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

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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 is 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 Desulvobibrio 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

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potential nitrogen base in close proximity to Fe₄.18–20 The metal ligand sphere is complemented by a terminal carbonyl (CO) and cyanide (CN⁻/CO) ligand at Fe₃ and Fe₄. In the following, pCO/CN⁻ and dCO/CN⁻ refer to the terminal ligands in the proximal or distal position. A third carbonyl ligand is found in the Fe–Fe bridging position (µCO), for example in the active-ready, oxidized state of the cofactor (Hox).21–23 Crystal structures have shown that Fe₄ exhibits a vacant, apical coordination site in EPR-active.

Fig. 1 Active site and catalytic cofactor in [FeFe]-hydrogenases. The crystal structure corresponds to the active-ready, oxidized state (Hox) of bacterial [FeFe]-hydrogenase CPI (PDB entry 4XDC).6 The catalytic cofactor comprises a [4Fe–4S] cluster and a diiron site, [FeFe], and Fe₃ and Fe₄ – proximal and distal iron ions. µCO–Fe₃–Fe₄ bridging CO ligand. N(adt) – nitrogen headgroup of the adt ligand. The dashed circle highlights an apical vacancy at Fe₄. C169 is a conserved cysteine residue that couples the H-cluster to the proton transfer pathway. Sulfur atoms are numbered 1–10 according to the DFT model (see the ESI†).

Table 1 Electronic configuration of different redox species

<table>
<thead>
<tr>
<th>Redox species</th>
<th>Δe⁻</th>
<th>[4Fe–4S] cluster</th>
<th>Diiron site</th>
<th>Alternative annotation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hox</td>
<td>—</td>
<td>+2</td>
<td>I/II</td>
<td>—</td>
<td>46–48</td>
</tr>
<tr>
<td>Hox-CO</td>
<td>—</td>
<td>+2</td>
<td>I/II</td>
<td>—</td>
<td>25–28</td>
</tr>
<tr>
<td>Hred¹</td>
<td>1</td>
<td>+1</td>
<td>I/II</td>
<td>H’red¹, Hred</td>
<td>29, 31 and 49</td>
</tr>
<tr>
<td>Hhyd</td>
<td>2</td>
<td>+1</td>
<td>I/II</td>
<td>—</td>
<td>37–40</td>
</tr>
<tr>
<td>Hred</td>
<td>1</td>
<td>+2</td>
<td>I/II</td>
<td>HredH⁺</td>
<td>21, 22 and 31</td>
</tr>
<tr>
<td>Hsred</td>
<td>2</td>
<td>+1</td>
<td>I/II</td>
<td>—</td>
<td>32 and 33</td>
</tr>
</tbody>
</table>

Formal charge is given for Fe₄/Fe₃. Only the mixed-valence state I/II is EPR-active.

was suggested.33,34 Reorientation of µCO has been discussed also for Hred.52,31,33–36 In amino acid variants of HYDA1, another two-electron reduced species has been observed. Hhyd carries a µCO ligand and was suggested to bind a terminal hydride in the apical position at Fe₄.37–40 It needs to be clarified whether and how the various redox species are involved in the catalytic cycle.41–45

Exchange of protons between the [FeFe]-hydrogenase active site and the aqueous solvent is supported by a chain of phylogenetically conserved amino acids. In HYDA1, the putative proton transfer pathway comprises residues E144, S189, E141, C169, and at least one water molecule.50–52 Side-directed mutagenesis studies identified C169 as the primary proton relay to the diiron site of the H-cluster51 and proton transfer is further facilitated by the N(adt) group (Fig. 1).18–26,35 Theoretical studies support a rapid exchange of protons via this ‘catalytic’ pathway.53–55 However, the interplay between proton transfer and electron transfer events in the hydrogen conversion reaction of [FeFe]-hydrogenases remains largely unclear.

Here, we describe in situ, real-time spectroscopy on [FeFe]-hydrogenase protein films under controlled variation of gas composition, sample pH, and reductant concentration. Shifts of the CO/CN⁻ vibrational bands were used to study electron and proton transfer at the active site via attenuated total reflection Fourier-transform infrared spectroscopy (ATR FTIR). Vibrational band patterns were characterized for three different [FeFe]-hydrogenases, proteins with amino acid exchanges in the proton path, and five cofactor variants comprising alternative dithiolate groups. Redox and pH titrations, element and isotope exchange studies, and density functional theory (DFT) calculations identified novel H-cluster species, most likely protonated at the [4Fe–4S] cluster.

