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Submitted date: 28/01/2020 •Posted date: 29/01/2020
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Citation information: Mészáros, Lívia S.; Ceccaldi, Pierre; Lorenzi, Marco; Redman, Holly J.; Pfitzner, Emanuel; Heberle, Joachim; et al. (2019): Spectroscopic investigations under whole cell conditions provide new insight into the metal hydride chemistry of [FeFe]-hydrogenase. ChemRxiv. Preprint.
https://doi.org/10.26434/chemrxiv.9778967.v2

Hydrogenases are among the fastest H2 evolving catalysts known to date and have been extensively studied under in vitro conditions. Here, we report the first mechanistic investigation of an [FeFe]-hydrogenase under in vivo conditions. Functional [FeFe]-hydrogenase from the green alga Chlamydomonas reinhardtii is generated in genetically modified Escherichia coli cells, by addition of a synthetic cofactor to the growth medium. The assembly and reactivity of the resulting semi-synthetic enzyme was monitored using whole-cell electron paramagnetic resonance as well as Fourier-transform infrared spectroscopy. Through a combination of gas treatments, pH titrations and isotope editing, we were able to corroborate the physiological relevance of a number of proposed catalytic intermediates, including reactive iron-hydride species. We demonstrate the formation of the so-called hydride state in vivo. Moreover, two previously uncharacterized redox species are reported herein, illustrating the complex metal hydride chemistry of [FeFe]-hydrogenase.

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Spectroscopic investigations under whole cell conditions provide new insight into the metal hydride chemistry of [FeFe]-hydrogenase

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Keywords:
metalloenzymes; reaction mechanism; infrared spectroscopy; EPR spectroscopy; biophysics; hydrogen
Abstract: Hydrogenases are among the fastest H₂ evolving catalysts known to date and have been extensively studied under *in vitro* conditions. Here, we report the first mechanistic investigation of an [FeFe]-hydrogenase under whole-cell conditions. Functional [FeFe]-hydrogenase from the green alga *Chlamydomonas reinhardtii* is generated in genetically modified *Escherichia coli* cells, by addition of a synthetic cofactor to the growth medium. The assembly and reactivity of the resulting semi-synthetic enzyme was monitored using whole-cell electron paramagnetic resonance and Fourier-transform infrared spectroscopy as well as scattering scanning near-field optical microscopy. Through a combination of gas treatments, pH titrations and isotope editing, we were able to corroborate the physiological relevance of a number of proposed catalytic intermediates in living cells. Moreover, a previously uncharacterized catalytic intermediate is reported herein, attributed to the formation of a protonated metal hydride species.
INTRODUCTION

Hydrogenases are gas processing metalloenzymes that interconvert protons (H\(^+\)) and molecular hydrogen (H\(_2\)) with remarkable efficiency. The so-called [FeFe]-hydrogenases are considered the most efficient H\(_2\) producers in nature, with reported turnover frequencies up to 10,000 H\(_2\) s\(^{-1}\).\(^1,2\) This reactivity makes [FeFe]-hydrogenases highly relevant for biotechnological H\(_2\) production as an alternative to platinum-based electrolysis\(^3-6\) and a biological blueprint for the design of synthetic catalysts.\(^7-9\) Consequently, intense efforts have been invested in elucidating the structure and catalytic mechanism of these enzymes.\(^10,11\)

The reactivity of [FeFe]-hydrogenases is enabled by a hexanuclear iron complex, referred to as the hydrogen-forming cluster or simply the “H-cluster” (Fig. 1A). This cofactor consists of a canonical iron-sulfur cluster ([4Fe-4S]\(_H\)) coupled to an organometallic diiron subsite ([2Fe]\(_H\)). The low-valent iron ions of the [2Fe]\(_H\) subsite are bridged by an azadithiolate group (\(\text{SCH}_2\text{NHCH}_2\text{S}^-\), adt) and further coordinated by strong-field ligands like carbon monoxide (CO) and cyanide (CN\(^-\)).\(^12-15\) Due to its unique nature, the biosynthesis of the [2Fe]\(_H\) subsite requires at least three hydrogenase specific maturation enzymes.\(^16-20\)

Despite challenges in preparing the enzyme, extensive \textit{in vitro} work has revealed a number of potential catalytic intermediates. The oxidized resting state of the enzyme (H\(_{\text{ox}}\)) exhibits a mixed-valence [2Fe]\(_H\) subsite and an oxidized [4Fe-4S]\(_H\) cluster ([4Fe-4S]\(_H\))\(^2^+\)--[Fe(I)Fe(II)]\(_H\)).\(^21,22\) Under acidic conditions H\(_{\text{ox}}\)H is formed, attributed to a protonation at the [4Fe-4S]\(_H\) cluster.\(^23,24\) Reduction of H\(_{\text{ox}}\) by one electron results in either H\(_{\text{red}}\)\(^\prime\) or H\(_{\text{red}}\) (the latter also referred to as “H\(_{\text{red}}\)H\(^{\text{++}}\)”).\(^23,25,26\) The H-cluster shows a reduced and possibly protonated [4Fe-4S]\(_H\) cluster in H\(_{\text{red}}\)\(^\prime\) whereas in H\(_{\text{red}}\) the [2Fe]\(_H\) site is reduced and protonated.\(^27\) Further reduction of H\(_{\text{red}}\) generates the “super-reduced” state, H\(_{\text{sred}}\), identified as a [4Fe-4S]\(_H^+\)--[Fe(I)Fe(I)]\(_H\) species.\(^28\)
The exact nature of these intermediate states and their relevance to the catalytic mechanism is under debate.\textsuperscript{10, 27, 29-31} However, all recent models agree that a terminal hydride is formed during catalysis. One such intermediate, denoted \( \text{H}_{\text{hyd}} \), has been detected \textit{in vitro} and shown to accumulate in amino acid variants with disrupted proton transfer networks, but also in the native enzyme under reducing conditions at low pH.\textsuperscript{32-38} The \( \text{H}_{\text{hyd}} \) state features a terminal hydride on the \([2\text{Fe}]_\text{H}\) subsite (Fig. 1A) and the electronic structure of this state comprises a diamagnetic \([\text{Fe(II)Fe(II)}]_\text{H}\) subsite coupled to a paramagnetic \([4\text{Fe-4S}]_\text{H}^+\) cluster.\textsuperscript{33, 36}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Catalytic mechanism and assembly of the semi-synthetic H-cluster. Herein, proposed key intermediates were observed in cells for the first time, and conditions for accumulating a protonated hydride state (denoted \( \text{H}_{\text{hyd}}H^+ \)) are reported. (A) Schematic representation of the oxidized H-cluster and the catalytic cycle of [FeFe]-hydrogenase. The catalytic cycle is derived from refs. 10 and 36, debated protonation steps shown in parenthesis. Note that in case of \( \text{H}_{\text{red}} \), different one-electron reduced H-cluster states are conceivable, i.e., \( \text{H}_{\text{red}} \) or \( \text{H}_{\text{red}}' \). (B) Genetic modification of \textit{E. coli} for expression of [FeFe]-hydrogenase apo-protein, followed by synthetic maturation generates functional hydrogenase (the H-cluster is shown in the \( \text{H}_{\text{ox}} \) state). The reactivity of this semi-synthetic enzyme is probed in whole-cells by EPR and ATR FTIR spectroscopy. In parallel, the integrity of the cells is verified by AFM imaging and near-field IR spectroscopy.}
\end{figure}
The biological maturation machinery required for H-cluster assembly can be circumvented by incubating [FeFe]-hydrogenase apo-protein with synthetic mimics of the [2Fe]H subsite. Under in vitro conditions it is well established that the incorporation of the [Fe2(adt)(CO)4(CN)2]2− complex ([2Fe]adt) results in the spontaneous assembly of semi-synthetic hydrogenases indistinguishable from the native enzyme ([2Fe]adt-HydA1). We recently adopted this strategy for in vivo applications, enabling the preparation of fully functional, semi-synthetic enzymes in both E. coli and cyanobacteria. Herein, we take advantage of this protocol to perform spectroscopic investigations of [FeFe]-hydrogenase in whole-cells (Fig. 1B). We combine whole-cell electron paramagnetic resonance (EPR) and Fourier-transform infrared (FTIR) spectroscopy to characterize the enzymatic activity and catalytic mechanism of an [FeFe]-hydrogenase from the photosynthetic green alga Chlamydomonas reinhardtii, HydA1. In parallel, individual cells were characterized by atomic force microscopy (AFM), scattering-type scanning near-field optical microscopy (sSNOM) and nano-FTIR spectroscopy, verifying cellular integrity and protein content. Through a combination of gas flushes and pH changes we detected a number of proposed catalytic intermediates, including $\text{H}_\text{ox}$, $\text{H}_\text{oxH}$, and $\text{H}_\text{red}$, supporting their catalytic relevance. The present study also provides the first demonstration of the formation of reactive metal hydride species in living cells, as the $\text{H}_\text{hyd}$ state accumulates in whole cells under reducing conditions mimicking the native environment of the enzyme, i.e. the weakly alkaline stroma of the chloroplast in C. reinhardtii. Moreover, the formation of a protonated hydride state is observed under mildly acidic and strongly reducing conditions.

