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Hydrogenases are microbial redox enzymes that catalyze H2 oxidation and proton reduction (H2 evolution). While all hydrogenases show high oxidation activities, the majority of [FeFe]-hydrogenases are excellent H2 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 that facilitate catalysis at low overpotential. Distinct proton transfer pathways connect the active site niche with the solvent, resulting in a non-trivial dependence of hydrogen turnover and bulk pH. To analyze the catalytic mechanism of [FeFe]-hydrogenase, we employ in situ infrared spectroscopy and infrared spectro-electrochemistry. Titrating the pH under H2 oxidation or H2 evolution conditions reveals the influence of site-selective protonation on the equilibrium of reduced cofactor states. Governed by pKa 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 natural pH dependence of hydrogen turnover as catalyzed by [FeFe]-hydrogenase.
Site-selective Protonation of the One-electron Reduced Cofactor in [FeFe]-Hydrogenase

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ABSTRACT

Hydrogenases are microbial redox enzymes that catalyze H\textsubscript{2} oxidation and proton reduction (H\textsubscript{2} evolution). While all hydrogenases show high oxidation activities, the majority of [FeFe]-hydrogenases are excellent H\textsubscript{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 that facilitate catalysis at low overpotential. Distinct proton transfer pathways connect the active site niche with the solvent, resulting in a non-trivial dependence of hydrogen turnover and bulk pH. To analyze the catalytic mechanism of [FeFe]-hydrogenase, we employ in situ infrared spectroscopy and infrared spectro-electrochemistry. Titrating the pH under H\textsubscript{2} oxidation or H\textsubscript{2} evolution conditions reveals the influence of site-selective protonation on the equilibrium of reduced cofactor states. Governed by pKa 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 natural pH dependence of hydrogen turnover as catalyzed by [FeFe]-hydrogenase.
INTRODUCTION

[FeFe]-hydrogenases are gas-processing metalloenzymes that have been found in bacteria and green algae.\textsuperscript{1–3} They serve various roles in the hydrogen metabolism of prokaryotes, including oxidation of H\textsubscript{2} as an energy source and proton reduction (H\textsubscript{2} evolution) to maintain the cellular redox equilibrium.\textsuperscript{4} In the chloroplast of green algae, they are part of the photosynthetic electron transport chain, coupling H\textsubscript{2}O oxidation and H\textsubscript{2} evolution at the reducing end of photosystem I.\textsuperscript{5} 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.\textsuperscript{6–12} Additionally, various biophysical techniques were employed to characterize the electronic structure of the active site cofactor, the so-called ‘H-cluster’ (Figure 1AB).\textsuperscript{1} This iron-sulfur compound is formed by a [4Fe-4S] cluster connected to a bimetallic iron site via a bridging cysteine residue. Carbon monoxide (CO) and cyanide ligands (CN\textsuperscript{–}) tune the redox potential of the H-cluster and anchor the diiron site within the protein.\textsuperscript{13–15} The secondary amine of the H-cluster’s aminodithiolate ligand (ADT) has been suggested to serve as proton relay between the diiron site and the amino acid residues of the catalytic PT pathway.\textsuperscript{16–21} Moreover, it acts as an inner-sphere hydrogen-bonding donor to a number of apical ligands at the distal iron ion (Fe\textsubscript{d}).\textsuperscript{22} Figure 1C depicts a schematic representation of the H-cluster with potential binding sites for hydrogen species.

![Figure 1. H-cluster and proton transfer pathways.](image)

(A) Homology model of [FeFe]-hydrogenase CrHydA1. The catalytic PT pathway (including C169 close to the H-cluster) is highlighted. Cysteine C417 at the [4Fe-4S] cluster is located close to the protein surface and available for proton exchange. (B)
The H-cluster comprises a [4Fe-4S] cluster linked to the catalytic diiron site via a bridging cysteine (*). The diatomic ligands are labelled (crystal structure of Hox 4XDC). Most likely, the ADT ligand serves as proton relay between H-cluster (dashed circle) and C169 of the catalytic PT pathway (blue labels). At the [4Fe-4S] cluster, C417 may receive a proton directly from the solvent via a second PT pathway (red labels). (C) Schematic representation of the H-cluster highlighting potential protonation site. This includes the [4Fe-4S] cluster {H+}, the ADT ligand {+NH2}, the apical binding site of Fe_d {H–}, and the Fe-Fe bridging site {µH}.

