Supporting Information

Protonation/Reduction Dynamics at the [4Fe-4S] Subsite of the Hydrogen-forming Cofactor of [FeFe]-Hydrogenases

Moritz Senger†, Stefan Mebs‡, Jifu Duan§, Olga Shulenina⊥, Konstantin Laun†, Leonie Kertess§, Florian Wittkamp§, Ulf-Peter Apfel§, Thomas Happe#, Martin Winkler#, Michael Haumann†, Sven T. Stripp†

† Department of Physics, Experimental Molecular Biophysics, Freie Universität Berlin, 14195 Berlin, Germany
‡ Department of Physics, Biophysics of Metalloenzymes, Freie Universität Berlin, 14195 Berlin, Germany
# Faculty of Biology and Biotechnology, Photobiotechnology, Ruhr-Universität Bochum, 44801 Bochum, Germany
⊥ Faculty of Physics, St. Petersburg State University, 198504 St. Petersburg, Russian Federation
§ Faculty of Chemistry and Biochemistry, Inorganic Chemistry I, Ruhr-Universität Bochum, 44801 Bochum, Germany
Contents

Table S1  Vibrational frequencies for Hox and HoxH

Figure S1  Reduction of methyl viologen via dithionite in the aerosol
Figure S2  Global fit analysis and experimental uncertainty
Figure S3  Verification of the aerosol-mediated pH titration
Figure S4  Influence of DT on Hox → HoxH conversion
Figure S5  Further kinetic studies
Figure S6  Influence of protein concentration, hydration level, and temperature
Figure S7  Formation of HoxH-CO and HoxH
Figure S8  Sulfur/ Selenium exchange
Figure S9  H/D exchange, KIE, and cofactor integrity
Figure S10  Population of reduced states in HYDA1adt in presence of H₂
Figure S11  Inter-molecular electron transfer in hydrogenase films
Figure S12  Model structures for DFT
Figure S13  Calculation results for model and theory level variation
Figure S14  DFT on Hox and HoxH
Figure S15  DFT on Hox-CO and HoxH-CO
Figure S16  DFT on Hred´ and Hred´H
Figure S17  Vibrational modes, spin densities, and charges from DFT
Figure S18  Putative proton path to the [4Fe-4S] cluster in CPI
Figure S19  Accumulation of HoxH and basic reaction cycle
Figure S20  Numerical simulations of HoxH formation
**Table S1A – Vibrational Frequencies of Hox and HoxH.** Native CPI, DDH, and HYDA1 as well as amino acid variant C169D and C169A of HYDA1.

<table>
<thead>
<tr>
<th>(A) enzyme</th>
<th>variation</th>
<th>vCN</th>
<th>vCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hox CPI</td>
<td>native</td>
<td>2081</td>
<td>2069</td>
</tr>
<tr>
<td></td>
<td>DDH native</td>
<td>2090</td>
<td>2078</td>
</tr>
<tr>
<td>HYDA1</td>
<td>native</td>
<td>2088</td>
<td>2017</td>
</tr>
<tr>
<td></td>
<td>C169D</td>
<td>2087</td>
<td>2071</td>
</tr>
<tr>
<td></td>
<td>C169A</td>
<td>2087</td>
<td>2068</td>
</tr>
<tr>
<td>Hox DDH</td>
<td>native</td>
<td>2090</td>
<td>2078</td>
</tr>
<tr>
<td>HYDA1</td>
<td>native</td>
<td>2088</td>
<td>2017</td>
</tr>
<tr>
<td></td>
<td>C169D</td>
<td>2087</td>
<td>2071</td>
</tr>
<tr>
<td></td>
<td>C169A</td>
<td>2087</td>
<td>2068</td>
</tr>
</tbody>
</table>

**Table S1B – Vibrational Frequencies of Hox and HoxH.** HYDA1 cofactor variants odt, sdt, pdt, and edt. Legend: p - proximal, d - distal, µ - bridging. See Fig. 3 for spectra.