**RESULTS AND DISCUSSION**

*In vivo assembly of the semi-synthetic H-cluster.* To generate active hydrogenase in concentrations suitable for whole-cell spectroscopy, the structural gene hyda1 was heterologously expressed in BL21(DE3) E. coli cells, similar to previous reports. The absence of the [FeFe]-hydrogenase specific
maturation machinery in *E. coli* results in the synthesis of an inactive form of the enzyme containing the [4Fe-4S]_H cluster but lacking the [2Fe]_H subsite (apo-HydA1). 50 mL cell cultures were concentrated to 2 mL and depleted of O_2 at which point the synthetic cofactor mimic [2Fe]^{adt} was added to the medium, and the H-cluster assembly monitored by EPR spectroscopy and H_2 production assays. The apo-enzyme remained undetected in our experiments, due to the intensity of underlying signals attributed to other iron-sulfur proteins in *E. coli* (Fig. S1). Conversely, the formation of the H-cluster was readily observable by whole-cell EPR spectroscopy (Fig. 2A). Incubation of the cell suspensions for 1 h in the presence of low concentrations (0.008 – 0.8 µM) of the synthetic [2Fe]^{adt} cofactor resulted in the appearance of a rhombic EPR signal attributable to H_ox (g = 2.100; 2.040; 1.998). For higher concentrations of [2Fe]^{adt} in the cell medium (8 – 80 µM), the overall intensity of the EPR signal increased. Quantification of the signals versus a copper standard showed that a final total spin concentration of up to 10 µM was obtained following this protocol (Fig. 2B). However, the spectra became dominated by an axial signal originating from the CO inhibited state H_ox-CO (g = 2.054; 2.007).
Figure 2. *In vivo* H-cluster assembly monitored by EPR spectroscopy and H\textsubscript{2} gas production. (A) Whole-cell EPR spectra of apo-HydA1 (control, 0 µM [2Fe]\textsuperscript{adt} added) and [2Fe]\textsuperscript{adt}-HydA1 containing cells. H-cluster assembly occurs spontaneously upon addition of [2Fe]\textsuperscript{adt} to the medium, with H\textsubscript{ox} being the favored state at low [2Fe]\textsuperscript{adt} concentrations. As the concentration of the synthetic cofactor increases, the cell samples become dominated by the H\textsubscript{ox}-CO state. The g-values of H\textsubscript{ox} (green dashed lines) and H\textsubscript{ox}-CO (blue dashed lines) states are based on simulations of the two samples at 0.8 and 8 µM (black dash-dotted lines).

All [2Fe]\textsuperscript{adt}-HydA1 spectra were corrected for contribution from the cells by subtracting the signal of the apo-HydA1 control sample. EPR experimental conditions: T = 20 K, P = 1 mW, ν = 9.28 GHz. (B) Concentrations of the H\textsubscript{ox} (green bars) and H\textsubscript{ox}-CO (blue bars) signals in each sample, extrapolated from the simulations. The full length of the bar reflects total spin count.

(C) Average rate of hydrogen gas production during 1 h from anaerobic 2 mL *E. coli* cultures.

The successful assembly of functional enzyme was further verified by monitoring H\textsubscript{2} production from recombinant *E. coli* cells suspended in fresh M9 minimal medium. Hydrogen gas formation became clearly observable after the addition of 0.8 µM [2Fe]\textsuperscript{adt} to the medium and increased up to 80 µM [2Fe]\textsuperscript{adt}, concomitantly with the overall EPR signal intensity (Fig. 2C). The same trend was observed in *in vitro* assays. Hydrogenase activity assays performed on lysed cells demonstrated that a major fraction (approx. 80%) of the total available apo-HydA1 pool had formed the active [2Fe]\textsuperscript{adt}-HydA1 enzyme after 1 h incubation with 80 µM [2Fe]\textsuperscript{adt} in the cell medium (Fig. S2). Increasing the concentration of [2Fe]\textsuperscript{adt} beyond this point resulted in a decline of *in vivo* H\textsubscript{2} production. Due to the dominance of H\textsubscript{ox}-CO at higher [2Fe]\textsuperscript{adt} concentration (Fig. 2B), the decreased H\textsubscript{2} productivity is attributed to release of CO from excess cofactor in the cell medium resulting in inhibition of the enzyme. The integrity and viability of the cells following anaerobic incubation in the presence of 80 µM [2Fe]\textsuperscript{adt} was verified in parallel assays via plating experiments in combination with SDS-PAGE (Figs S3 and S4). The effect of the hydrogenase activity on the cytoplasmic pH was found to be negligible, with a decrease from pH 7.6 (± 0.2) to 7.5 (± 0.3) after incubation of the cells with [2Fe]\textsuperscript{adt} for 60 minutes observed in separate assays using pH dependent fluorescent dyes. Consequently, a concentration of 80 µM [2Fe]\textsuperscript{adt} in the cell medium was determined as optimal for the following mechanistic studies.
Investigating the integrity and enzymatic activity of single cells. The integrity of individual cells following incubation with [2Fe]adl was monitored by atomic force microscopy (AFM) on diluted *E. coli* samples (factor $5 \times 10^5$). The topography of the cells suggested viable bacteria and only a limited number of cells were found to be morphologically altered (Figs 3A and S5). Subsequently, the local IR absorption of single cells was recorded using scattering-type scanning near-field optical microscopy (sSNOM). This label-free technique provides a chemical image of the cell at a spatial resolution of 30 nm. To visualize the protein content in a number of representative cells, the amide I band ($v_1 = 1660$ cm$^{-1}$) was corrected for a topography feature at $v_2 = 1710$ cm$^{-1}$ (Fig. 3A and Fig. S6 for additional examples). We found that the protein distribution was largely homogenous within the cells and no indication of secreted protein outside the cell was detected. Nano-FTIR near-field phase spectra showed typical amide I and amide II absorption within the cell, while such bands were not observed outside of the cell (Fig. 3B and Fig. S7 for additional examples). Moreover, only limited loss of H-cluster signal was observed upon washing *E. coli* cells in up to $10^8$ time the volume of buffer (Fig. S8). In combination with the aforementioned SDS-PAGE and plating experiments (Figs. S3 and S4), these findings underscore that our whole-cell spectroscopy reports on [FeFe]-hydrogenase located inside the cells.

Figure 3. Analysis of individual cells. (A) Upper left: AFM topography of a [2Fe]adl - HydA1 containing *E. coli* cell. Lower left: sSNOM image mapped at $v_1 = 1660$ cm$^{-1}$ (amide I absorption). Lower right: sSNOM image mapped at $v_2 = 1710$ cm$^{-1}$ (topography artifact). Upper right: $v_1 - v_2$ sSNOM difference image. The protein is clearly localized to the cellular environment. See Figs. S6 and S7 for additional examples. (B) Nano FTIR near field phase spectrum from 2000 – 1300 cm$^{-1}$. (C)
Spectra were recorded on the cell (spot 1, blue) and next to the cell (spot 2, red) in the AFM topography (panel A). (C) ATR FTIR difference spectra showed an HDO band (2515 cm\(^{-1}\)) increase in the presence of \(\text{D}_2\) (exposure time 0 s – 90 s increasing from black to purple). Inset: A simultaneous enrichment of \(\text{H}_{\text{red}}\) (1891 cm\(^{-1}\)) over \(\text{H}_{\text{ox}}\) (1940 cm\(^{-1}\)) was observed.

In the next step, we used attenuated total reflection Fourier-transform infrared (ATR FTIR) spectroscopy to probe the catalytic competence of hydrated \textit{E. coli} films. Cells containing [2Fe]\(\text{adt}\)-HydA1 were deposited on the silicon crystal of the ATR optic, dried under \(\text{N}_2\), and rehydrated to form a film that can interact with gases. When the film was kept under 100 mbar deuterium gas (\(\text{D}_2\)) instead of pure \(\text{N}_2\), deuterium ions (\(\text{D}^+\)) were released into bulk \(\text{H}_2\text{O}\). The clear appearance of the HDO band (2515 cm\(^{-1}\)) in ATR FTIR difference spectra served as direct verification of \(\text{D}_2\) oxidation activity (Fig. 3C), confirming that the [FeFe]-hydrogenase located inside the cells retained its activity in hydrated films. Additionally, H-cluster band shifts in the CO regime suggest reduction of \(\text{H}_{\text{ox}}\) into \(\text{H}_{\text{red}}\) (Fig. 3C, inset).