**Experimental**

**Protein sample preparation**

[FeFe]-hydrogenase HYDA1 and CPI apo-proteins were over-expressed in *Escherichia coli*, purified, quantitatively reconstituted in vitro with synthetic diiron complexes (Fe₂x(CO)₃(CN)₂), and concentrated to 500 μM protein (~ 25 g L⁻¹).20 The typical H₂ release activity was ~ 800 μmol H₂ mg⁻¹ HYDA1adt min⁻¹ and ~ 2500 μmol H₂ mg⁻¹ CPIadt min⁻¹. Analyzed cofactor variants included adt = (SCH₂)₂NH, odt = (SCH₂)₂O, sdt = (SCH₂)₂S, pdt = S₂(CH₂)₃, or edt = S₂(CH₂)₃ as bridging dithiolate groups (x) at the diiron site. Site-directed mutagenesis of HYDA1 at position 169 yielded variants C169D and C169A as described earlier.50 Selenium substitution at the [4Fe–4S] cluster was performed as recently published.56 Wildtype DDH enzyme was purified from *D. desulfuricans* in a functional form.57 All protein preparation and handling procedures were carried out at room temperature, under strictly anoxic conditions, and dim light.

**Fourier-transform infrared spectroscopy**

Fig. 2 shows a schematic drawing of the experimental ATR FTIR set-up for exposure of protein films to an aerosol with defined humidity, pH, and reactant concentration. In section (A), gasses
Sierra, USA). Reactant gases (12CO, 13CO, H2 from Linde, Germany) were adjusted using digital mass flow controllers (MFC, SmartTrak, ultra-high purity (5.0) was provided by a nitrogen generator (PN1450, 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.²⁸ Fig. S2 (ESI†) shows the accumulation of Hox-CO upon exchange of N2 to CO gas. Here, the Hox → Hox-CO conversion rate determined by ATR FTIR largely exceeds the value suggested by flash-photolysis IR spectroscopy (τ₁/₂ ≈ 3 s and 15 ms, respectively).²⁸⁻⁶⁰ 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 μL protein sample as reported earlier (500 μM 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 species at a given time point was derived as a specific H-cluster species at a given time point was derived as a sum of the contributions (liquid H2O) by subtraction of polynomial spline functions. The resulting spectra were normalized to unity area in the CO/CN⁻ frequency range were corrected for background contributions (liquid H2O) by subtraction of polynomial spline functions. The resulting spectra were normalized to unity area in the CO/CN⁻ spectral region and the CO/CN⁻ 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⁻ band intensities of the total (normalized) spectral intensity. We measured an experimental value 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.²⁸ Fig. S2 (ESI†) shows the accumulation of Hox-CO upon exchange of N2 to CO gas. Here, the Hox → Hox-CO conversion rate determined by ATR FTIR largely exceeds the value suggested by flash-photolysis IR spectroscopy (τ₁/₂ ≈ 3 s and 15 ms, respectively).²⁸⁻⁶⁰ 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 μL protein sample as reported earlier (500 μM 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 species at a given time point was derived as a specific H-cluster species at a given time point was derived as a sum of the contributions (liquid H2O) by subtraction of polynomial spline functions. The resulting spectra were normalized to unity area in the CO/CN⁻ spectral region and the CO/CN⁻ 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⁻ band intensities of the total (normalized) spectral intensity. We measured an experimental
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 Gaussian09.61–64 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.28 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 restrained at their crystallographic positions.6 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 bands, i.e. 2 CN⁻ plus 3 CO = 5 bands for, e.g., Hox) and the sox and oox values (meaning that rmsd values for all species were directly comparable to the rmsdF for Hox).

\[
\text{rmsd} = \sqrt{\frac{\sum_{i=1}^{n} (F_i^{\text{exp}} - F_i^{\text{cal}})^2}{n}}
\] (1)

\[
F_i^{\text{cal}} = o_{ox} + s_{ox}F_i^{\exp}
\] (2)

\[
F_i^{\text{exp}} = (F_i^{\text{cal}} - o_{ox})s_{ox}
\] (3)