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

Both Hred’ and Hred are enriched upon proton-coupled electron transfer (PCET). Previously, we suggested that a cysteine residue coordinating the [4Fe-4S] cluster may bind a proton in Hred’ (Figure 1B); however, there is no consensus regarding the nature of protonation and cofactor geometry in Hred. Sommer et al. presumed protonation of the ADT ligand (+NH2) and a shift of the µCO ligand into a ‘semi-bridging’ position at Fe_d as seen in a crystal structure grown under H2. Ratzloff et al. proposed a +NH2 geometry with a conserved µCO ligand in Hred, 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. In contrast, our infrared evaluation of Hred and Hsred at ambient temperatures implied the formation of a bridging hydride species (µH) and an apical CO ligand at Fe_d. Such changes would
exclude Hred and Hsred from the catalytic cycle as a \( \mu \)H geometry was calculated to be rather unreactive.\cite{40-42} Following the time-dependent evolution of individual H-cluster bands by transient infrared spectroscopy at ambient temperature, Sanchez et al. proposed the kinetic competence of both Hred\(^{'\prime}\) and Hred.\cite{43} Unfortunately, the spectroscopic marker bands that were used to follow Hred in this study are nearly identical for the \( \mu \)CO and the \( \mu \)H geometry, impeding any kinetic discrimination.\cite{38,39} We presume that the cryogenic states may represent kinetically trapped intermediates.\cite{44}

In this study, we investigate the pH-dependent accumulation of 1e\(^{-}\)-reduced H-cluster states in the [FeFe]-hydrogenase from *Chlamydomonas reinhardtii*, CrHydA1, to understand the equilibrium of Hred\(^{'\prime}\) and Hred under turnover conditions. Making use of *in situ* attenuated total reflection Fourier-transform infrared (ATR FTIR) spectroscopy and spectro-electrochemistry under \( \text{H}_2 \) oxidation or \( \text{H}_2 \) evolution conditions, we found consistent trends for an accumulation of Hred\(^{'\prime}\) 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 the 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.

**EXPERIMENTAL**

*Protein purification and activation.* All experiments involving CrHydA1 were performed under strictly anaerobic conditions. Native apo-protein and amino acid variants C417SDH were expressed in *Escherichia coli* host strain BL21 (DE3) \( \Delta \)iscR and purified by strep-tactin affinity chromatography as described previously.\cite{45,46} The synthetic mimics of the native ADT complex and artificial propanedithiolate complex (PDT) were synthesized following literature procedures.\cite{17} Apo-protein was activated in the presence of ADT or PDT at a 10-fold molar excess. After an incubation period of at least 1 h at 25 °C, size-exclusion chromatography was employed to remove redundant complex.\cite{18} Activated CrHydA1 was eluted in 10 mM Tris/HCl (pH 8). For native CrHydA1, sodium dithionite was avoided to
prevent accumulation of HoxH and Hhyd at low pH values. Enzyme was concentrated to ~1 mM and stored anaerobically at ~80 °C.

**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. Each sample was diluted 1:1 (~0.5 mM CrHydA1) with mixed buffer containing 50 mM Tris, MES, and PIPPS to adjust the desired pH value.

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 N₂ aerosol for 30 – 60 minutes. A constant gas stream (1.5 L min⁻¹) was adjusted with digital mass flow controllers (SmartTrak, Sierra). Then, H₂ was added to the N₂ stream via separate flow controllers and passed through a wash bottle containing 150 mL mixed buffer (0.1 – 100% at ambient pressure). 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. For each H₂ concentration step, the film was equilibrated for 2.5 min (Figure S1).

