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Site-selective protonation of the one-electron reduced cofactor in [FeFe]-hydrogenase†

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Hydrogenases are bidirectional redox enzymes that catalyze hydrogen turnover in archaea, bacteria, and algae. While all types of hydrogenase show H_2 oxidation activity, [FeFe]-hydrogenases are excellent H_2 evolution catalysts as well. Their active site cofactor comprises a [4Fe–4S] cluster covalently linked to a diiron site equipped with carbon monoxide and cyanide ligands. The active site niche is connected with the solvent by two distinct proton transfer pathways. To analyze the catalytic mechanism of [FeFe]-hydrogenase, we employ *operando* infrared spectroscopy and infrared spectro-electrochemistry. Titrating the pH under H_2 oxidation or H_2 evolution conditions reveals the influence of site-selective protonation on the equilibrium of reduced cofactor states. Governed by pK_a differences across the active site niche and proton transfer pathways, we find that individual electrons are stabilized either at the [4Fe–4S] cluster (alkaline pH values) or at the diiron site (acidic pH values). This observation is discussed in the context of the complex interdependence of hydrogen turnover and bulk pH.

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[FeFe]-Hydrogenases are gas-processing metalloenzymes that have been found in bacteria and green algae.[1–4] They serve various roles in the hydrogen metabolism of prokaryotes, including oxidation of H_2 as an energy carrier and proton reduction (H_2 evolution) to maintain the cellular redox equilibrium.[5] In the chloroplast of green algae, they are part of the photosynthetic electron transport chain, coupling H_2O oxidation and H_2 evolution at the reducing end of photosystem I.[6] The first crystal structures of [FeFe]-hydrogenase helped identifying accessory and catalytic iron-sulfur clusters as well as gas channels and potential proton transfer (PT) pathways.[7–13] Additionally, various biophysical techniques were employed to characterize the electronic structure of the active site cofactor, the so-called 'H-cluster' (Fig. 1).[1] This iron-sulfur compound is formed by a [4Fe–4S] cluster connected to a bimetallic iron site *via* a bridging cysteine residue. The diiron site has been shown to bind carbon monoxide (CO) and cyanide ligands (CN^-) that are a unique feature of hydrogenase.[14–16] The aminodithiolate ligand (ADT) that bridges the metal ions of the diiron site was suggested to act as an inner-sphere hydrogen-bonding donor to a number of apical ligands at the distal iron ion (Fe_d).[17]

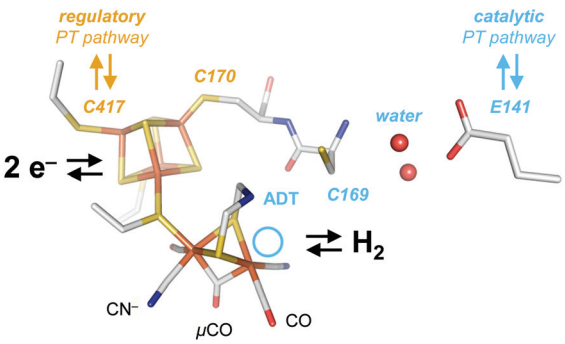

Fig. 1 Cofactor and active site niche. The H-cluster comprises a [4Fe–4S] cluster linked to the catalytic diiron site. In the crystallized Hox state, the H-cluster carries two terminal CO and CN^- ligands and a single μCO ligand (pdb ID 4XDC). The ADT ligand serves as a proton relay between the distal iron ion (Fe_d) and the catalytic PT pathway (light blue) including C169, a water cluster, E141, and other residues. The light blue circle marks the open coordination site at Fe_d in the oxidized state, **Hox**. At the [4Fe–4S] cluster, C417 may receive a proton directly from the solvent *via* the regulatory PT pathway (brown).

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Moreover, the secondary amine of the ADT ligand serves as proton relay between the diiron site and the amino acid residues of the catalytic proton transfer pathway (Fig. 1).[18–23] The latter includes arginine and glutamic acid residues connected by a cysteine, a serine, and a small water cluster.[24]

The diiron site and the [4Fe–4S] cluster exist in an oxidized and reduced form each, resulting in a total of four different H-cluster species: the oxidized state, **Hox**, the $1e^-$-reduced states **Hred′** (reduced [4Fe–4S] cluster) and **Hred** (reduced diiron site) as well as the $2e^-$-reduced state, **Hsred**.[25–32] An additional state, **Hhyd**, is comprised of a reduced [4Fe–4S] cluster and a formally over-oxidized diiron site with a terminal hydride ligand (H^-).[33–35] Additional states include the CO-inhibited states **Hox-CO** and **Hred′-CO** and the oxidized protonated state, **HoxH**.[29,32] While most authors agree on the importance of **Hred′** and **Hhyd** in hydrogen turnover (Fig. 2), the protonation state, cofactor geometry, and involvement in catalysis of **Hred** and **Hsred** are under discussion.[36]