Results and discussion

IR band shifts upon acidification of [FeFe]-hydrogenase

Fully active [FeFe]-hydrogenase HYDA1 as reconstituted with the native adt cofactor19,20 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 studies33,32 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 DDD 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 accessory iron–sulfur clusters in the prokaryotic enzymes or amino acid differences near the H-cluster. We note that HYDA1 and CPI apo-enzymes were artificially maturated19,20 whereas DDD 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 (adt, std) 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
observed in both variants (Fig. 3E), the rate of HoxH formation in the C169A variant was smaller than in wild-type HYDA1adt 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 H2 release activity significantly similar to native enzyme (up to 80%) while HYDA1 C169A did not evolve H2 in the presence of DT and MV. We therefore assume that H2 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 → 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 → HoxH conversion with individual protein films after a change of the aerosol from pH 8 to pH 6–4 with 10 mM DT. To elucidate the influence of DT, HoxH formation was monitored in a concentration range of 0–1000 mM DT at pH 6 (Fig. 4B). Aqueous solutions of DT were freshly prepared and the pH was checked before and after each experiment, which excluded any decomposition of DT. Additional experiments with the highest available DT concentration showed no enrichment of reduced HoxH when the pH was increased from pH 6 to pH 8 (Fig. 4B). Aqueous solutions of DT were freshly prepared and the pH was checked before and after each experiment, which excluded any decomposition of DT.67,68 Clearly, the formation rate of HoxH depends on the DT concentration as well. The sigmoidal time course of the Hox → 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 ↔ 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 → HoxH conversion and linear regression

Table 2: IR band frequencies of CO and CN− ligands in HYDA1adt

<table>
<thead>
<tr>
<th>H-Cluster species</th>
<th>CO bands</th>
<th>CN− bands</th>
<th>Additional reference</th>
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<tr>
<td>HoxH</td>
<td>2088</td>
<td>2070</td>
<td>1964 1940 1802 21 22 28</td>
</tr>
<tr>
<td>HoxH′</td>
<td>2092</td>
<td>2074</td>
<td>1970 1946 1812 This study</td>
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<td>Hred′</td>
<td>2084</td>
<td>2066</td>
<td>1962 1933 1792 29 31 49</td>
</tr>
<tr>
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<td>2086</td>
<td>2068</td>
<td>1966 1938 1800 This study</td>
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<td>2070</td>
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<td>1961 1941 1891 21 22 31</td>
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<td>2068</td>
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<td>1953 1918 1882 32</td>
</tr>
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<td>1968 1962 1808 25 28</td>
</tr>
<tr>
<td>HoxH-Co</td>
<td>2084</td>
<td>2086</td>
<td>1972 1966 1816 This study</td>
</tr>
</tbody>
</table>

a The bridging carbonyl (μCO) is moved to a terminal position at FeS in Hred and Hsred. b In the CO-inhibited states, the coupled pCO/dCO stretching vibration gives rise to an additional IR band (HoxH-CO: 2012 cm−1, HoxH-CO: 2006 cm−1). IR frequencies were determined from the spectra shown in Fig. 3, 6 and Fig. S7, S10 (ESI).
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− 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 \([4\text{Fe}–4\text{Se}]\) cluster should influence the formation rate of \(\text{HoxH}\), the latter was not found to be affected (Fig. S8, ESI†). Therefore, direct protonation of the bridging sulfur atoms in the \([4\text{Fe}–4\text{S}]\) cluster seems to be less likely. Exposure of \([\text{FeFe}]-\text{hydrogenase}\) to an aerosol containing \(\text{D}_2\text{O}\) instead of \(\text{H}_2\text{O}\) facilitated quantitative deuteration of the protein film (Fig. S9, ESI†). For diiron model complexes, protonation at the bridging thiolate sulfur atoms has been considered.72–74 The frequency down-shift of the S–H vibration upon deuteration77 from \(~2550\) to \(~1850\ \text{cm}^{-1}\) could help in identifying protonation of the dithiolate ligand at the diiron site due to vibrational coupling with the CO/\(\text{CN}^-\) ligands. However, formation of \(\text{HoxH}\) in the presence of 0.5% \(\text{D}_2\text{SO}_4\) and 2 mM DT did not result in altered \(\text{Hox}\) and \(\text{HoxH}\) IR-band patterns (Fig. S9, ESI†), even when HYDA1 was isolated and activated in the presence of \(\text{D}_2\text{O}\) 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 \(\text{HoxH}\) less likely.