**ATR FTIR spectro-electrochemistry.** The pH-dependent reduction of CrHydA1 in the absence of H₂ was analyzed by ATR FTIR spectro-electrochemistry. 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 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 N₂ throughout
the whole experiment. After 60 – 90 minutes, the film was fully hydrated. The gold mesh was connected with the working electrode, 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). 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 (Figure S2) until no further spectral changes were observed. Midpoint potentials were estimated from bi-sigmoidal fits. Although the lack spectral changes after 20 min suggested steady-state conditions, smaller changes in current hinted at imperfect equilibria, in particular at strongly reducing potential (Figure S2). Thus, we refrained from Nernst-fitting and a quantitative, comparative analysis of midpoint potentials.

**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. In the frequency regime of the H-cluster (2150 – 1750 cm\(^{-1}\)), absorbance spectra were subtracted with a polynomial function simulating the broad combination band of liquid water underneath the sharp CO/CN\(^-\) bands of the H-cluster (Figures S1A and S2A). This gave rise to baseline-corrected spectra as shown in Figures S1B and S2B. Reference spectra (Figure 2 and Figure S3) allowed determining fit parameters for all observed redox states (frequency, intensity, band width, and peak ratio, see Table S1), as described earlier. The sum of peak area (2 CN\(^-\) + 3 CO) for a given redox state was obtained by simulation of spectral data with the fixed fit parameters that represent the population in relation to the population of 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 Figure S1C or electrochemical potential in Figure S2C). For the last step of data evaluation, 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 CrHydA1 under ambient conditions. In the first step, ATR FTIR spectroscopy and spectro-electrochemistry\textsuperscript{27–29} were employed to extract the IR signatures of all relevant redox states (Table 1 and Figure S3). Figure 2A shows how ATR FTIR spectro-electrochemistry at alkaline or acidic conditions facilitated recording difference spectra for the transition from Hox into Hred\textsuperscript{′} (top, pH 9, -650 minus -450 mV vs SHE) and Hred into Hsred (bottom, pH 5, -750 minus -550 mV vs SHE). The overall downshift of the cofactor bands from Hox → Hred\textsuperscript{′} and Hred → Hsred has been attributed to a reduction of the [4Fe-4S] cluster (Figure 2B).\textsuperscript{25,26} Opposed to the Hox → Hred\textsuperscript{′} difference spectrum, the Hred → Hsred difference spectrum shows no signal around 1800 cm\textsuperscript{-1} (yellow mark-up, dashed line). This highlights the lack of a μCO ligand at the reduced diiron site (Figure 2B). Moreover, note the absence of other H-cluster species, which confirms the assignment of bands at 1961 cm\textsuperscript{-1} and 1953 cm\textsuperscript{-1} to Hred and Hsred, respectively (blue mark-up). The small band at 1972 cm\textsuperscript{-1} is unrelated to any known redox state. It has been assigned to Hhyd:red under cryogenic conditions\textsuperscript{39}; however, the spectrum lacks the respective μCO band at 1851 cm\textsuperscript{-1}. Therefore, Hred and Hsred are depicted with a terminal CO ligand and a μH ligand instead of a μCO ligand in Figure 2B.\textsuperscript{28}

Figure 2. Characterization of key H-cluster states. (A) FTIR spectro-electrochemistry difference spectra for the transitions Hox → Hred\textsuperscript{′} (top spectrum, pH 9, -650 minus -450 mV vs SHE) and Hred → Hsred (bottom spectrum, pH 5, -750 minus -550 mV vs SHE). The overall downshift has been attributed to redox changes at the [4Fe-4S] cluster. In contrast to the Hox → Hred\textsuperscript{′} difference spectrum
(yellow mark-up), no $\mu$CO band is observed for Hred and Hsred (dashed line). Instead terminal CO bands appear at 1961 cm$^{-1}$ and 1953 cm$^{-1}$ for Hred and Hsred (yellow mark-up). (B) Proposed H-cluster geometries. The rectangle represents the diiron site including ADT ligand (NH), Fe-Fe bridging ligand ($\mu$CO, $\mu$H), and the apical binding site of Fe$_4$ (vacant or occupied with CO). The cube represents the [4Fe-4S] cluster. Colors hint at differences relative to Hox.