Fig. 2 highlights that the $1e^-$-reduced H-cluster states **Hred′** and **Hred** are enriched upon coupled or sequential electron- and proton transfer (PCET or ETPT). Due to the transient nature of electron tunnelling *via* the [4Fe–4S] cluster reduction, we will only use the PCET nomenclature in the following. In previous work, we suggested that a cysteine residue coordinating the [4Fe–4S] cluster may bind a proton in **Hred′** (Fig. 1);[31,38] however, there is no consensus regarding the nature of protonation and cofactor geometry in **Hred**. Sommer *et al.* presumed protonation of the ADT ligand ($^+NH_2$) and a shift of the μCO ligand into a 'semi-bridging' position at Fe_d as seen in hydrogenase crystals grown under 10% H_2 and pressurized with 6 bar H_2.[39,40] Ratzloff *et al.* proposed a $^+NH_2$ geometry with a conserved μCO ligand in **Hred**,[41] which was supported in recent infrared studies by Birrell *et al.* and Lorent *et al.* that identified a μCO ligand for **Hred** and **Hsred** at cryogenic temperatures.[37,42] In contrast, our infrared evaluation of **Hred** and **Hsred** at ambient temperature implied the formation of a bridging hydride species (μH) and an apical CO ligand at Fe_d.[30] A μH geometry was calculated to be rather unreactive.[43–45] Therefore, such changes would exclude **Hred** and **Hsred** from the catalytic cycle (in contrast to Model 1 in Fig. 2) and favor a catalytic mechanism without the reduction of the diiron site (*i.e.*, Model 2 in Fig. 2). Sanchez *et al.* demonstrated the kinetic competence of both **Hred′** and **Hred** at ambient temperature[46] but the spectroscopic marker bands that were used to follow **Hred** in their study are nearly identical for the μCO and the μH geometry. This impedes a kinetic discrimination of these isomers.[37,42] We presume that the cryogenic states may represent kinetically trapped intermediates.[24,47]

The influence of bulk pH on **Hox**, **Hred**, and **Hsred** in the native [FeFe]-hydrogenase from *Chlamydomonas reinhardtii* (*Cr*HydA1)[32,39] and **Hred′** in cofactor variant *Cr*HydA1^{PDT} was analysed before.[31,48] To understand the equilibrium of **Hred′** and **Hred** in native *Cr*HydA1, we now investigate the pH-dependent accumulation of both $1e^-$-reduced H-cluster states under turnover conditions. Making use of *operando* attenuated total reflection Fourier-transform infrared (ATR FTIR) spectroscopy and spectro-electrochemistry under H_2 oxidation or H_2 evolution conditions, we found consistent trends for an accumulation of **Hred′** towards alkaline pH values whereas the accumulation of **Hred** increases towards acidic pH values. This observation is explained by site-selective PCET to either the diiron site or the [4Fe–4S] cluster, guided by differences in proton affinity. Our findings are employed to distinguish catalytic from regulatory H-cluster states and inspire a molecular understanding of the pH-dependent hydrogen turnover of [FeFe]-hydrogenase.

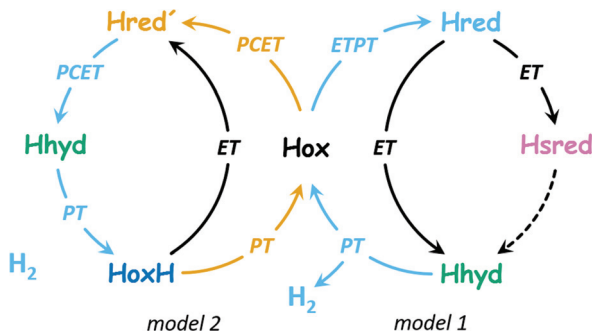

Fig. 2 Two proposals of the catalytic cycle. In H_2 evolution direction, both cycles start from **Hox**. Model 1 (right side, based on ref. 37) is characterized by ETPT to the diiron site in the 1st step (formation of **Hred**, protonation of the diiron site). This includes transient electron transfer (ET) *via* the [4Fe–4S] cluster and proton transfer (PT) in the formation of **Hred**. A subsequent reduction step (ET) may form **Hhyd** either directly or *via* **Hsred** by an unknown mechanism (dashed arrow). With a 2nd proton, H_2 is released and **Hox** is restored. Model 2 (left side, based on ref. 36) is characterized by PCET to the [4Fe–4S] cluster in the 1st step (formation of **Hred′**, binding of a regulatory proton at the [4Fe–4S] cluster). Subsequently, PCET to the diiron site promotes formation of **Hhyd**, and with a 3rd proton, H_2 is released and **HoxH** is formed. The latter may lose the regulatory proton to restore **Hox** or accept an electron to form **Hred′** directly.

