (<1 cm⁻¹), in good agreement with the experimental data of HoxH in D₂O buffer and HoxH-CO (Fig. S14 and S15, ESI†).

Infrared spectra were calculated for H-cluster structures of the one-electron reduced Hred⁰ state. A Hox-like structure with a μCO ligand and apical vacancy at the distal iron ion yielded a mean frequency down-shift of ~35 cm⁻¹ of the CO/CN⁻ bands, far larger than experimentally observed (Fig. S16, ESI†). Excess frequency down-shifts (rmsdμ > 30 cm⁻¹) were also observed for protonation of the reduced H-cluster at N(adt) or S1/S2. Inclusion of an apical hydride at the distal iron ion produced excess frequency up-shifts (rmsdμ ~ 40 cm⁻¹), in particular for the μCO ligand, resembling the μCO shift in Hlyyd.³⁷–⁴⁰ Protonation at one of the sulfur atoms of the [4Fe–4S] cluster yielded small IR band down-shifts in good agreement with the experiment, but led to structural distortions, whereas protonation at a cysteine sulfur preserved the cubane geometry in Hred⁰. Best agreement between calculated and experimental IR frequencies of Hred⁰ was obtained for protonation at S9 (rmsdμ ~ 9 cm⁻¹, see Fig. 8). These results showed that the IR spectrum of Hred⁰ is well compatible with a one-electron reduced H-cluster, which carries an additional proton and adopts a similar geometry to Hox. Structures for Hred'H were calculated comprising a second-site protonation. Similar trends with respect to structural changes and IR patterns in response to protonation at various sites as for HoxH and Hred⁰ were observed (Fig. S16, ESI†). Protonation at two cysteine S-atoms of the [4Fe–4S] cluster (i.e., S8 and S9) yielded IR frequencies in good agreement with the Hred'H spectrum (Fig. 8). Accordingly, Hred'H was attributed to a double-protonated H-cluster species with the same electron count and cofactor geometry as Hred⁰.

Good agreement between calculated and experimental IR band patterns for Hox/HoxH, and Hred/Hred'H facilitated assignment of the IR bands of the CO/CN⁻ ligands to individual vibrational modes. In addition, our simulations provided access to the spin and charge distribution of the cofactor (Fig. S17, ESI†). Protonation (i.e. at S9) and protonation/reduction affected the largely uncoupled behavior of the diatomic ligand vibrations only marginally. In the four states, the spin density distribution at the diiron site was similar, showing that the apparent mixed-valence Fe(i)Fe(ii) configuration was preserved. The net spin thus resided largely on the diiron site in Hox/HoxH, in agreement with EPR data.⁴⁶–⁴⁸ For Hred/Hred'H, the spin density implied that the surplus electron was localized mainly at the [4Fe–4S] cluster, as supported by the increased negative charge density on the [4Fe–4S] cluster. Anti-ferromagnetic coupling between the unpaired spins at the diiron site and at the reduced [4Fe–4S] cluster was expected to render these states EPR-silent.²⁹ The minor charge variations at the iron ions of the diiron site correlate well with the small experimental IR band frequency differences of the CO ligands in the four states (Fig. S17, ESI†).

### Protonation at the H-cluster

Real-time ATR FTIR spectroscopy on [FeFe]-hydrogenase protein films identified four states of the H-cluster that show similar CO/CN⁻ band patterns but differ in the redox and/or protonation state (Hox/HoxH and Hred/Hred'H). The oxidized state, Hox, has been characterized extensively. In agreement with crystallographic assignments⁴⁷ and previous FTIR studies,²¹–²⁸ DFT favored a μCO ligand and an apical vacancy at the distal iron ion in Hox.⁷⁷ Our calculations suggest similar cofactor structures in all four species. Furthermore, both Hox and HoxH can be assigned to a mix-valence diiron site with an oxidized [4Fe–4S] cluster⁴⁶–⁴⁸ while in Hred⁰ and Hred'H a reduced [4Fe–4S] cluster was identified.²⁹ The mixed-valence state clearly distinguishes Hred⁰ from Hred and Hsred (see Table 1). While Hox represents the unprotonated H-cluster, protonation at the diiron site has been suggested for Hred⁰ and Hsred.³¹–³⁴,³⁸ We found that a satisfactory calculation of the Hred'H band pattern also requires addition of a proton. This view is supported by the experimentally observed pH-dependence of Hred'H formation in both HYDA1pdH and HYDA1adH. However, protonation of the diiron site or at surrounding amino acid residues cannot explain the observed IR band shifts. Our computational results favor a terminal cysteine ligand of the [4Fe–4S] cluster as a common protonation site in HoxH and Hred/Hred'H. In HYDA1, cysteine 417 (S9) is the most likely candidate. Additional protonation occurs in Hred'H, presumably at C170 (S8). Iron–sulfur clusters are typically involved in electron transfer processes, but their participation in proton transfer has been documented as well.⁷⁸–⁸² More specifically, protonation at a terminal cysteine ligand has been proposed to be involved in the catalytic mechanism of [NiFe]-hydrogenases.⁸³–⁸⁶

Formation of HoxH and Hred/Hred'H was found to be independent of the proton pathway to the active site that includes C169, N(adt), and Fe(II). The crystal structure of the HYDA1 apoprotein⁸⁷ suggests that the [4Fe–4S] cluster in HYDA1 is in direct contact with bulk water while in bacterial [FeFe]-hydrogenases, the H-cluster is buried deeper in the catalytic domain.⁴⁷ However, a chain of water molecules extends from the surface close to the cysteine that corresponds to our favored protonation site at the
[4Fe–4S] cluster (Fig. S18, ESIf). Two proton trajectories thus may be present in [FeFe]-hydrogenases: the 'catalytic' proton pathway that shuttles protons to the diiron site and a pathway that facilitates protonation at the [4Fe–4S] cluster.