Mechanistic investigations on cell films using in situ ATR FTIR spectroscopy. The H-cluster-specific CO and CN\(^-\) vibrations in the region from 2150 – 1750 cm\(^{-1}\) were clearly discernible in ATR FTIR spectroscopy. Films of [2Fe]\(\text{adt}\)-HydA1-containing \textit{E. coli} showed predominantly oxidized enzyme with only minor contaminations of CO-inhibited cofactor (Fig. S9). No trace of the free [2Fe]\(\text{adt}\) cofactor was detected. Exposing the film to 1% \(\text{H}_2\) resulted in an enrichment of \(\text{H}_{\text{red}}\) over \(\text{H}_{\text{ox}}\) and \(\text{H}_{\text{ox}}\)-CO on a time-scale of seconds, and increasing \(\text{H}_2\) content to 100% had no further effects on the final spectra (Fig. 4A, spectrum a and Fig. S9). Notably, neither \(\text{H}_{\text{red}}\)’ (CO marker band 1933 cm\(^{-1}\)) nor \(\text{H}_{\text{red}}\) (CO marker band 1882 cm\(^{-1}\)) were detected in whole-cell samples, in contrast to \(\text{H}_2\)-exposed films of purified [2Fe]\(\text{adt}\)-HydA1 (Fig. S10). When \(\text{H}_2\) was removed from the atmosphere (\(\text{N}_2\) purging), the H-cluster converted back into \(\text{H}_{\text{ox}}\) (Fig. 4A, spectrum b). The fact that \(\text{H}_{\text{ox}}\)-CO does not re-appear suggests a high degree of cofactor stability on the time-scale of the FTIR measurements. When exposed to 1% CO, the oxidized whole-cell film converted to \(\text{H}_{\text{ox}}\)-CO (Fig. 4A, spectrum c).
**Figure 4.** ATR FTIR analysis of [2Fe]^{adm}-HydA1 containing cells. All difference spectra show the CO/CN\textsuperscript– regime of the H-cluster. (A) In the presence of 1% H\textsubscript{2} (pH 8, 2 mM NaDT) freshly prepared cells converted from H\textsubscript{ox} and H\textsubscript{ox}-CO to H\textsubscript{red} (spectrum a). * The band at 2032 cm\textsuperscript{–1} was attributed to a CN\textsuperscript– band of H\textsubscript{red}. In the absence of H\textsubscript{2} quantitative enrichment of H\textsubscript{ox} was observed (spectrum b). Exposure to 1% CO resulted in population of H\textsubscript{ox}-CO (spectrum c). Under non-reducing, acidic conditions (N\textsubscript{2}, pH 4, 2 mM NaDT) H\textsubscript{ox} converted into H\textsubscript{ox}H (spectrum d). (B) In the presence of 1% H\textsubscript{2} (pH 4, 2 mM NaDT) formation of H\textsubscript{hyd} over H\textsubscript{ox}H was observed (spectrum a, black line). Reduction with D\textsubscript{2} gas reproduced the hydride-specific downshift of the \(\mu\text{CO}\) band from 1860 to 1855 cm\textsuperscript{–1} (spectrum a, dark yellow line). Increasing the NaDT in the aerosol to 100 mM (pH 4, 1% H\textsubscript{2}) facilitated accumulation of a novel state, H\textsubscript{hyd}H\textsuperscript{+} (spectrum b, black line). The downshift of the \(\mu\text{CO}\) band under D\textsubscript{2} (1875 to 1870 cm\textsuperscript{–1}) verified the presence of a hydride ligand (spectrum b, purple line). * Small fraction of H\textsubscript{red} at low pH.
Acidification of the cell film via the aerosol (mixed buffer set to pH 4) and addition of 2 mM sodium dithionite (NaDT) caused a complete conversion of $\text{H}_{\text{ox}}$ into $\text{H}_{\text{oxH}}$ (Fig. 4A, spectrum d and Fig. S9).\textsuperscript{23} The cytoplasmic pH was determined in separate assays, and incubation in pH 4 media resulted in a decrease in cytoplasmic pH from 7.5 ($\pm$ 0.3) to 6.1 ($\pm$ 0.1) of $[2\text{Fe}]^{\text{adt}}$-HydA1 containing cells. In the presence of 1% $\text{H}_2$ or $\text{D}_2$, a quantitative enrichment of $\text{H}_{\text{hyd}}$ over $\text{H}_{\text{oxH}}$ was observed (Fig. 4B, both spectra a). Increasing the NaDT concentration in the medium enabled detection of $\text{H}_{\text{hyd}}$ also at pH 8 (Fig. S11), suggesting that strongly reducing conditions allow the accumulation of this reactive intermediate also at weakly alkaline pH. Similar trends have been observed for the \textit{in vitro} enrichment of $\text{H}_{\text{oxH}}$ and $\text{H}_{\text{red'}}$.\textsuperscript{23, 26} The combination of high NaDT concentrations and acidic pH resulted in the formation of a new species, shifted to higher frequencies by up to 15 cm$^{-1}$ as compared to the previously reported $\text{H}_{\text{hyd}}$ state (Fig. 4B, both spectra b). Low pH reference experiments on purified HydA1 in the presence of 100 mM NaDT and 1% $\text{H}_2$ confirmed the possibility of generating this species also \textit{in vitro}, which facilitated a precise assignment of the band positions (Fig. S11). To verify that this new signal reflected a hydride-binding form of the H-cluster, the same species was generated using $\text{D}_2$, which resulted in the expected downshift of the bridging carbonyl band\textsuperscript{48} from 1875 to 1870 cm$^{-1}$ (Fig. 4B, purple spectrum b).

Making use of \textit{in situ} ATR FTIR spectroscopy on whole-cells, we were able to observe several H-cluster states previously identified on purified hydrogenase. It is worth noting that, in contrast to \textit{in vitro} conditions, $\text{H}_{\text{red}}$ and $\text{H}_{\text{red'}}$ were never observed, neither in alkaline nor acidic media. This stark difference in reactivity further solidifies the notion that our data report on whole-cell samples. Conversely, $\text{H}_{\text{hyd}}$ as well as the up-shifted $\text{H}_{\text{hyd}}$-like state readily accumulated under acidic conditions and could be selectively enriched as a function of NaDT concentration. A similar up-shifted hydride state was reported earlier\textsuperscript{32, 48} but has not been characterized in detail. Comparing its IR signature to that of $\text{H}_{\text{hyd}}$, the observed spectral up-shifts are larger than those previously attributed to protonation changes at the $[4\text{Fe}-4\text{S}]_1$ cluster (Fig. S11).\textsuperscript{23, 26} DFT calculations carried out by Mulder et al. have attributed shifts of similar magnitude to
protonation changes at the [2Fe]H subsite. Thus, we assign this species to a novel hydride state featuring a nitrogen-protonated azadithiolate ligand, H_{hyd}H^+.

Two different hydride-like states observed by EPR as a function of pH. The formation of H_{hyd}, as well as an alternative hydride-like state formed at low pH, was corroborated by EPR spectroscopy. A rhombic signal \((g_{xyz} = 2.079, 1.935, 1.878)\) in good agreement with the \textit{in vitro} identified H_{hyd} state was observed for weakly alkaline [2Fe]^{adt}-HydA1 containing \textit{E. coli} suspensions, rapidly frozen after incubation with NaDT and H\(_2\) (Fig. 5, spectrum a and Fig. S12, table S3). The \(g_y\) and \(g_x\) positions of the H_{hyd} signal were readily apparent in spectra recorded at both 10 and 20 K (Fig. S13), while the broad nature of the feature observed at \(g \approx 2.08 - 2.07\) complicated an exact assignment of the \(g_z\) position. Spectra recorded at higher power (\(P \geq 8\)mW) simplified the low-field region and were consequently used for the \(g_z\) assignment (Fig. S14). Small contributions from H\(_{ox}\) were still visible in the spectrum, in combination with an additional minor rhombic signal (Figs.5 and S12). Conversely, including an H\(_{ox}\)-CO component in the simulation did not improve the overall fit. Thus, the central \(g \approx 2.0\) feature is most likely attributable to EPR active species native to \textit{E. coli}. Finally, it is noteworthy that the H_{hyd} signal was readily observable in cells incubated under an H\(_2\) atmosphere even in the absence of NaDT, underscoring its physiological relevance.
**Figure 5.** Whole-cell generation of the H$_{\text{hyd}}$ and H$_{\text{hyd}+}$ states under different conditions probed by EPR spectroscopy. (a): The H$_{\text{hyd}}$ state was observable in EPR spectra recorded on [2Fe]$_{\text{adt}}$-HydA1 containing cells at pH 7.5, complemented with 10 mM NaDT, flushed with H$_2$ for 15 min and rapidly frozen (dark yellow spectrum); (b): A new signal attributed to H$_{\text{hyd}+}$ was observed in samples prepared as lane a but at pH 4 (purple spectrum); (c): Samples prepared as lane b but excluding NaDT generated a weaker H$_{\text{hyd}+}$ signal (green spectrum); (d): Samples prepared as lane b but excluding H$_2$ flushing did not generate any discernable H$_{\text{hyd}+}$ signal (blue spectrum). The g-values of H$_{\text{ox}}$/H$_{\text{ox}+}$ (green dashed vertical lines), H$_{\text{hyd}}$ (dark yellow dashed vertical lines) and H$_{\text{hyd}+}$ (purple dashed vertical lines) states are based on the simulations of the spectra (black dash-dotted lines), for details see Fig. S12. Unassigned weak signals potentially arising from an H$_{\text{trans}}$-like state indicated with asterisks. EPR experimental conditions: T = 20 K, P = 1 mW, ν = 9.38 GHz.