Experimental

Protein purification and activation

All experiments involving *Cr*HydA1 were performed under strictly anaerobic conditions. *Cr*HydA1 apo-protein (wild-type and amino acid variants)[49–51] and the synthetic mimics of the diiron site (ADT and PDT)[19,20] were prepared as described previously. Activated *Cr*HydA1 was eluted in 10 mM Tris/HCl (pH 8). Sodium dithionite was avoided to prevent accumulation of **HoxH** and **Hhyd** at low pH values.[32,34] Each sample was diluted 1 : 1 (~0.5 mM *Cr*HydA1) with mixed buffer containing 50 mM Tris, MES, and PIPPS to adjust the desired pH value.

ATR FTIR spectroscopy

The FTIR spectrometer (Tensor27, Bruker) was equipped with a triple-reflection ZnSe/Si crystal ATR cell (Smith Detection) and placed in an anaerobic chamber. Infrared spectra were recorded with 80 kHz scanning velocity at a spectral resolution of 2 cm^{-1} (MCT detection). Under these conditions, the time-resolution of data acquisition is in the range of seconds (*i.e.*, five interferometer scans in forward/backward direction). ATR

FTIR measurements were performed at 25 °C and on hydrogenase films derived by controlled dehydration and rehydration of 1 µl protein sample as reported earlier.[29]

The anaerobically purified and activated [FeFe]-hydrogenase typically contained the H-cluster in various states. **Hox** was enriched in the film under a constant stream of N_2 for 30–60 minutes. A constant gas stream (1.5 L min^{-1}) was adjusted with digital mass flow controllers (SmartTrak, Sierra). Then, H_2 was added to the N_2 stream *via* separate flow controllers and passed through a wash bottle containing 150 mL mixed buffer (0.1–100% at ambient pressure). The resulting aerosol was fed into a gas-tight PCTFE compartment, attached on top of the ATR crystal plate and equipped with six optional gas inlets, a manometer for pressure control, and a glass window for UV/vis irradiaton.[29] For each H_2 concentration step, the film was equilibrated for 2.5 min to ensure a sufficiently stable composition of H-cluster states (Fig. S1†).

ATR FTIR spectro-electrochemistry

The pH-dependent reduction of *Cr*HydA1 in the absence of H_2 was analyzed by ATR FTIR spectro-electrochemistry.[30,31] For this, 1 µL protein sample (diluted with 50 mM mixed buffer pH 9–5) was injected into a 9 µm thin gold mesh on top of an ATR silicon crystal. The mesh was covered with an 8 kDa dialysis membrane to protect the film from dilution. A custom-made PCTFE electrochemical cell was attached to the ATR crystal plate and filled with 3 mL electrolyte buffer (50 mM mixed buffer pH 9–5 including 500 mM KCl as electrolyte) that was purged with N_2 throughout the whole experiment. After 60–90 minutes, the film was fully hydrated and stable. The gold mesh was connected with the working electrode, a platinum wire was used as counter electrode, and an Ag/AgCl electrode served as reference (+230 mV *vs.* SHE, as determined with 1 mM methyl viologen at pH 7).[31] After complete oxidation at −100 mV *vs.* SHE, the potential was lowered incrementally from −150 mV to −850 mV in steps of 50 mV with a fixed duration of 20 minutes for each step (Fig. S2†) until no further spectral changes were observed. Midpoint potentials were estimated from bi-sigmoidal fits. At strongly reducing potentials, smaller changes in current hinted at imperfect equilibria (Fig. S2†).

Data treatment

All absorbance spectra were derived from single channel spectra of 'reference' (ZnSe/Si) and 'sample' (ZnSe/Si + protein) in OPUS software. Then, data was exported to a home-written routine as described previously.[32] In the frequency regime of the H-cluster (2150–1750 cm^{-1}), absorbance spectra were subtracted with a polynomial function simulating the low-frequency combination band of liquid water underneath the 'sharp' CO/CN^- bands of the H-cluster. This gave rise to background-corrected spectra as shown in Fig. S1–S3.† Reference spectra of pure redox states (Fig. 3 and Fig. S3†) allowed determining fit parameters for all observed redox states (frequency, intensity, bandwidth, and peak ratio, see Table S1†), as described earlier.[32] The sum of band area (2 CN^- + 3 CO) for a given redox state was obtained by simulation of spectral data with a fixed set of parameters that represent the population in relation to the other redox states. This value (%) and was plotted against time illustrating how the system converges into new redox equilibria upon disturbance (*i.e.*, changes in H_2 concentration in Fig. S1† or electrochemical potential in Fig. S2†). In the final step, the population of redox states was plotted as a function of H_2 concentration or electrochemical potential.