### Table 1. Vibrational and electronic properties of different H-cluster states.

|           | CN$^{-}$/ cm$^{-1}$ | CO / cm$^{-1}$ | [4Fe-4S] | [FeFe] |
|-----------|---------------------|----------------|----------|--------|
| Hox       | 2088 2070           | 1964 1940      | 1802     | +2     | +3     |
| Hox-CO    | 2092 2082           | 1968 1962*     | 1812     | +2     | +3     |
| Hred$'$   | 2084 2066           | 1962 1933      | 1793     | +1     | +3     |
| Hhyd      | 2082 2068           | 1978 1960      | 1860     | +1     | +2     |
| Hred      | 2072 2034           | 1961** 1915    | 1891     | +2     | +2     |
| Hsred     | 2068 2026           | 1953** 1918    | 1882     | +1     | +2     |

To address the pH-dependent population of H-cluster states under H$_2$ oxidation conditions, we investigated CrHydA1 by ATR FTIR spectroscopy at different H$_2$ concentration without external potential control. Direct proof for the hydrogenase-catalyzed cleavage of H$_2$ came from D$_2$ oxidation experiment in aqueous environment.$^{32}$ 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, Figure 3A depicts a series of baseline-corrected ATR FTIR absorbance spectra in the CO regime of the H-cluster recorded after 2.5 min under 0 – 100% H$_2$ (steady-state conditions, compare Figure S1). We note that residual, unidentified H-cluster states were
present in the spectra. As these contribution did not to interfere with the global fit analysis (i.e., $\chi^2 < 10^{-4}$) we did consider them any further. Figure 3B illustrates how increasing the H$_2$ concentration from 0 – 0.1% resulted in an accumulation of 1e$^-$-reduced states H$_{\text{red}}$´ and H$_{\text{red}}$, which remained fairly stable between 0.1 – 3% H$_2$. At higher concentrations of H$_2$, the 2e$^-$-reduced H$_{\text{sred}}$ state dominated the spectrum. Only minor traces of the 2e$^-$-reduced H$_{\text{hyd}}$ state were observed, most likely due to the lack of sodium dithionite in the sample (see Experimental section).$^{32}$

**Figure 3. Composition of H-cluster states under H$_2$ oxidation conditions (pH 7).** All data recorded on a hydrated film of CrHydA1 at room temperature and 0 – 100% H$_2$ in the gas phase. (A) Series of baseline-corrected FTIR absorbance spectra in the CO regime of the H-cluster obtained after 2.5 min at each step. (B) State populations as a function of H$_2$ concentration. Between 0.1 – 3% H$_2$, the population of H$_{\text{red}}$´ and H$_{\text{red}}$ was relatively stable whereas H$_{\text{sred}}$ dominated for H$_2 > 10\%$.