A new rhombic, H$_{\text{hyd}}$-like signal was observed when the analogous experiment was performed in media acidified to pH 4 (Fig. 5, spectrum b and Figs S12-S14). As the signal accumulates under conditions similar to those of H$_{\text{hyd}+}$ in ATR-FTIR, we assign this signal to the same protonated hydride state. An exact assignment of the g$_z$ position was challenging also for H$_{\text{hyd}+}$, due to the complex nature of the g$_z$ region at low microwave powers (Figs. S12 and S14). Still, in comparison to H$_{\text{hyd}}$ the low pH spectrum featured a clearly discernible decreased anisotropy (g$_{\text{xy}}$ = 2.073, 1.935, 1.881, vertical purple dashed lines.
We assign the observed shift of the $H_{ox} g_z$ position from 2.100 to 2.101 at low pH to the formation of $H_{ox}$. Attempts at simulating the high pH $H_{hyd}$ spectrum as a combination of $H_{hyd}H^+$ and $H_{red}$ did not yield reasonable results, further supporting that these three rhombic EPR signals arise from different states (table S3). Based on the similarity of the rhombic $H_{hyd}H^+$ signal to that observed for the $H_{hyd}$ state in particular, but also the $H_{trans}$ state, it is attributed to a species featuring a reduced [4Fe-4S]$_1$ cluster coupled to a diamagnetic [Fe(II)Fe(II)]$_1$ subsite, closely resembling the $H_{hyd}$ state.

Exposing apo-HydA containing cells to $H_2$ in the presence or absence of NaDT did not result in any spectral features similar to $H_{hyd}H^+$, neither did BL21(DE3) E. coli cells lacking the hyda1 gene. The transient nature of the $H_{hyd}H^+$ state in cell suspensions was verified by exchanging the $H_2$ atmosphere with Ar for 30 minutes, which resulted in a loss of $H_{hyd}H^+$ and conversion back to a mixture of oxidized states ($H_{ox}$ and $H_{ox}$-CO). Interestingly, the presence of NaDT in the cell medium during $H_2$ treatment was not strictly required for the generation of the $H_{hyd}H^+$ state (Fig. 5, compare spectra a and c). Increasing the NaDT concentration in the cell suspension up to 100 mM, i.e. conditions shown to favor accumulation of the $H_{hyd}H^+$ state under ATR-FTIR conditions (Fig. 4B), resulted in a more intense $H_{hyd}H^+$ signal but no distinct new spectral features were observed. Conversely, no $H_{hyd}H^+$ signal was discernible in the absence of $H_2$, irrespective of NaDT concentration (Fig. 5, spectrum d).

CONCLUSIONS

The artificial maturation of apo-HydA in E. coli provides access to high concentrations of [FeFe]-hydrogenase in vivo. Furthermore, cellular $H_2$ oxidation and production verify that the resulting semi-synthetic HydA enzyme is functional and connects to the cell metabolism, most likely via ferredoxins. This has facilitated the first mechanistic investigation of an [FeFe]-hydrogenase under whole-cell conditions, employing EPR and FTIR spectroscopy as complementary techniques. Despite differences in
sample preparation and experimental conditions (i.e. cell suspension vs cell film, cryogenic vs ambient temperatures) both techniques reveal the same trends.

The dominant oxidation states under alkaline conditions are $\text{H}_{\text{ox}}$ and $\text{H}_{\text{ox}-\text{CO}}$. In addition, FTIR spectroscopy on hydrated films showed that $\text{H}_{\text{red}}$ was enriched in the presence of $\text{H}_2$. This transition was rapid and quantitative but the enzyme converted back to $\text{H}_{\text{ox}}$ when $\text{H}_2$ was removed from the gas phase. Similar reactivity (“auto-oxidation”) is generally observed for HydA1 in vitro and is clearly retained in vivo. It is noteworthy that diverging reactivity was recently reported for two putative sensory [FeFe] hydrogenases of different origin, which appear to stabilize in an $\text{H}_{\text{red}}$-like state both in vitro and in whole cells.\textsuperscript{44, 49} This arguably reflects the different physiological roles of sensory hydrogenases and the prototypical HydA1 enzyme.\textsuperscript{50}

In addition to the relatively stable catalytic intermediates $\text{H}_{\text{ox}}$ and $\text{H}_{\text{red}}$, the study revealed complex metal-hydride chemistry occurring in living cells and two different hydride states were observed. The EPR spectra recorded on cell suspensions incubated under a neat $\text{H}_2$ atmosphere in weakly alkaline buffer revealed the formation of the $\text{H}_{\text{hyd}}$ state. This finding verify that this proposed catalytic intermediate is generated under physiologically relevant conditions, i.e. in the absence of the artificial reductant dithionite and in weakly alkaline cell medium where the cytoplasmic pH is $\approx 7.5$. The whole-cell formation of $\text{H}_{\text{hyd}}$ was also confirmed by FTIR spectroscopy. Acidification of the cell medium resulted in decrease of cytoplasmic pH and rapid formation of $\text{H}_{\text{oxH}}$. Exposing the acidified cells to reducing conditions resulted in accumulation of $\text{H}_{\text{hyd}}$, previously only observed in vitro. Moreover, the complementary nature of EPR and FTIR spectroscopy has allowed us to detect and characterize an additional hydride-like state, denoted $\text{H}_{\text{hyd}}\text{H}^+$. The existence of alternative hydride-like state has been suggested earlier from work on HydA1, often in amino acid variants with impaired proton transfer capacity, but has so far been challenging to accumulate and consequently not characterized in detail.\textsuperscript{31-33, 35, 48} Herein, we show how an alternative hydride-like state, $\text{H}_{\text{hyd}}\text{H}^+$, can be selectively generated also in the fully functional enzyme, not only in
in vitro but also under whole-cell conditions. The assignment of $H_{hyd}H^+$ as a hydride state is confirmed by
the observed H/D isotopic shift of the μ-CO ligand. The pH dependence and spectral up-shift relative to
$H_{hyd}$, as well as earlier DFT calculations, supports that this hydride state features a protonated nitrogen
bridgehead atom.\textsuperscript{48} Additionally, the relatively small change observed in the corresponding EPR spectra
supports a model in which the electronic configuration of $H_{hyd}$ remains unchanged (i.e. [4Fe-4S]$H^+$-
[Fe(II)Fe(II)]$_{H}$ upon protonation, as the latter technique reports primarily on the paramagnetic [4Fe-4S]$H^+$
cluster. The possibility to accumulate this state also \textit{in vitro} should enable more detailed studies.

In conclusion, the expansion of artificial maturation to \textit{in vivo} conditions in combination with our capacity
to manipulate and spectroscopically investigate [FeFe]-hydrogenases in whole cells has allowed us to
support the physiological relevance of a number of proposed catalytic intermediates. In the context of
mechanistic understanding, $H_{hyd}H^+$ potentially represent the last isolatable intermediate in the transition
from from $H_{hyd}$ to $H_{ox}$ with concomitant H$_2$ formation. Deciphering and mapping out the reactivity of this
hydride species represents an exciting future prospect, expected to provide critical insight into H$_2$
formation and cleavage.

\section*{EXPERIMENTAL}

\textbf{General.} All chemicals were purchased from Sigma-Aldrich or VWR and used as received unless
otherwise stated. All anaerobic work was performed in an MBRAUN glovebox ([O$_2$] < 10 ppm). The
expression vector encoding the \textit{hydA1} gene (pETDuet-CrHydA1-His) was kindly provided by Prof. Marc
Fontecave (Collège de France, Paris/CEA, Grenoble). (Et$_4$N)$_2$[Fe$_2$(adt)(CO)$_4$(CN)$_2$] ([2Fe$^{\text{adt}}$]) was
synthesized in accordance to literature protocols with minor modifications, and verified by FTIR
spectroscopy.\textsuperscript{51-54} The complex was dissolved in anaerobic potassium phosphate buffer (100 mM, pH 6.8)
at 10 ng/µL - 10 µg/µL concentration and used directly. Protein content was analyzed by 10% SDS- PAGE
minigels in a BioRad Mini-PROTEAN Tetra Cell system. The proteins were stained with Page Blue protein staining solution (Thermo Fisher Scientific) according to the supplier’s instructions.