Results

All experiments were performed with the [FeFe]-hydrogenase *Cr*HydA1 under ambient conditions. In the first step, ATR FTIR spectroscopy and spectro-electrochemistry[29–31] were

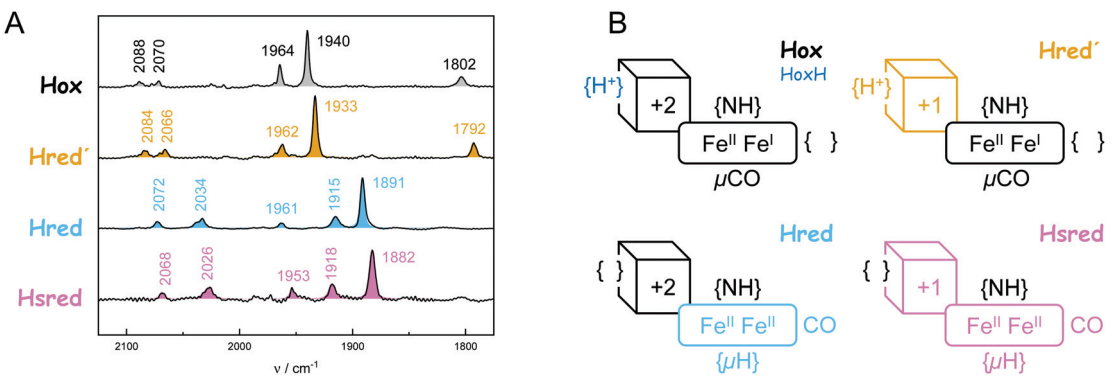

Fig. 3 Characterization of key H-cluster states. (A) Background-corrected spectra of **Hox** (grey) and **Hred'** (brown) recorded under alkaline conditions[:] (pH 9) at −300 mV *vs.* SHE and −600 mV *vs.* SHE, respectively. Additionally, the figure shows background-corrected spectra recorded either upon illumination in the presence of eosin Y and EDTA (**Hred**, light blue) or under acidic conditions (pH 5) at −800 mV *vs.* SHE (**Hsred**, rose). (B) Proposed H-cluster geometries. The rectangle represents the diiron site including the ADT ligand (NH), the Fe–Fe bridging ligand (µCO, µH), and the apical binding site of Fe_d (vacant or occupied with CO). Observed charges are formally +4 to +2. The cube represents the [4Fe–4S] cluster (charges +2 or +1). Colors hint at differences relative to **Hox** (color code: **Hox** (black), **HoxH** (dark blue), **Hred'** (brown), **Hred** (light blue), **Hsred** (rose).

Table 1 Vibrational and electronic properties of different H-cluster states. In **Hox-CO**, vibrational coupling results in an additional IR band at 2012 cm^{-1}. In **Hred** and **Hsred** the low-frequency μCO ligand moved into a terminal position at 1961 and 1953 cm^{-1}. **Hhyd** is best described with an 'over-oxidized' diiron site (+4) and a terminal hydride ligand that accounts for two electrons

	CN^-/cm^{-1}		CO/cm^{-1}			[4Fe]	[2Fe]
Hox	2088	2070	1964	1940	1802	+2	+3
Hox-CO	2092	2082	1968	1962	1812	+2	+3
Hred′	2084	2066	1962	1933	1792	+1	+3
Hhyd	2082	2068	1978	1960	1860	+1	+4
Hred	2072	2034	1961	1915	1891	+2	+2
Hsred	2068	2026	1953	1918	1882	+1	+2

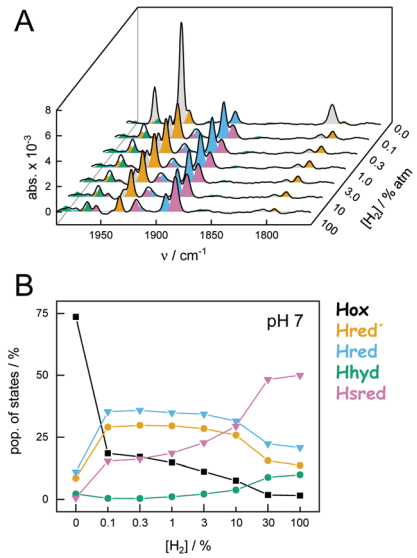

Fig. 4 Composition of H-cluster states under H_2 oxidation conditions (pH 7). All data recorded on a hydrated film of *Cr*HydA1 at ambient temperature and 0–100% H_2 in the gas phase. (A) Series of baseline-corrected FTIR absorbance spectra of the H-cluster obtained after 2.5 min at each step. (B) State populations as a function of $[H_2]$. Between 0.1–3% H_2, the population of **Hred′** and **Hred** was relatively stable. **Hsred** dominated for $[H_2] > 10\%$.