Analogous to the experiment shown in Figure 3, six individual CrHydA1 protein films between pH 10 – 5 were analyzed (Figure 4). Largely independent of pH, the oxidized state H$_{\text{ox}}$ was the most prominent species in the absence of H$_2$ while H$_{\text{sred}}$ dominated the spectrum for H$_2 > 10\%$ (Figure S4). The steady-state population of H$_{\text{red}}$´ and H$_{\text{red}}$ is plotted as a function of H$_2$ and at different pH values in Figure 4AB. Here, we observed diverging trends for the accumulation of the 1e$^-$-reduced states as highlighted
by the arrows: \textbf{Hred} dominated at acidic conditions whereas \textbf{Hred}´ was promoted under alkaline values. Figure 4C depicts the accumulation of \textbf{Hred}´ and \textbf{Hred} at 3\% \textit{H}$_2$ as a function of pH, which clearly illustrates this trend. At low pH and \textit{H}$_2$ > 10\%, an increasing accumulation of \textbf{Hhyd} was observed, which may explain the mild suppression of \textbf{Hsred} that was otherwise expected to follow the same pH dependence as \textbf{Hred} (Figure S4). Upon removal of \textit{H}$_2$ from the gas stream, the 2e\textsuperscript{−}-reduced states \textbf{Hhyd} and \textbf{Hsred} converted transiently into the 1e\textsuperscript{−}-reduced states \textbf{Hred}´ and \textbf{Hred}, indicating intermolecular electron transfer in the dense films\textsuperscript{30}, before the equilibrium shifted back towards \textbf{Hox} upon auto-oxidation.\textsuperscript{22} The diverging pH dependence for the steady-state accumulation of \textbf{Hred}´ and \textbf{Hred} in the presence of \textit{H}$_2$ was found to be well conserved in this transient increase, emphasizing the robustness of all trends observed for of the 1e\textsuperscript{−}-reduced states (Figure S5).

![Figure 4. Accumulation of Hred´ and Hred as a function of H\textsubscript{2} and pH. Population of Hred´ (panel A) and Hred (panel B) for six different pH values (pH 5 – 10) at 0.1 – 100\% \textit{H}$_2$. The arrows at 3\% \textit{H}$_2$ hint at an opposite pH dependence, which is clearly illustrated plotting the population of Hred´ and Hred at 3\% \textit{H}$_2$ against pH (panel C).](image)

The simultaneous presence of \textbf{Hred}´ and \textbf{Hred} complicates unique conclusions regarding the mechanism of H-cluster protonation. Therefore, additional experiments were performed. First, we probed the pH dependence of \textit{H}$_2$ oxidation with \textit{CrHydA1}\textsuperscript{PDT}. This cofactor variant lacks the secondary amine of the natural ADT ligand (Figure 1B) and allows analyzing the reduction of the [4Fe-4S] cluster (i.e., the \textbf{Hox/Hred}´ transition) independent of redox chemistry at the diiron site.\textsuperscript{26,29} Figure S6 shows that the \textit{H}$_2$ oxidation activity of \textit{CrHydA1}\textsuperscript{PDT} increases between pH 10 – 8. While this cannot be explained by the
stoichiometry of the catalyzed reaction, the results are in agreement with earlier experiments under H₂ evolution conditions and support PCET chemistry at the [4Fe-4S] cluster.²⁹

The influence of cysteinyln ligand C417 on the catalytic properties of CrHydA1 has been addressed by site-directed mutagenesis earlier.⁴⁶,⁴⁷ Here, we analyzed three cysteine variants to compare the composition of H-cluster states under H₂ oxidation conditions (Figure S7). (i) C417S behaved much like wild-type CrHydA1 but showed a reduced percentage of Hred` under H₂. (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 CrHydA1.⁴⁷ In agreement with earlier observations, C417H adopted Hred` as a resting state and even after 12 – 18 h under N₂ we did not observe accumulation of Hox, reflecting the lack of H₂ evolution activity of CrHydA1 C417H.⁴⁷ In the presence of H₂, the variant converted into Hsred (at pH 8) or Hhyd (pH 4) with no detectable traces of Hred. In the absence of H₂, 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, e.g., due to hydrogen-bonding between the aspartic acid side chain and the [4Fe-4S] cluster. In variance to the histidine variant, C417D converted into Hox (pH 8) or HoxH (pH 4) after 12 – 18 h, reflecting the low but significant H₂ evolution activity of CrHydA1 C417D.⁴⁶