**Overexpression of the apo-HydA1 hydrogenase.** *Escherichia coli* BL21(DE3) cells containing the HydA1 plasmid were grown in 50 mL M9 medium [22 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 85 mM NaCl, 18 mM NH$_4$Cl, 0.2 mM MgSO$_4$, 0.1 mM CaCl$_2$, 0.4% (v/v) glucose] under aerobic conditions until O.D.$_{600}$=0.6 – 0.8 in the presence of ampicillin. The protein overproduction was induced with 1 mM IPTG and persisted at 20°C for 16-18 h with continuous aeration. The media was supplemented with 100 µM FeSO$_4$ at the time of the induction. Final O.D.$_{600}$ of the cultures were 1.4±0.2.

**In vivo formation of [2Fe]$^{adt}$-HydA1.** The preparation of the semi-synthetic hydrogenase was performed following a literature protocol with minor modifications.$^{42, 43}$ The apo-HydA1 protein was expressed in 50 mL *E. coli* cultures as described in the “Overexpression of the apo-HydA1 hydrogenase” section. After the 16 – 18 h expression period the cells were harvested, deaerated and transferred to the glove-box. The cells were re-suspended in fresh M9 medium (2 mL final volume), and formation of [2Fe]$^{adt}$–HydA1 was achieved by treating the cell suspensions with 100 µg (156 nmol) [2Fe]$^{adt}$ complex (80 mM final conc.), unless otherwise stated, for 1 h at 37°C under strictly anaerobic conditions.

**Whole-cell EPR and FTIR sample preparation.** The 2 mL concentrated cell suspensions generated via the “In vivo formation of [2Fe]$^{adt}$-HydA1” protocol were centrifuged and the cell pellet washed with 1 mL TRIS-HCl buffer (100 mM TRIS, 150 mM NaCl pH 7.5) three times under anaerobic conditions. For EPR samples the cells were diluted to a final volume of 400 µL with TRIS-HCl buffer after the washing protocol and transferred into EPR tubes. The tubes were capped and directly frozen in liquid nitrogen. In case of FTIR samples four separate 2 mL sample preparations were combined, concentrated and diluted to 400 µL with TRIS-HCl buffer and frozen in liquid nitrogen under anaerobic conditions.

**Generation of the H$_{hyd}$ EPR samples.** The apo-HydA1 containing cells were activated as described above. After activation the cells were washed with TRIS-HCl buffer (100mM TRIS, 150mM NaCl) and
re-suspended 400 µL buffer under anaerobic conditions. The pH of the TRIS-HCl buffer was either pH 7.5 or pH 4 according to the experiment, and verified before and after each flushing experiment. When indicated, the TRIS-HCl buffer was complemented with 10 or 100 mM Na-dithionite. The H₂ treated samples were flushed with H₂ gas for 15 min under anaerobic conditions. After flushing the cells were quickly transferred into EPR tubes. The tubes were capped and flash frozen in liquid nitrogen. Plating the cells on ampicillin enriched LB-agar plates as well as SDS-PAGE gel analysis was used to ensure the viability and integrity of the cells also under low pH conditions (Fig. S3).

**Hydrogenase activity measurements.** *In vivo* and *in vitro* activity measurements were performed according to published protocols.⁴² Hydrogen production was determined by analyzing the head-space gas, using a gas chromatograph (GC; PerkinElmer LLC, MA, USA) equipped with a thermal conductivity detector (TCD) and a stainless-steel column packed with Molecular Sieve (60/80 mesh). A calibration curve was established by injecting known amounts of hydrogen. The operational temperatures of the injection port, the oven and the detector were 100 °C, 80 °C and 100 °C, respectively. Argon was used as the carrier gas at a flow rate of 35 mL min⁻¹.

**EPR measurements.** The EPR spectra shown are representative signals from at least two individual experiments. The individual experiments show some preparation dependent differences, but the amplitude of these background signals are negligible compared to the signal intensity of the [2Fe]²⁺⁻ activated HydA1. Measurements were performed on a Bruker ELEXYS E500 spectrometer using an ER049X SuperX microwave bridge in a Bruker SHQ0601 cavity (Figure 2) or a Bruker EMX micro equipped with an EMX Premium bridge and an ER4119 HS resonator (Figure 5 + S12-14), both equipped with an Oxford Instruments continuous flow cryostat and using an ITC 503 temperature controller (Oxford Instruments). Measurement temperatures ranged from 10 to 20 K, using liquid helium as coolant, with the following EPR settings unless otherwise stated: microwave power 1 mW, modulation amplitude 1 mT, modulation frequency 100 kHz. The spectrometer was controlled by the Xepr software package (Bruker).
EPR spectra processing, simulations and Spin quantification. The EPR spectra were processed using the softwares Matlab (Mathworks, Inc) and QSoas. Matlab served for converting the EPR files to ascii format, while QSoas was used to display the spectra as a function of g values, for visual inspection and subtraction of background signals emerging from the cells. The processed signals were used for figures 2, 5 and S12. The simulations were performed using the easyspin toolbox (5.2.23) within Matlab. The positions and line-width of each signature were manually adjusted to fit the experimental features, and kept the same across all datasets. Other details of the procedure can be found in ref 43.

Infrared measurements. For whole-cell ATR FTIR spectroscopy, 1 μL E. coli suspension was deposited on the silicon crystal of an ATR cell in the beam path of a commercial FTIR spectrometer (Bruker). All experiments were performed at ambient temperature (~24 °C) and pressure (~1 atm), in the dark, and on hydrated films. The cell suspension was dried under 100% N2 gas and re-hydrated with buffer solution (100 mM TRIS, MES, and PIPPS) in the humidified gas stream (aerosol), similar to what was reported for purified protein earlier. The utilized buffer mix allowed titrating the cell film between pH 9 and pH 3. Reduction of [FeFe]-hydrogenase in the cells was induced by adding 1% - 100% H2 to the N2 gas stream (flow volume 1.5 L min⁻¹). In the absence of H2 (100% N2), the oxidized resting state (Hox or HoxH) recovered due to auto-oxidation. Plating experiments were performed to verify cell viability after the FTIR experiments (Fig. S4) Transitions were followed with a spectral precision of 2 cm⁻¹ and 1,000 averages of interferometer scans per spectrum. Difference spectra were calculated by subtraction of single absorbance spectra recorded under varying conditions (e.g., H2 – N2, pH 4 – pH 8, etc.).

Single cell analysis. For single-cell AFM, sSNOM and nano-FTIR cell suspensions were diluted by a factor of 5 x 10⁵ (1:20,000) and a 1 μL droplet was dried on template stripped gold substrate. Topographies, near-field maps and spectra were acquired under ambient conditions. For other technical details, the setup is described in the supplementary material section, Fig. S15.
Cytoplasmic pH quantification. The intracellular pH of the pH 4 and pH 7.5 incubated cells were quantified with pH Rhodo™ Green AM Intracellular pH Indicator and with Intracellular pH Calibration Buffer Kit (Thermo Fisher Scientific). The E.coli cells containing the overproduced apo-HydA1 protein with and without [2Fe]_{ad} activation were incubated in pH 4 or pH 7.5 TRIS-HCl media for 60 minutes under anaerobic conditions. Detection of the intracellular pH was performed in accordance to the manufacturer’s instructions using a Hidex Chameleon plate reader with 495 nm excitation and 590 nm emission filter.

Supporting Information

Electronic Supplementary Information (ESI) available: Including additional EPR and FTIR data, AFM images of E. coli cells, cell fitness data, in vitro enzymatic assay data, as well as an overview of the sSNOM and nanoFTIR setup.

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Conflicts of Interest

There are no conflicting interests to declare.
ACKNOWLEDGMENT

The European Research Council (GB, StG contract no. 714102) and the Olle Engkvist Byggmästare foundation (GB and LM) are gratefully acknowledged for funding. The Deutsche Forschungsgemeinschaft (DFG) is acknowledged for financial support to STS (STR 1554/5-1) and JH (HE 2063/5-1). Part of the project is funded by the DFG under Germany’s Excellence Strategy – EXC 2008/1 (UniSysCat) – 390540038 to JH.