employed to extract the IR signatures of all relevant redox states (Table 1 and Fig. S3†). Fig. 3A shows spectra of **Hox** and **Hred′** recorded under alkaline conditions (pH 9) at −300 mV *vs.* SHE and −600 mV *vs.* SHE, respectively. A pure spectrum of **Hred′** in native *Cr*HydA1 has not been reported before. Additionally, Fig. 3A shows spectra recorded either upon illumination in the presence of eosin Y and EDTA (**Hred**, see ref. 52 for the protocol) or under acidic conditions (pH 5) at −800 mV *vs.* SHE (**Hsred**). The overall downshift of the cofactor bands from **Hox** → **Hred′** and **Hred** → **Hsred** has been attributed to a reduction of the [4Fe–4S] cluster (depicted as a cubane in Fig. 3B).[27,28] Fig. S4† emphasized that the **Hred** → **Hsred** difference spectrum shows no signal around 1800 cm^{-1}. This highlights the lack of a μCO ligand at the reduced diiron site (depicted as a rectangle in Fig. 3B) and confirms the assignment of bands at 1961 cm^{-1} and 1953 cm^{-1} to **Hred** and **Hsred**, respectively. A detailed discussion of the IR spectrum of **Hred** can be found in ref. 47. Based on previous work,[30] **Hred** and **Hsred** are depicted with a terminal CO ligand and a μH ligand in Fig. 3B.

To address the pH-dependent population of H-cluster states under H_2 oxidation conditions, we investigated *Cr*HydA1 by ATR FTIR spectroscopy at different H_2 concentration (without external potential control). Direct proof for the hydrogenase-catalysed cleavage of H_2 came from D_2 oxidation experiment in an aqueous environment.[34] Moreover, H_2 oxidation induces an accumulation of reduced H-cluster states so that we were able to follow the increase and decrease of redox state populations as a function of atmospheric H_2 and time. As an example, Fig. 4A depicts a series of background-corrected ATR FTIR absorbance spectra of the H-cluster recorded after 2.5 min under 0–100% H_2 (compare Fig. S1†). We note that residual, unidentified H-cluster states may be present in the spectra. As these contributions did not to interfere with the global fit analysis (*i.e.*, $\chi^2 < 10^{-4}$) we did consider them any further. Broader features in the corrected spectra stem from the 'combination band' of liquid water. For a spectroscopically unique identification of H-cluster states, see Fig. 3A and ESI.† Fig. 4B illustrates how increasing the H_2 concentration to 0.1% resulted in an accumulation of $1e^-$-reduced states **Hred′** and **Hred**, which remained fairly stable between 0.1–3% H_2. At higher concentrations of H_2, the $2e^-$-reduced **Hsred** state dominated the spectrum. Only minor traces of the $2e^-$-reduced **Hhyd** state were observed, most likely due to the lack of sodium dithionite in the sample (see Experimental section).[34]

Analogous to the experiment shown in Fig. 4, six individual *Cr*HydA1 protein films between pH 10–5 were analyzed (Fig. 5). Largely independent of pH, the oxidized state **Hox** was the most prominent species in the absence of H_2 while **Hsred** dominated the spectrum for $[H_2] > 10\%$ (Fig. S5†). The steady-state population of **Hred′** and **Hred** is plotted as a function of $[H_2]$ and at different pH values in Fig. 5AB. Here, we observed diverging trends for the accumulation of the $1e^-$-reduced states: **Hred** dominated at acidic conditions whereas **Hred′** was promoted under alkaline pH values. Fig. 5C depicts the accumulation of **Hred′** and **Hred** at 3% H_2 as a function of pH, which clearly illustrates this trend. At low pH and $[H_2] > 10\%$, an increasing accumulation of **Hhyd** was observed, which may explain the mild suppression of **Hsred** that was otherwise expected to follow the same pH dependence as **Hred** (Fig. S5†). Upon removal of H_2 from the gas stream, the $2e^-$-reduced states **Hhyd** and **Hsred** converted transiently into the $1e^-$-reduced states **Hred′** and **Hred**, indicating intermolecular electron transfer in the dense films,[32] before the equilibrium shifted back towards **Hox** upon auto-oxidation.[17] The diverging pH dependence of **Hred′** and **Hred** was found to be well conserved in this transient increase, emphasizing the robustness of all observed trends (Fig. S6†).