**Reaction sequence of electron and proton transfer**

While the exponential $H_{ox}H \rightarrow H_{ox}$ conversion implies a direct deprotonation of the [4Fe–4S] cluster, the sigmoidal time course of $H_{ox}H$ formation suggests at least two successive reaction steps to be involved in the ‘blue shift’ reaction. $H_{ox}H$ was found to accumulate under $H_2$ release conditions, that is, for increasing concentrations of the reductant (such as the one-electron donor sodium dithionite, DT). This places $H_{ox}H$ towards the end of a multi-step reaction cycle (Fig. S19, ESIf).

Under conditions of maximal $H_2$ release (e.g. pH 7–8, 200 mM DT as in Fig. S6, ESIf) the prevalence of $H_{red}$ over $H_{red}'$ suggests $H_{red}'$ as an intermediate in the $H_{ox} \rightarrow H_{ox}H$ conversion. $H_{ox}$ dominates at alkaline pH so that we assume that deprotonation of $H_{ox}H$ is required to regain $H_{ox}$. In contrast, the quantitative accumulation of $H_{ox}H$ suggests that this deprotonation was slow or impaired at low pH. Numerical simulations show that the above considerations can qualitatively reproduce the pH and DT dependence of $H_{ox}H$ formation (Fig. S20, ESIf). This model also consistently explains the formation of $H_{oxH}-CO$ due to reductive conversion of $H_{oxH}$ into $H_{red}'$, $H_2$ release, and subsequent rebinding of CO to $H_{oxH}$ (Fig. S7, ESIf). It has been shown that reduction of the [4Fe–4S] cluster weakens the binding of external CO.4–7

Our data provide evidence that protonation at the same cysteine ligand of the [4Fe–4S] cluster occurred in both $H_{ox}H$ and $H_{red}'$. We assume that this protonation occurs from a putative donor group (BH’), such as a water molecule consistently observed in CPI and DDH structures (Fig. S18, ESIf).4–7 Deprotonation of $H_{oxH}$ likely involves the same molecule, now serving as a proton acceptor (B). Re-protonation of this group at acidic pH (i.e. via the adjacent chain of water molecules) would impair the deprotonation reaction so that $H_{oxH}$ accumulates (Fig. S19, ESIf). $H_{red}'$ is populated upon reduction and protonation of $H_{oxH}$ at low pH, but seems not to advance further in the reaction cycle, possibly because second-site protonation interferences with the $H_2$ forming reactions. Further studies are required to clarify this point. Accumulation of $H_{red}'$ under $H_2$ likely involves inter-molecular electron transfer between oxidized (and transiently formed) two-electron reduced [FeFe]-hydrogenase proteins (either in the $H_{sred}$ or $H_{hyd}$ states). We note that inter-molecular electron transfer seems to require a densely packed protein film and is unlikely to occur under physiological conditions.

**Conclusions**

In contrast to $H_{ox}$ and $H_{red}'$, $H_{red}$ and $H_{sred}$ carry an additional electron at the diiron site and lack the Fe–Fe bridging CO ligand.4–7 Respective ligand rotation into a terminal or “semi-bridging” position has been considered difficult to reconcile with the large hydrogen turnover rates of [FeFe]-hydrogenases.4–7 An involvement of $H_{red}$ and $H_{sred}$ in hydrogen turnover is certainly not excluded at present.31,38,60 However based on the differences in ligand orientation (bridging vs. terminal), these states can be considered unlikely intermediates in the reaction cycle of rapid $H_2$ formation.34 In HYDA1 hesitant, formation of $H_{red}$ was suppressed at alkaline pH in favor of $H_{red}'$ while for acidic pH values $H_{red}$ dominated over $H_{red}'$. Thus, the relative population of $H_{red}$ and $H_{red}'$ in HYDA1 critically depends on pH. This suggests that the rates of proton delivery either to the [4Fe–4S] cluster or to the diiron site may be decisive for the location of electrons at the H-cluster. In addition to the ‘catalytic’ proton pathway towards the diiron site,50–55 we propose a ‘regulatory’ proton pathway to the [4Fe–4S] cluster. Concomitant protonation and reduction at the [4Fe–4S] cluster in $H_{red}'$ stabilize the mixed-valence state and prevent ligand rotation and premature protonation at the diiron site. Whether this is a sequential or concerted process remains to be elucidated.58–90

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

The authors thank J. Fontecilla-Camps (Institut de Biologie Structurale, Grenoble) for generously providing a sample of DHH protein and P. Chernev (Freie Universität Berlin) for kindly providing data analysis software. M. S. and T. S. thank the International Max Planck Research School (IMPRS) on Multiscale Biosystems and the Focus Area NanoScale (Freie Universität Berlin) for financial support. M. H. gratefully acknowledges funding by the Bundesministerium für Bildung und Forschung (Grant 05K14KE1). M. W. and T. H. thank the Volkswagen Foundation for funding (Grant LighH2) and the Deutsche Forschungsgemeinschaft (DFG) for support within the Cluster of Excellence RESOLV (EXC 1069). U.-P. A. gratefully acknowledges financial support by the Fonds der Chemischen Industrie (Liebig Grant) and the DFG (Emmy Noether Grant AP242/2-1). J. D. thanks the China Scholarship Council (CSC) for support and L. K. acknowledges funding by the Fonds der Chemischen Industrie (Kekulé Mobility Fellowship).

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