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For Table of Contents Only
in vivo spectroscopy (EPR, FTIR, sSNOM)
Supplementary Material

Spectroscopic investigations under whole cell conditions provide new insight into the metal hydride chemistry of [FeFe]-hydrogenase

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Figure S1. *In vivo* H-cluster assembly monitored using X-band EPR spectroscopy.

Whole-cell EPR spectra recorded on *E. coli* cells expressing apo-HydA1. Samples collected from cells incubated in the absence (apo-HydA1) and presence of 0.008 – 80 µM [2Fe]adt, as described for Figure 2 (main text), but shown with a wider scan window. EPR spectra were recorded at 20 K, 1 mW microwave power, at a microwave frequency of 9.28 GHz.
The fraction of available apo-HydA1 activated \textit{in vivo} was determined using standard enzymatic assays, by comparing the maximum activity observed for enzymes activated either \textit{in vivo} or \textit{in vitro} as previously described. The total amount of apo-HydA1 available for activation was determined through \textit{in vitro} activation assays. Cultures were prepared in an identical fashion to the EPR sample preparation protocol, but lysed prior to addition of the cofactor. Under such \textit{in vitro} conditions, activation of apo-HydA1 is expected to proceed quantitatively and the resulting hydrogenase activity was determined in standard methyl viologen/dithionite assays (grey bars). This \textit{in vitro} activation protocol resulted in a maximum H$_2$ production rate of 93.0 ($\pm$ 14\%) nmol H$_2$ min$^{-1}$ mL$^{-1}$, reached after addition of 1-10 $\mu$g [2Fe]$^{\text{adt}}$ (0.8-8 $\mu$M final concentration). In parallel, apo-HydA1 expressing cells were treated with varying amounts of the synthetic cofactor analogously to the EPR sample preparation protocol, washed and lysed into KPi buffer as previously described. Following this \textit{in vivo} activation a maximum activity of 73.0 ($\pm$ 23\%) nmol H$_2$ min$^{-1}$ mL$^{-1}$ was reached, after addition of 100 $\mu$g [2Fe]$^{\text{adt}}$ (80 $\mu$M final concentration) (blue bars). In combination, the enzymatic assays reveal a high degree of activation of the enzyme also under \textit{in vivo} conditions (approx. 80\% of total enzyme content) albeit with a requirement for larger excess of the synthetic cofactor as compared to the \textit{in vitro} protocol.

\textbf{Figure S2. Extent of activation determined through \textit{in vitro} enzymatic H$_2$ release assays.}
Figure S3. Cellular integrity at pH 7.5 and at pH 4.

[2Fe]\textsuperscript{adl}-HydA1 containing cells were incubated in TRIS-HCl buffer at pH 7.5 and at pH 4 respectively for 30 minutes. In the case of pH 4 medium, the TRIS-HCl buffer was acidified by HCl addition and the pH stability verified after the 1 h incubation. Then the cells were separated from the buffer with high-speed centrifugation. The whole-cell samples and the soluble fractions were analyzed in 1:1 ratio using 10% SDS-PAGE gel. (M): PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) as reference. No distinct difference in cell lysis was observed between acidic and weakly alkaline medium. Additionally, growing the cells on ampicillin containing LB-agar plates, the pH 4 incubated cells showed growth comparable to cells in pH 7.5 medium, in agreement with other reports on the acid stability of \textit{E. coli}. \textsuperscript{3}
Figure S4. Fitness of E. coli cell cultures.

To probe whether recombinant E. coli cell cultures suffer from the different modes of sample preparation, three experiments were performed. (I) AFM/ sSNOM conditions, left column. 1 μL standard cell culture was pipetted on the silicon crystal of the ATR cell and kept under dry N₂ for 1h. (II) ERP conditions. As a reference, 1 μL cell culture was kept at room temperature for 1 h (liquid sample, no film formation). (III) ATR FTIR conditions, right column. 1 μL cell culture was pipetted on the silicon crystal of the ATR cell, dried, and rehydrated for 1 h with an aerosol based on N₂ and 10 mM TRIS-HCl buffer (pH 8). After each experiment, cells were carefully re-suspended in 3 x 3 μL LB-amp medium and diluted to 200 μL.

(A) FTIR spectra of E. coli cell cultures (I) – (III). The inset shows the CO regime of the H-cluster. (B) LB-amp agar plates for 20 μL diluted cell cultures (I) – (III) after 48 h at 37°C. Plate (II) serves as reference (100% growth efficiency). Comparing plate (I) and (II) suggests slightly diminished growth of cell cultures that were kept under dry N₂ for 1 h (~90% fitness). Comparing plate (III) and (II) suggests significantly diminished growth of cell cultures that were kept under “wet” N₂ (~30 % fitness), presumably due to osmotic shock in the presence of low salt buffer. While AFM/ sSNOM experiments were performed over 2 – 3 hours, typical ATR FTIR experiments took 10 - 20 minutes. Therefore, we assume an overall comparable fitness of cells in all experiments.
Figure S5. AFM topography of *E. coli* cell suspension dried on template-stripped gold.

(A) AFM height profile of *E. coli* suspension diluted 1:20000 in deionized water and dried on template-stripped gold. Inset: 3x3 µm² region of a freshly cleaved, clean template-stripped gold surface. (B) Cross-section of a cell (along the red line in (A)). (C) Representative AFM topographies of damaged cells. Typically, two out of the seven cells (~30%) that were investigated in detail showed morphological alteration (e.g. protuberances, indicated by arrow). These cells might be considered as partially disintegrated with defects in the cell membrane.
Figure S6. Near-field imaging of five representative *E. coli* cells.

(A) AFM topographies as in Fig. S5. (B) ssNOM near-field amplitude (|σ₃|, n=3) maps recorded at 1660 cm⁻¹. (C) ssNOM near-field phase (Arg{σ₃}, n=3) maps recorded at 1660 cm⁻¹. The phase is sensitive to near-field absorption of the amide I mode of proteins but might be affected by the samples’ topography.⁴ ⁵ (D) ssNOM near-field phase (Arg{σ₃}, n=3) maps recorded at 1710 cm⁻¹. No major absorption band contributes to the phase at this frequency. Only the topography artifact is displayed. As ssNOM predominantly probes the surface of cells we speculate that this may be related to the C=O ester vibration of lipids in the outer *E. coli* membrane.⁶ (E) Subtraction of the phase maps shown in (C) and (D). No major protein content outside the cells is visible. All phase maps were aligned according to their topography prior to subtraction.
Figure S7. Analysis of further cells by AFM and nanoFTIR.

(A) 12 Topographies of *E. coli* containing overexpressed HydA1. The cells were diluted by 1:20000 in pure H₂O, spread on template stripped gold, and dried under ambient conditions. The field of view (2x2 µm²) and color scale is identical for all data set. (B) & (C) Unreferenced nanoFTIR phase Arg{σ₃} spectra (demodulated at the third harmonic of the tip frequency) recorded at the positions indicated by the colored circles (B, high features) or squares (C, lower features). The dashed lines represent reference spectra recorded next to each cell. All spectra are subject to a linear baseline and an offset. (D) Two selected topographies from panel A (lower right column, see asterisk) with a different height scale emphasizing subtle features next to the cell (green and red circle) and on the cell (blue circle). Colored dashed squares: background (template stripped gold). The right panel depicts unreferenced nanoFTIR phase Arg{σ₃} spectra at the positions indicated by the colored circles. The data show that these features may comprise of background artifacts (red trace) or protein (green trace, e.g. from disintegrated cells). The blue trace is for comparison with panels B and C. Dashed traces represent a “clean” background.
Figure S8. FTIR verification that the activated hydrogenase is located within the cells.

To probe whether recombinant *E. coli* cells eject the heterologously synthesized and activated HydA1 enzyme, cells were washed four times. For this, 1 mL standard cell culture was pelleted at 3000 rpm for 5 minutes. The supernatant was removed and the pellet was suspended in ~10 µL 10 mM TRIS-HCl buffer (pH 8). Then, 1 µL of this suspension was analyzed by ATR FTIR spectroscopy as described in the main text. The remaining suspension was diluted to 1 mL with fresh medium (factor 100) and pelleted again.

(A) FTIR spectra of rehydrated cell suspensions at wash steps 1 – 5, corresponding to relative volumes of fresh medium between $10^2$ and $10^8$. Comparing the amide II signal intensity at 1545 cm$^{-1}$ (inset) allows estimating the concentration of functional hydrogenase. (B) Baseline-corrected FTIR spectra in the CO regime of the H-cluster, weighted for the amide II signal intensity differences detected in panel (A). The inset depicted the H-cluster signal intensity ($\text{H}_{\text{ox}} + \text{H}_{\text{red}}$) relative to the undiluted reference sample (= 100%). We observed ~50% stability of hydrogenase in *E. coli* cells that were washed in up to $10^8$ times their volume, which suggest only insignificant secretion of hydrogenase enzyme into the medium. The H-cluster signals decrease may be related to osmotic shock and cell lysis (compare Fig. S4). Notably, there is no loss of H-cluster intensity in the first step of dilution.
Figure S9. Absolute FTIR spectra as a function of pH and H₂.