The simultaneous presence of **Hred′** and **Hred** complicates unique conclusions regarding the mechanism of H-cluster protonation. Therefore, additional experiments were performed.

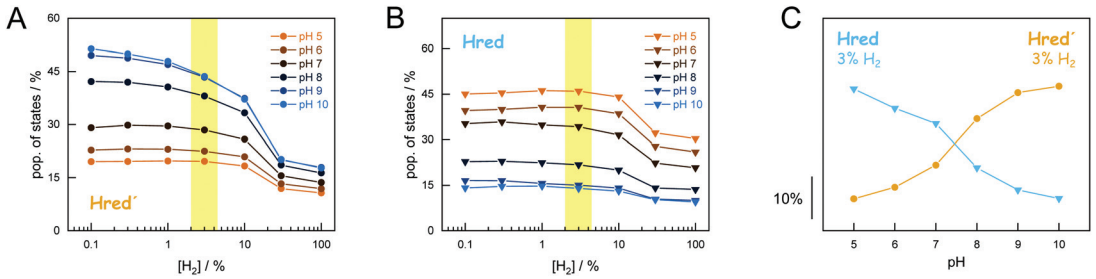

Fig. 5 Accumulation of **Hred′** and **Hred** as a function of H_2 and pH. Population of **Hred′** (panel A) and **Hred** (panel B) for six different pH values (pH 10–5) under 0.1–100% H_2. Plotting the population of **Hred′** and **Hred** at 3% H_2 (yellow mark-up) against pH clearly illustrates the opposing pH dependency (panel C). For $[H_2]$ > 10%, **Hsred** and **Hhyd** are accumulated.

First, we probed the pH dependence of H_2 oxidation with *Cr*HydA1^{PDT}. This cofactor variant lacks the secondary amine of the native ADT ligand (Fig. 1) and allows analysing the reduction of the [4Fe–4S] cluster (*i.e.*, the **Hox** → **Hred′** transition) independent from redox chemistry at the diiron site.[28,31] Fig. S7† shows that the H_2 oxidation activity of *Cr*HydA1^{PDT} increases between pH 10–8. While this cannot be explained by the stoichiometry of the catalysed reaction, our results support PCET chemistry at the [4Fe–4S] cluster.[31] Earlier, the influence of cysteinyl ligand C417 on the catalytic properties of *Cr*HydA1 has been addressed by site-directed mutagenesis.[50,51] Now, we analyzed three cysteine variants to compare the composition of H-cluster states under H_2 oxidation conditions (Fig. S8†). We made the following observations. (i) C417S behaved much like wild-type *Cr*HydA1 but showed a reduced percentage of **Hred′** under H_2. (ii) Due to electron withdrawal from the [4Fe–4S] cluster by the imidazole ligand, C417H was reported with a less negative redox potential than wild-type *Cr*HydA1.[51] In agreement with earlier observations C417H adopted **Hred′** as a resting state, reflecting the lack of H_2 evolution activity of *Cr*HydA1 C417H.[51]

In the presence of H_2, the variant converted into **Hsred** (at pH 8) or **Hhyd** (pH 4) with no detectable traces of **Hred**. In the absence of H_2, low pH conditions resulted in an accumulation of **Hred′H**. (iii) The spectral behavior of C417D was surprisingly similar to C417H, indicative of electron withdrawal. We speculate that this may be due to hydrogen-bonding between the aspartic acid side chain and the [4Fe–4S] cluster. In variance to the histidine variant, C417D slowly converted into **Hox** (pH 8) or **HoxH** (pH 4), reflecting the low but significant H_2 evolution activity of *Cr*HydA1 C417D.[50]

Overall, our data demonstrate how the equilibrium of redox states is affected by cluster ligation, proton concentration (pH), and the percentage of H_2 in the gas phase. Under H_2 oxidation conditions, virtually all H-cluster species were present at every step of the experiment, impeding an individual analysis of states. Thus, we investigated the pH-dependent population of redox states in *Cr*HydA1 under H_2 evolution conditions by injecting electrons into the systems in the absence of H_2. We employed ATR FTIR spectro-electrochemistry to follow the evolution of state populations as a function of electrochemical potential. In contrast to conventional, Mosstype[53] transmission cells, the ATR FTIR spectro-electrochemistry approach allowed H_2 to be released from the protein-modified working electrode hence precluding product oxidation or product inhibition. Fig. 6 depicts how the oxidized states **Hox** and **Hox-CO** were lost at reductive potentials, followed by accumulation of the 1e$^-$-reduced states **Hred′** and **Hred**. Upon further reduction, accumulation of **Hsred** was observed; however, in contrast to the experiments performed under H_2 oxidation conditions (Fig. 4), **Hhyd** was not observed.