Overall, our data demonstrate how the equilibrium of redox states is affected by cluster ligation, proton concentration (pH), and the percentage of H₂ in the gas phase. Under H₂ 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 CrHydA1 under H₂ evolution conditions by injecting electrons into the systems in the absence of H₂. We employed ATR FTIR spectro-electrochemistry to follow the evolution of state populations as a function of electrochemical potential. In contrast to conventional, Moss-type⁴⁸ transmission cells, the ATR FTIR spectro-electrochemistry approach allowed H₂ to be released from the protein film which precluded product oxidation. Figure 5
depicts how the oxidized states $\text{Hox}$ and $\text{Hox-CO}$ were lost at reductive potentials, followed by accumulation of the $1e^-$-reduced states $\text{Hred}^-$ and $\text{Hred}$. Upon further reduction, accumulation of $\text{Hsred}$ was observed; however, in contrast to the experiments performed under $\text{H}_2$ oxidation conditions (Figure 3), $\text{Hhyd}$ was not observed at all. The latter observation may hint at different catalytic pathways for $\text{H}_2$ oxidation and $\text{H}_2$ evolution.\textsuperscript{21}

**Figure 5. Composition of H-cluster states under electrochemical control (pH 6).** Exemplary data recorded on a hydrated film of $\text{CrHydA1}$ between -50 – -750 mV vs SHE and constant $\text{N}_2$ purging. (A) Series of baseline-corrected ATR FTIR absorbance spectra in the CO regime 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: $\text{Hox/Hred}^-$ -375 mV; $\text{Hox/Hred}$ -345 mV; $\text{Hred/Hsred}$ -605 mV and -585 mV vs SHE.

Analogous to the experiment shown in Figure 5, nine individual protein films were analyzed (pH 9 – 5, in steps of 0.5 pH units). Reductive currents at potentials more negative than -550 mV vs SHE (Figure S2) restricted the analysis of midpoint potentials. Instead, the maximal population of H-cluster states as a function of potential 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 $\text{H}_2$ oxidation conditions (Figure
4). The oxidized state \textbf{Hox} was the most prominent species at potentials more positive than -350 mV vs SHE, largely independent of pH. The accumulation of \textbf{Hsred} was found to be affected by pH more drastically, with a higher population at acidic pH values that reflects the lack of \textbf{Hhyd} in the experiment (Figure S8). Overall, we observed a mean midpoint potential around -650 mV vs SHE for \textbf{Hsred}, which leaves a potential window of ~300 mV to analyze the accumulation of the 1e\textsuperscript{-}-reduced states. In Figure 6AB the steady-state population of \textbf{Hred\textsuperscript{'}} and \textbf{Hred} is plotted as a function of potential and at different pH values. Similar to what has been observed under H\textsubscript{2} oxidizing conditions, \textbf{Hred} dominated at acidic conditions whereas \textbf{Hred\textsuperscript{'}} was promoted under alkaline values. The arrows highlight these trends at reducing potentials; here, the population of states varies from 30 – 80 % (-550 mV vs SHE, \textbf{Hred\textsuperscript{'}}) and 40 – 5% (-350 mV vs SHE, \textbf{Hred}) for increasing pH values. Figure 6C depicts the accumulation of \textbf{Hred\textsuperscript{'}} and \textbf{Hred} as a function of pH. This trend is strictly conserved in the aforementioned potential window (Figure S8) and facilitated an estimation of apparent proton affinities for the accumulation of \textbf{Hred\textsuperscript{'}} (pK\textsubscript{a} > 8) and \textbf{Hred} (pK\textsubscript{a} < 6).

\textbf{Figure 6. Accumulation of Hred\textsuperscript{'} and Hred as a function of electrochemical potential and pH.}

Population of \textbf{Hred\textsuperscript{'}} (panel A) and \textbf{Hred} (panel B) for nine different pH values (pH 9 – 5) between -50 and -750 mV vs SHE. The arrows hint at an opposite pH dependence, which is clearly illustrated plotting the population of \textbf{Hred\textsuperscript{'}} (-550 mV vs SHE) and \textbf{Hred} (-350 mV vs SHE) as function of pH (panel C).
DISCUSSION