(A) Absorbance spectra of samples of *E. coli* cells containing [2Fe]\textsuperscript{adt}-HydA1 exposed to different gas atmospheres and pH. Hydration level and protein content are comparable for these conditions. (B) Zoom in the CO/CN ligand regime of HydA1. Ligand bands are well observable in absolute spectra.

Figure S10. Reactivity differences between purified and whole-cell samples of [2Fe]\textsuperscript{adt}-HydA1 monitored by FTIR spectroscopy.

Purified [2Fe]\textsuperscript{adt}-HydA1 enzyme was diluted with BSA (bovine serum albumin) as an unreactive protein standard (red spectrum) to obtain a protein to hydrogenase ratio comparable to *E. coli* cells containing [2Fe]\textsuperscript{adt}-HydA1. Exposed to 100% H₂, H\textsubscript{red} (CO marker bands at 1915 and 1891 cm\textsuperscript{-1}) and H\textsubscript{red} (1882 cm\textsuperscript{-1}) are co-populated in the diluted, purified enzyme sample (red spectrum) whereas only H\textsubscript{red} is visible in *E. coli* cells (black spectrum). H\textsubscript{red} (1933 cm\textsuperscript{-1}) is not expected to be enriched under these conditions.\textsuperscript{7}
Figure S11. Influence of pH and reductant on the formation of H\textsubscript{hyd} and H\textsubscript{hydH+} monitored by FTIR spectroscopy.

(A) In vivo H\textsubscript{2}-N\textsubscript{2} difference spectra for various pH values and dithionite (NaDT) concentrations. Formation of H\textsubscript{red} (1915 and 1891 cm\textsuperscript{-1}) and depopulation of H\textsubscript{ox} (1940 cm\textsuperscript{-1}) is observed at pH 8 and 2 mM NaDT (black difference spectrum). Formation of H\textsubscript{red} and H\textsubscript{hyd} (1978, 1960 and 1860 cm\textsuperscript{-1}) with depopulation of H\textsubscript{ox} and H\textsubscript{ox}H (1946 cm\textsuperscript{-1}) is observed at pH 8 and 100 mM NaDT (dark yellow difference spectrum). Formation of H\textsubscript{hyd} and depopulation of H\textsubscript{ox}H is observed at pH 4 and 2 mM NaDT (light blue difference spectrum). This data show how high NaDT concentrations can compensate for high pH values in the enrichment of H\textsubscript{hyd}, at least partly.

(B) At pH 4 and in the strict absence of NaDT, purified [2Fe]\textsuperscript{adt}-HydA1 adopts a mixture of reduced states under 1% H\textsubscript{2}. The black trace shows the corresponding H\textsubscript{2} – N\textsubscript{2} difference spectrum. Negative bands are assigned to H\textsubscript{ox}, positive bands include H\textsubscript{red} (black labels), H\textsubscript{hyd} (dark yellow labels), and traces of H\textsubscript{red}\textsuperscript{'} and H\textsubscript{hyd} (not annotated). At pH 4 and in the presence of ~2 mM NaDT, purified [2Fe]\textsuperscript{adt}-HydA1 converts from H\textsubscript{ox}H into H\textsubscript{hyd} with a small percentage of H\textsubscript{red} (dark yellow trace), as previously reported.\textsuperscript{8} At pH 4 and in the presence of ~100 mM NaDT, purified [2Fe]\textsuperscript{adt}-HydA1 converts from H\textsubscript{ox}H into H\textsubscript{hydH+} (pink trace). This species was observed in [2Fe]\textsuperscript{adt}-HydA1 C169S previously.\textsuperscript{9}

(C) Careful subtraction of H\textsubscript{ox}H from the dark yellow and pink traces in (B) allows the generation of relatively pure spectra of H\textsubscript{hyd} and H\textsubscript{hydH+}, respectively (contributions by H\textsubscript{red}}
and $H_{\text{red}}H$ are indicated). While most bands are strongly shifted towards higher wavenumbers in $H_{\text{hyd}}H^+$ relative to $H_{\text{hyd}}$, we found no significant difference between the (putative) dCO band at 1960 cm$^{-1}$ ($H_{\text{hyd}}$) and 1959 cm$^{-1}$ ($H_{\text{hyd}}H^+$), this specific peak assignment is in contrast to an earlier assignment of the proposed $H_{\text{hyd}}H^+$ state.$^9$

(D) Subtraction of vibrational frequencies of CO/CN$^-$ normal modes for $H_{\text{ox}}$, $H_{\text{ox}}$-CO, $H_{\text{red}}$', and their protonated counterparts $H_{\text{ox}}H$, $H_{\text{ox}}$-H-CO, and $H_{\text{red}}H$ (compare Table S2). The coupled $\delta$-mode of $H_{\text{ox}}$-CO is excluded. Index p (proximal) and d (distal) refers to the relative position of the Fe ion to the [4Fe-4S]$_{\text{H}}$ cluster. Index $\mu$ marks the Fe-Fe bridging CO ligand. The mean differences are $\sim$3 cm$^{-1}$ for p/dCN, $\sim$5 cm$^{-1}$ for p/dCO, and $\sim$9 cm$^{-1}$ for $\mu$CO. These shifts have been attributed to a protonation of the [4Fe-4S] cluster.$^7$ In comparison, the differences between the hydride states (blue bars) are significantly larger (or smaller, i.e. for dCO). This observation inspired our decision to assign the blue-shifted IR signature of $H_{\text{hyd}}$ to a hydride-binding H-cluster geometry with a protonated adt ligand, $H_{\text{hyd}}H^+$. A similar assignment was suggest by Mulder et al. previously.$^9$
Figure S12. Simulation details of EPR spectra recorded at 20 K showing two hydride-like states detected at high and low pH.

(Top panel): High pH EPR spectrum (dark yellow solid line) simulated as a combination of $\text{H}_{\text{ox}}$ (cyan dash-dotted line) and $\text{H}_{\text{hyd}}$ (dash-dotted dark yellow line). Potential contributions from the previously reported “rhombic 2.06 signal” indicated with asterisks (reported g = 2.061, 1.968, 1.900).$^9,10$ Previously reported g-values for the “rhombic 2.08 signal” assigned to the $\text{H}_{\text{hyd}}$ state: 2.078; 1.935; 1.880.$^9,11$ (Bottom panel): Low pH EPR spectrum (purple solid line) simulated as a combination of $\text{H}_{\text{ox}H}$ (cyan dash-dotted line) and $\text{H}_{\text{hyd}}H^+$ (purple dash-dotted line), also shown is a simulation highlighting an additional feature at g = 2.066 visible at low microwave power.

In both spectra the low field region is complex suggesting the presence of additional minor species, however, at high microwave power these contributions become negligible (Fig. S14). Contributions from $\text{H}_{\text{sred}}$ are unlikely due to the absence of the expected high field feature at g = 1.868.$^{12}$ see also Fig. S10. The final simulations are shown as black dash-dotted lines. The hydride species are generated under H$_2$ atmosphere in the presence of 10 mM NaDT, experimental parameters as in main text Fig. 5.
Figure S13. Temperature effects on the EPR signals attributed to hydride states detected at low and high pH.

**Upper panel** (pH 4): EPR spectra of apo-HydA1 (lane a) and [2Fe]^{adt}-HydA1 (lane b) containing cells buffered at pH 4, recorded at 10 K (blue spectra) and 20 K (orange spectra); also shown are the background subtracted spectra of [2Fe]^{adt}-HydA1 (lane c, denoted b-a).

**Lower panel** (pH 7.5): EPR spectra of apo-HydA1 (lane a) and [2Fe]^{adt}-HydA1 (lane b) containing cells buffered at pH 7.5, recorded at 10 K (blue spectra) and 20 K (orange spectra); also shown are the background subtracted spectra of [2Fe]^{adt}-HydA1 (lane c, denoted b-a).

The background corrected spectra are normalized at the g_z through to facilitate comparison. The g-values for H_{hyd}^+ (2.073, 1.935 and 1.881, purple vertical lines) and H_{hyd} (2.079, 1.935 and 1.878, yellow vertical lines) are indicated for clarity. Spectra recorded at 10 and 20 K reveal only negligible temperature induced differences in the signals attributed to H_{hyd} and H_{hyd}^+. Spectra recorded at 10 μW, other experimental parameters as in Figure 5.
Figure S14. Power dependence of the hydride states detected at high and low pH monitored at 20 K.