**Reduction of HYDA1 with \(\text{H}_2\)**

The \(\text{H}_2\) uptake activity of HYDA1\textsuperscript{adt} was exploited to enrich reduced H-cluster species in the protein film. In the presence of 10% \(\text{H}_2\), the IR signature of \(\text{Hox}\) was lost in favor of band patterns mainly attributable to reduced species (Fig. 6 and Table 2). In the \(\text{H}_2\text{–N}_2\) difference spectrum at pH 8, prominent CO bands at 1933 cm\(^{-1}\) and 1792 cm\(^{-1}\) resembled a pattern previously observed in reduced HYDA1\textsuperscript{pdt} enzyme.29 These bands have been assigned to \(\text{Hred}\) in HYDA1\textsuperscript{adt} recently.31,49 Interestingly, we found that \(\text{Hred}^+\) was favored over \(\text{Hred}\) 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 \(\text{Hred}\) and destabilization of the reduced diiron site at alkaline pH.31 For a distinct assignment of IR band patterns, we exchanged the bridging and distal \(^{13}\text{CO}\) ligands in HYDA1\textsuperscript{adt} to \(^{13}\text{CO}\), following a previously established protocol.28 Reduction of the isotope-edited enzyme with \(\text{H}_2\) facilitated direct assignment of the bands at 1891 cm\(^{-1}\) and 1932 cm\(^{-1}\) to \(\text{dCO}\) of \(\text{Hred}\) and \(\text{Hred}^+\), respectively (Fig. 6). Whether the \(^{13}\text{CO}\) band at 1792 cm\(^{-1}\) belonged to \(\text{Hred}\) or \(\text{Hred}^+\) still remains unclear. To refine the assignment, HYDA\textsuperscript{adt} was reduced under 10% \(\text{H}_2\) and titrated from pH 8 to pH 6 (Fig. 6 and Fig. S10, ESI†). Although the \(\text{H}_2\) concentration was kept constant, acidification caused pronounced changes in the relative population of redox species and \(\text{Hsred}\) and \(\text{Hred}^+\) were lost in favor of \(\text{Hred}\) and \(\text{Hox}\). Due to the concomitant decrease of bands at 1933 cm\(^{-1}\) (dCO) and 1793 cm\(^{-1}\) (\(^{13}\text{CO}\)), the experiment facilitated a clear assignment of these bands to \(\text{Hred}^+\) (Table 2). The deviating pH-dependence of bands at 1933 cm\(^{-1}\) and 1891 cm\(^{-1}\) further distinguishes \(\text{Hred}^+\) from \(\text{Hred}\) (Fig. S10, ESI†). In agreement with former observations,\textsuperscript{10} an increase of \(\text{Hhyd}\) was observed when decreasing the pH from 8 to 6 (Fig. 6). We observed no ‘blue shift’ of the marker bands of \(\text{Hred}\) (1891 cm\(^{-1}\)) and \(\text{Hsred}\) (1882 cm\(^{-1}\)) at acidic pH.

To investigate \(\text{Hred}^+\) in greater detail, we focused on HYDA1 cofactor variant pdt. This variant does not form \(\text{Hred}\) and \(\text{Hsred}\) and therefore represents an excellent model to study the relation between \(\text{Hox}\) and \(\text{Hred}^+\).29 Although HYDA1\textsuperscript{pdt} has been reported to show only minimal \(\text{H}_2\) uptake activity,\textsuperscript{66} we observed quantitative \(\text{Hox} \rightarrow \text{Hred}^+\) conversion under 100% \(\text{H}_2\) (Fig. 7). At pH 8, both states showed similar IR band patterns as in HYDA1\textsuperscript{adt} with relative frequency shifts of less than 5 cm\(^{-1}\). When HYDA1\textsuperscript{pdt} was purged with \(\text{N}_2\) at pH 4 and in the presence of 2 mM DT, an IR spectrum similar to the one of

![Fig. 6 Reduction of HYDA1\textsuperscript{adt} in the presence of \(\text{H}_2\). (A) IR difference spectra in the CO region collected during a change from 100% \(\text{N}_2\) to 10% \(\text{H}_2\) on native (top) and \(^{13}\text{CO}\)-edited HYDA1\textsuperscript{adt} (bottom). Spectra show an increase of \(\text{Hred}\) (green) and \(\text{Hred}^+\) (red) at the expense of \(\text{Hox}\) (black). Isotope editing of the \(^{13}\text{CO}\) and \(\text{dCO}\) ligands leads to \(~40\ \text{cm}^{-1}\) frequency down-shifts in \(\text{Hox}, \text{Hred}\), and \(\text{Hred}^+\). (B) Difference spectra for changing the pH from 8 to 6 in the presence of 10% \(\text{H}_2\) for absolute spectra see Fig. S10, ESI†). Here, \(\text{Hox}\) (green) and \(\text{Hox}\) (black) are populated at the expense of \(\text{Hred}^+\) (red) and \(\text{Hsred}\) (magenta). Note the additional formation of \(\text{Hhyd}\) (grey) and decrease of the minor \(\text{Hox}\)-CO population (asterisks).](image-url)
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 HYDA₁pd as well, although this state was more difficult to detect in native enzyme due to competing population of Hred/Hsred 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†). 40 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). 28 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