Analogous to the experiment shown in Fig. 6, nine individual protein films between pH 9–5 were analyzed (Fig. 7). The maximal population of H-cluster states as a function of poten-

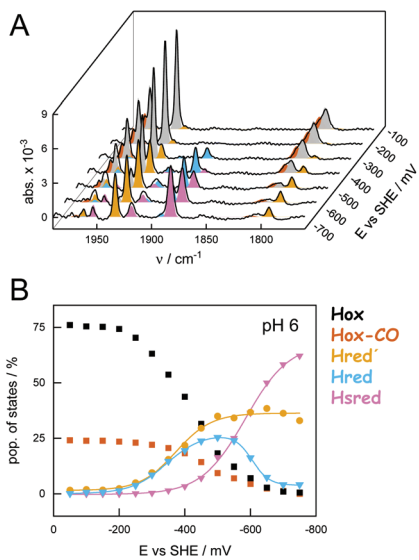

Fig. 6 Composition of H-cluster states under H_2 evolution conditions (pH 6). Exemplary data recorded on a hydrated film of *Cr*HydA1 between −50 and −750 mV *vs.* SHE and constant N_2 purging. (A) Series of baseline-corrected ATR FTIR absorbance spectra of the H-cluster obtained after 20 min at each increment of 50 mV (steady-state conditions). (B) State populations as a function of electrochemical potential. Sigmoidal fits allowed approximating the following midpoint potentials (*vs.* SHE): **Hox** → **Hred′** −375 mV; **Hox** → **Hred** −345 mV; **Hred** → **Hsred** −585 mV (a second fit component at −605 mV may account for **Hred′** → **Hsred**).

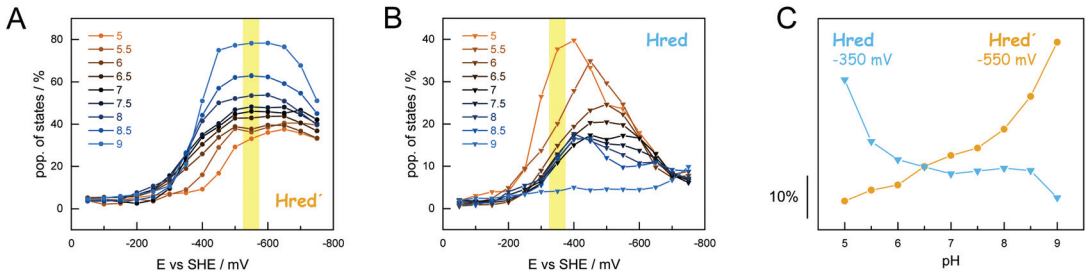

Fig. 7 Accumulation of **Hred′** and **Hred** as a function of electrochemical potential and pH. Population of **Hred′** (panel A) and **Hred** (panel B) for nine different pH values (pH 9–5) between −50 and −750 mV *vs.* SHE. The opposite pH dependence is clearly illustrated plotting the population of **Hred′** (−550 mV *vs.* SHE) and **Hred** (−350 mV *vs.* SHE) as function of pH in panel C (the yellow mark-up highlights the respective potentials).

tial and pH value was used as the main observable. This approach allowed analyzing the $1e^-$- and $2e^-$-reduced states separately, which was not possible under H_2 oxidation conditions (Fig. 5). The oxidized state **Hox** was the most prominent species at potentials more positive than −350 mV *vs.* SHE, largely independent of pH (*i.e.*, in the absence of dithionite[32]). The accumulation of **Hsred** was found to be affected by pH more drastically, with a higher population at acidic pH values that reflects the lack of **Hhyd** in the experiment (Fig. S9†). Overall, we observed a mean midpoint potential around −650 mV *vs.* SHE for **Hsred**, which leaves a potential window of ∼300 mV to analyze the accumulation of the $1e^-$-reduced states.

In Fig. 7AB the steady-state population of **Hred′** and **Hred** is plotted as a function of potential and at different pH values. Like what has been observed under H_2 oxidizing conditions, **Hred** dominated under acidic conditions whereas **Hred′** was promoted at alkaline pH values. The population of states varies from 30–80% (−550 mV *vs.* SHE, **Hred′**) and 40–5% (−350 mV *vs.* SHE, **Hred**) for increasing pH values. Fig. 7C depicts the accumulation of **Hred′** and **Hred** as a function of pH. This trend is strictly conserved in the aforementioned potential window (Fig. S9†) and facilitated an estimation of apparent proton affinities for the accumulation of **Hred′** and **Hred**. Moreover, the Pourbaix diagram in Fig. S9† highlights the decrease in electrochemical driving force for **Hox** → **Hred′** between pH 9–8 and **Hox** → **Hred** between pH 6–5. Interestingly, no pronounced pH dependency is observed around pH 7, which may reflect the concomitant formation of **Hred′** and **Hred**.