We analyzed the [FeFe]-hydrogenase HYDA1 from *Chlamydomonas reinhardtii* by ATR FTIR spectroscopy and spectro-electrochemistry under ambient conditions. Our in-depth characterization by infrared difference spectroscopy proved the lack of cryogenic H-cluster states. We analyzed the [FeFe]-hydrogenase HYDA1 from *Chlamydomonas reinhardtii* by ATR FTIR spectroscopy and spectro-electrochemistry under ambient conditions. Our in-depth characterization by infrared difference spectroscopy proved the lack of cryogenic H-cluster states.\(^{37-39}\) This facilitated a unique assignment of the IR pattern of H\textsubscript{red} and H\textsubscript{sred}, notably without a bridging CO ligand and in agreement with former reports.\(^{23-26}\) Moreover, we addressed the pH-dependent accumulation of various H-cluster states. Varying the sample pH under H\textsubscript{2} oxidation and H\textsubscript{2} evolution conditions established consistent trends for the accumulation of 1e\textsuperscript{−}-reduced H-cluster states: we observed enrichment of H\textsubscript{red} under acidic conditions whereas H\textsubscript{red}´ 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 H\textsubscript{red}´ formed upon reduction of the [4Fe-4S] cluster and protonation of a nearby cysteine, C417 in CrHydA1.\(^{29}\) Moreover, the H\textsubscript{red}´ state is involved in the steady-state accumulation of HoxH,\(^{30}\) which proceeds under reducing conditions exclusively and represents the starting state for an enrichment of Hhyd in the presence of H\textsubscript{2}.\(^{32,49}\) 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 non-trivial pH dependence of H\textsubscript{red}´ and H\textsubscript{red} under turnover conditions is yet to be accomplished. Due to the simultaneous presence of various reduced H-cluster states under H\textsubscript{2} (including Hhyd and H\textsubscript{sred}), unraveling the PCET chemistry of H\textsubscript{red}´ and H\textsubscript{red} was found to be challenging.\(^{35}\) To this end, ATR FTIR spectro-electrochemistry facilitated analyzing the population of 1e\textsuperscript{−}- and 2e\textsuperscript{−}-reduced H-cluster states individually. The accumulation of H\textsubscript{red} at acidic pH values and mildly reducing conditions (e.g., -350 mV vs SHE) suggests an apparent pK\textsubscript{a} < 6\(^{19}\), in agreement with the involvement of glutamic acid residues as ‘bottle neck’ in the catalytic PT pathway.\(^{19}\) In contrast, our data for the population of H\textsubscript{red}´ at more reducing potentials (e.g., -550 mV vs SHE) hints at an apparent pK\textsubscript{a} > 8, which reflects the greater ease of proton transfer to the [4Fe-4S] cluster via bulk solvent\(^{30,34}\) and is in
excellent agreement with the formal $pK_a$ of 8.1 for a cysteine sidechain. This observation is direct evidence for the pH dependence of $H_{\text{red}}^-$ in native [FeFe]-hydrogenase, in particular because the $H_{\text{red}}$ state does not show dedicated pH dependence in this pH window. We propose to distinguish a ‘high pressure’, catalytic PT pathway to the diiron site from a ‘low pressure’, 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. This has recently been confirmed by bulk electrochemistry. While the increase in $H_2$ evolution activity between pH 9 – 7 can be attributed to rising proton concentration, our data now allows correlating the activity decrease between pH 7 – 5 to the formation of $H_{\text{red}}$, which clearly dominated over $H_{\text{red}}^-$ at acidic pH values. This behavior is in agreement with $H_{\text{red}}$ and $H_{\text{sred}}$ as ‘$H_2$-inhibited’ states that bind a bridging hydride at the diiron site and have been shown to play a key role in sensory [FeFe]-hydrogenases. The ligand flip required to form a reactive terminal hydride geometry disfavors fast catalysis. 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.

We suggest that similar concepts may give rise to a novel generation of biomimetic hydrogen catalysts.

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$2 \text{e}^- \rightleftharpoons \mu\text{CO} \rightleftharpoons \text{H}_2$