![Fig. 7 Reduction of HYDA₁pd in the presence of H₂. IR difference spectra collected during a change in the aerosol from N₂ to H₂. (A) At pH 8 and 2 mM DT, Hox (black) was found to be de-populated in favor of Hred' (red). (B) At pH 4 and 2 mM DT, HoxH (blue) converted into Hred'H (orange).](image1)

![Fig. 8 Comparison of IR spectra from experiment and theory. (A) Plots of calculated (cal) vs. experimental (exp) IR frequencies and intensities (inset) for Hox, HoxH, Hred', and Hred'H. (B) Experimental normalized IR spectra (black thin lines) and fit curves (smooth lines). (C) Calculated normalized IR spectra (from DFT at the TPSSH/TZVP level and after correction of band frequencies for systematic effects, eqn (1)–(3)). Dashed lines in (B) and (C) emphasize band shifts relative to Hox; CN⁻ bands were scaled by factors of 2 (exp) or 4 (cal) for clarity. Experimental and calculated IR data of Hox-CO and HoxH-CO are shown in Fig. S15 (ESI†).](image2)
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 rmsdF 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 (rmsdF 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 (rmsdF 7–9 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 Hred state. A Hox-like structure with a μS 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 (rmsdF > 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 (rmsdF ~ 40 cm⁻¹), in particular for the μSCO ligand, resembling the μSCO shift in Hhyd.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 Hred'. Best agreement between calculated and experimental IR frequencies of Hred' was obtained for protonation at S9 (rmsdF ~ 9 cm⁻¹, see Fig. 8). These results showed that the IR spectrum of Hred' 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 Hred' 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 Hred'.

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 resides 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 assignments⁴⁶–⁷⁷ and previous FTIR studies,²¹–²⁸ DFT favored a μSCO 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 Hred' and Hred'H a reduced [4Fe–4S] cluster was identified.²⁹ The mixed-valence state clearly distinguishes Hred' from Hred' and Hsred' (see Table 1). While Hox represents the unprotonated H-cluster, protonation at the diiron site has been suggested for Hred' and Hsred'.³¹–³⁴,³⁸ We found that a satisfactory calculation of the Hred' band pattern also requires addition of a proton. This view is supported by the experimentally observed pH-dependence of Hred' formation in both HYDA1pdT and HYDA1Nat. 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.²⁸–⁸² 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 Fe₄. The crystal structure of the HYDA1 apo-protein suggested 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.⁴⁻⁵ However, a chain of water molecules extends from the surface close to the cysteine that corresponds to our favored protonation site at the
An involvement of $H_{\text{red}}$ and $H_{\text{sred}}$ in hydrogen turnover is certainly not excluded at present. 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. In HYDA1$^{\text{adt}}$, formation of $H_{\text{red}}$ was suppressed at alkaline pH in favor of $H_{\text{red}}'$ while for acidic pH values $H_{\text{red}}$ dominated over $H_{\text{red}}'$. Thus, the relative population of $H_{\text{red}}$ and $H_{\text{red}}'$ in HYDA1$^{\text{adt}}$ 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, we propose a ‘regulatory’ proton pathway to the [4Fe–4S] cluster. Combinant protonation and reduction at the [4Fe–4S] cluster in $H_{\text{red}}'$ 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.

Conflicts of interest

There are no conflicts to declare.

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Conclusions

In contrast to $H_{\text{ox}}$ and $H_{\text{red}}'$, $H_{\text{red}}$ and $H_{\text{sred}}$ carry an additional electron at the diiron site and lack the Fe–Fe bridging CO ligand. 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. An involvement of $H_{\text{red}}$ and $H_{\text{sred}}$ in hydrogen turnover is certainly not excluded at present. 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. In HYDA1$^{\text{adt}}$, formation of $H_{\text{red}}$ was suppressed at alkaline pH in favor of $H_{\text{red}}'$ while for acidic pH values $H_{\text{red}}$ dominated over $H_{\text{red}}'$. Thus, the relative population of $H_{\text{red}}$ and $H_{\text{red}}'$ in HYDA1$^{\text{adt}}$ 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, we propose a ‘regulatory’ proton pathway to the [4Fe–4S] cluster. Combinant protonation and reduction at the [4Fe–4S] cluster in $H_{\text{red}}'$ 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.

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