Discussion

We analyzed the [FeFe]-hydrogenase HYDA1 from *Chlamydomonas reinhardtii* by ATR FTIR spectroscopy and spectro-electrochemistry under ambient conditions. We addressed the pH-dependent accumulation of various H-cluster states. Varying the sample pH under H_2 oxidation and H_2 evolution conditions established consistent trends for the accumulation of $1e^-$-reduced H-cluster states: we observed enrichment of **Hred** under acidic conditions whereas **Hred′** prevailed at alkaline conditions. Our data on cofactor and amino acid variants highlight the importance of the [4Fe–4S] cluster for catalysis and the equilibrium of redox species.

In earlier work, we identified the pH dependence of **Hred′** formed upon reduction of the [4Fe–4S] cluster and protonation of a nearby cysteine, C417 in *Cr*HydA1.[31] Moreover, the **Hred′** state is involved in the steady-state accumulation of **HoxH**,[32] which proceeds under reducing conditions exclusively and represents the starting state for an enrichment of **Hhyd** in the presence of H_2.[34,54] While these data indicate PCET to the [4Fe–4S] cluster and emphasize the importance of redox chemistry adjacent to the diiron site, an understanding of the pH dependence of **Hred′** and **Hred** with respect to hydrogen turnover is yet to be accomplished. Due to the simultaneous presence of various reduced H-cluster states under H_2 (including **Hhyd** and **Hsred**), unraveling the PCET chemistry of **Hred′** and **Hred** was found to be challenging.[39] To this end, ATR FTIR spectro-electrochemistry facilitated analyzing the population of $1e^-$- and $2e^-$-reduced H-cluster states individually. The accumulation of **Hred** at acidic pH values and mildly reducing conditions (*e.g.*, −350 mV *vs.* SHE) suggests a slightly acidic pK_a, in agreement with the involvement of glutamic acid residues as 'bottle neck' in the catalytic PT pathway.[21] In contrast, our data for the population of **Hred′** at more reducing potentials (*e.g.*, −550 mV *vs.* SHE) hints at a pK_a in the alkaline, which reflects the greater ease of proton transfer to the [4Fe–4S] cluster *via* bulk solvent[32,36] and is in excellent agreement with the formal pK_a of 8.1 for a cysteine sidechain. This observation is evidence for the pH dependence of **Hred′** in native [FeFe]-hydrogenase, in particular because the **Hred** state does not show dedicated pH dependence in this pH window. Surprisingly, Rodríguez-Macia *et al.* found the redox potential of the [4Fe–4S] cluster in *Cr*HydA1^{PDT} to be independent from bulk pH.[48] The reasons for this discrepancy are unclear. In our hands, present and previous data[31,38] support PCET in the formation of **Hred′**. We propose to distinguish a catalytic PT pathway to the diiron site from a regulatory pathway to the [4Fe–4S] cluster as a common feature of [FeFe]-hydrogenases.

Both *in vivo* and *in vitro*, the pH dependence of H_2 evolution of [FeFe]-hydrogenase shows a bell-shaped distribution with a maximal activity around neutral or mildly alkaline pH values.[55–58] This has recently been confirmed by bulk electro-

chemistry.[59] While the increase in H_2 evolution activity between pH 9–7 can be attributed to rising proton concentration, our data on *Cr*HydA1 now allows correlating the activity decrease between pH 7–5 to the formation of **Hred**, which clearly dominated over **Hred′** at acidic pH values. Although our data does not report on enzymatic activity directly, this behavior is in agreement with **Hred** and **Hsred** as 'H_2-inhibited' states[60] that bind a bridging hydride at the diiron site[30] and have been shown to play a key role in sensory [FeFe]-hydrogenases.[61–63] The ligand flip required to form a reactive terminal hydride geometry disfavors fast catalysis.[43–45] The present data support the theory that reduction and site-selective protonation at the [4Fe–4S] cluster adjusts the redox potential of the H-cluster to stabilizes a reactive geometry necessary for efficient hydrogen catalysis.[36] We suggest that similar concepts may give rise to a novel generation of biomimetic hydrogen catalysts.

Conflicts of interest

There are no conflicts to declare.

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