Both the H$_{\text{hyd}}$ (top) and H$_{\text{hyd}}$H$^+$ (bottom) signal increase as a function of microwave power and neither signal saturates rapidly at 20 K, in good agreement with the behavior reported at 20 K for the “rhombic g = 2.08” signal previously assigned to the H$_{\text{hyd}}$ state.$^{9-11}$ The g-values are obtained from simulations (see Fig. S12) and the respective simulated spectra are redrawn from Figure 5 for clarity (black dashed lines). It is noteworthy that in particular the low field tensor becomes more distinct at higher power, albeit the exact g-values are potentially slightly shifted due to power saturation. The hydride species are generated under H$_2$ atmosphere in the presence of 10 mM NaDT, microwave power indicated in figure, and spectra shown without background subtraction, other experimental parameters as in main text Figure 5.
The light of either a quantum-cascade laser (QCL, attenuated by a pair of polarizers, POL) or fiber-based fs laser system (selectable by a flip mirror, FM) is focused by an off-axis parabolic mirror (OAP) on the tip of an atomic force microscope (AFM). The scattered light interferes with phase-modulated light from a reference arm (attenuated by a gold grid attenuator, ATT) at a ZnSe beam-splitter (BS) and is detected by a mercury-cadmium-telluride detector (MCT) and demodulated by a lock-in amplifier (LIA). For nanoFTIR the light in the reference arm travels through a retroreflector (RR) and is back-reflected by a folding mirror.

AFM topographies were recorded on a commercial AFM (NanoWizard II, JPK Instruments AG, Germany) with a Pt coated cantilever (Arrow NCPt, NanoWorld GmbH, Germany) in intermittent contact mode under ambient conditions.

The scattering-type scanning near-field optical microscope (sSNOM) is conceptually setup as described above. Either a quantum-cascade laser (Daylight Solutions, USA) or fiber-based fs-laser system (NeaSpec GmbH, Germany) is focused by an off-axis parabolic mirror onto a metalized AFM tip (Arrow NCPt, NanoWorld GmbH, Germany) oscillating close to its first mechanical resonance Ω. The scattered light is collected by the same mirror and is directed by a beam-splitter (ZnSe plate, Ø25.4, thickness 3 mm, Eksma Optics, Lithuania) towards a mercury-cadmium-telluride detector (KLD-0.1-J1/11/DC, Kolmar Technologies, Inc., USA). There the scattered light interferes with light from the reference arm whose optical path difference is modulated by the mirror mounted on a piezo actuator (P-843.10, Physik
Instrumente (PI) GmbH & Co. KG, Germany) sinusoidally displacing it at a frequency of $M \sim 300$ Hz. The doubly modulated signal is then demodulated by a lock-in amplifier (HF2LI, Zurich Instruments, Switzerland) at the frequencies $n\Omega \pm mM$ similar as described in ref. 13 and registered while the sample is being moved in x-y plane underneath the AFM tip. The demodulated maps are then phase corrected as described in ref. 14. Additionally, a constant phase is subtracted line-by-line such that the phase at the maximal amplitude in each line equals to zero. Two maps were recorded per cell: one at 1660 cm$^{-1}$ (Fig. S6) displaying the amide I contribution and potential topography artifacts and one at 1710 cm$^{-1}$ (Fig. S6) which is only affected by the topography artifact. Those artifacts may be related to differences in illumination or loading of the antenna while scanning across the sample.\textsuperscript{4,5} The map recorded at 1710 cm$^{-1}$ was then subtracted from the map at 1660 cm$^{-1}$.

For broadband nano-Fourier transform infrared (nanoFTIR) spectroscopy\textsuperscript{15} the broadband fs-laser source is selected via the flip mirror. The optical path in the reference arm is changed such that the light is reflected off a retroreflector mounted on a voice-coil stage (V-524, Physik Instrumente (PI) GmbH & Co. KG, Germany) and being folded back by a folding mirror. While the tip sits on a specific location on the sample the reference mirror is periodically driven 0.4 mm with a velocity 0.05 mm/s. The relative position of the reference mirror is determined by an auxiliary HeNe-laser Michelson interferometer. Both, IR and HeNe interferograms are simultaneously recorded by the LIA and stored (for the IR interferogram only the demodulated signal at $n=1, 2$ and 3 is recorded). The IR interferogram is sampled at the zero-crossings of the HeNe interferogram and saved. Such treated interferograms are first aligned relative to their white light position by cross-correlation during post processing. All spectra are apodized by a 4-term Blackman-Harris apodization function resulting in spectra with a spectral resolution of $\Delta\nu \sim 16$ cm$^{-1}$. All spectra were phase corrected by a Mertz-type phase correction to compensate for slowly varying phase changes. During the phase correction a spectrally low-resolution ($\Delta\nu \sim 658$ cm$^{-1}$) phase spectrum was generated by applying a 12 point wide 4-term Blackman-Harris apodization before Fourier-transform. This low-resolution phase spectrum was then subtracted from the phase of the high-resolution spectrum. This allowed us to display the near-field phase spectra of \textit{E. coli} cells and the template-stripped gold substrate separately (Figure 3 in main text) without referencing it to a reference spectrum as typically done in near-field spectroscopy.\textsuperscript{13-15}
Supporting Tables

Table S1: CO band frequencies of the H-cluster in HydA1 as observed *in vivo*. The CN⁻ frequencies were not resolved at sufficient signal-to-noise.

| State     | νCO / cm⁻¹ |
|-----------|------------|
| H_ox      | 1964 1940 1802 |
| H_oxH     | 1970 1946 1812 |
| H_red⁻H   | n.d. n.d. n.d. |
| H_red⁺H   | n.d. n.d. n.d. |
| H_hyd     | 1978 1960 1860 |
| H_hydH⁺   | 1988 1959 1875 |
| H_red     | 1961 1915 1891 |
| H_sred    | n.d. n.d. n.d. |
| H_ox-CO   | 1968 1962* 1808 |
| H_oxH-CO  | n.d. n.d. n.d. |

* In the CO-inhibited state, the coupled pCO/ dCO stretching vibration gives rise to an additional IR band at 2012 cm⁻¹ (H_ox-CO); n.d. = not detected.
Table S2: CO/CN\(^{-}\) band frequencies of the H-cluster in HydA1 as observed \textit{in vitro}.
Data taken from reference \textsuperscript{7}.

| Species          | \(\nu\text{CN}^- / \text{cm}^{-1}\) | \(\nu\text{CO} / \text{cm}^{-1}\) |
|------------------|---------------------------------|--------------------------------|
| \(\text{H}^{\text{ox}}\) | 2088 2070                      | 1964 1940 1802                  |
| \(\text{H}^{\text{ox}}\text{H}^{\text{red}}\) | 2092 2074                      | 1970 1946 1812                  |
| \(\text{H}^{\text{red}}\text{H}\) | 2084 2066                      | 1962 1933 1792                  |
| \(\text{H}^{\text{hyd}}\) | 2088 2074                      | 1978 1960 1860                  |
| \(\text{H}^{\text{hyd}}\text{H}^{\text{red}}\) | 2092 2082                      | 1988 1959 1875                  |
| \(\text{H}^{\text{red}}\) | 2070 2033                      | 1961 1915 1891                  |
| \(\text{H}^{\text{sred}}\) | 2068 2026                      | 1953 1918 1882                  |
| \(\text{H}^{\text{ox}}\text{CO}\) | 2092 2082                      | 1968 1962\* 1808               |
| \(\text{H}^{\text{ox}}\text{H}-\text{CO}\) | 2094 2086                      | 1972 1966\* 1816               |

* In the CO-inhibited state, the coupled pCO/ dCO stretching vibration gives rise to an additional IR bands at 2012 cm\(^{-1}\) (\(\text{H}^{\text{ox}}\text{CO}\)) and 2006 cm\(^{-1}\) (\(\text{H}^{\text{ox}}\text{H}-\text{CO}\)).

Table S3: g-values of selected states reported for the H-cluster in HydA1

| Species          | \(g_z\)  | \(g_y\)  | \(g_x\)  | reference          |
|------------------|----------|----------|----------|--------------------|
| \(\text{H}^{\text{ox}}\) | 2.100-2.101 | 2.04     | 1.998    | 16 and this work   |
| \(\text{H}^{\text{ox}}\text{CO}\) | 2.054 | 2.007 | 2.007 | 15                  |
| \(\text{H}^{\text{sred}}\) | 2.076 | 1.943 | 1.868 | 12                  |
| \(\text{H}^{\text{hyd}}\) | 2.077-8 | 1.935 | 1.880 | 9-11                |
| \(\text{H}^{\text{hyd}}\) | 2.078 | 1.935 | 1.878 | this work           |
| \(\text{H}^{\text{hyd}}\text{H}^{+}\) | 2.073 | 1.935 | 1.881 | this work           |
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