<table>
<thead>
<tr>
<th>(B) enzyme</th>
<th>variation</th>
<th>vCN</th>
<th>vCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hox HYDA1</td>
<td>odt</td>
<td>2088</td>
<td>2070</td>
</tr>
<tr>
<td></td>
<td>sdt</td>
<td>2087</td>
<td>2069</td>
</tr>
<tr>
<td></td>
<td>pdt</td>
<td>2090</td>
<td>2073</td>
</tr>
<tr>
<td></td>
<td>edt</td>
<td>2088</td>
<td>2073</td>
</tr>
<tr>
<td>HoxH HYDA1</td>
<td>odt</td>
<td>2094</td>
<td>2078</td>
</tr>
<tr>
<td></td>
<td>sdt</td>
<td>2093</td>
<td>2076</td>
</tr>
<tr>
<td></td>
<td>pdt</td>
<td>2092</td>
<td>2075</td>
</tr>
<tr>
<td></td>
<td>edt</td>
<td>2094</td>
<td>2076</td>
</tr>
</tbody>
</table>
Fig. S1 – Reduction of methyl viologen via dithionite in the aerosol. 2 µL oxidized methyl viologen (MV, 500 mM in oxygen-free water (Millipore), see photo with the red frame) was used to form a film on the ATR crystal. The red trace shows the UV/vis spectrum recorded prior to film formation (Ocean Optics mini-spectrometer USB2000 equipped with an IMPLEN Sub-Micro Liter Cell). Methyl viologen was dried and rehydrated under N₂ aerosol enriched with 200 mM sodium dithionite (DT) solution. The aerosol treatment was continued with 1.5 L min⁻¹ for 30 minutes. Afterwards, the film was removed from the ATR crystal with 2 µL oxygen-free water. Visual inspection suggested reduction of MV (photo with the blue frame) and the spectrum identified the absorption bands of MV (most prominent at 395 nm and 605 nm) and DT (310 nm). This proof-of-concept experiment shows how soluble components like DT “fly” with the aerosol and can be deposited in the film on the ATR crystal, e.g. MV or protein.
Fig. S2 – Time resolution, global fit analysis, and experimental uncertainty. (A) CO binding to a film of oxidized HYDA1 followed by ATR FTIR. The increase of the marker band at 2012 cm\(^{-1}\) is followed over time (80 kHz, single scan forward/ backward, spectral resolution 8 cm\(^{-1}\)). Accumulation of Hox-CO shows a biphasic behavior with \(t_{1/2} \approx 3\) s (yellow area) and \(\sim 35\) s. Making use of flash-photolysis QCL IR spectroscopy, Mirmohades et al. measured \(t_{1/2}\)\(\approx 3\) s. (B) data treatment

\[
\text{redox state population in \% by area of fitted data}
\]

\[
\text{frequency / cm}^{-1}
\]

\[2100\quad 2050\quad 2000\quad 1950\quad 1900\quad 1850\quad 1800\]

\[
\text{population HoxH / \%}
\]

\[
\text{time / min}
\]

\[0\quad 0.5\quad 1.0\quad 1.5\quad 2.0\quad 2.5\quad 3.0\]

\[
\text{population HoxH / \%}
\]

\[
\text{time / min}
\]

\[0\quad 1\quad 2\quad 3\quad 4\]

\[
\text{Hox HoxH}
\]
~15 ms for CO binding to Hox. Clearly, CO inhibition cannot be followed by conventional FTIR spectroscopy. The value of ~3 s thus represents the timescale of equilibration in the aerosol set-up (the latter process might be associated with diffusion of gas through the thick protein film and similar “bulk” phenomena). Data acquisition (~200 ms for 80 kHz scanning velocity, 8 cm⁻¹ spectral resolution, and individual analysis of spectra) is not limiting to this process. (B) Second derivative spectra (magenta) were used to identify band positions. Fitting of raw spectra (black) using a polynomial defined the baseline (red). Baseline-corrected spectra (blue) were fitted with sets of Voigt functions (50% Gaussian/ 50% Lorentzian character, dark blue) to obtain band amplitudes, widths, and relative intensities of individual H-cluster species as input parameters for the global fit analysis of spectra from H-cluster state mixtures. The summed area of observed bands (2 CN, 3 CO) is referred to as “population” and given in % throughout the text (the oscillator strengths are approximately the same in all analyzed redox states). Data processing and curve fitting was performed with inhouse software. (C) Baseline-corrected spectra for a typical Hox to HoxH conversion (from top to bottom and red to blue colours). Band widths and relative intensities for each H-cluster species were kept constant in the fit. Lower panel: H-cluster state populations plotted over reaction time. (D) Estimation of experimental uncertainty from repeated population of HoxH from Hox for five individual protein films. The relative error in the determination of the halftime (or rate) of HoxH formation was estimated as <25 %.

Reference:
Fig. S3 – Verification of the aerosol-mediated pH titration. (A) Population yield of HoxH as a function of pH in solution (black) and via aerosol (red). The pH difference was estimated as ~0.25 units in the aerosol vs. solution experiments. (B) For the solution-based titration, a pH electrode was used to directly measure the pH in the medium covering the enzyme (the buffer penetrated a semi-permeable 8 kDa dialysis membrane to protect the protein film from dilution). (C) Standard HYDA1 preparations as titrated to pH 4 (black) or 1 M DT (blue) via the aerosol (aero) are compared to protein as isolated at pH 4 (red) or pre-adjusted to 0.5 M DT (magenta). The respective spectra predominantly show HoxH in all cases. (D) Transmission spectra of 200 µM HYDA1 in solution (pH 8/ pH 4). Right panel: pCO/ dCO spectrum. At pH 4, a mixture of Hox and HoxH is observed (blue trace) whereas at pH 8 (black trace) Hox dominates.
Fig. S4 - Influence of DT on Hox → HoxH conversion. (A) In presence of 2 mM DT (black trace), a change from pH 8 to pH 4 induced a steep increase of HoxH in HYDA1\textsuperscript{adt}. The process is reversible; conversion back to Hox was achieved when the buffer pH was changed back to pH 8. HYDA1 as isolated in the absence of DT (red trace) did not show significant HoxH population in response to sample acidification (the small IR change around t = 0 is due to a minor transient change in sample hydration due to the aerosol change). (B) Time course of HYDA1\textsuperscript{adt} HoxH formation at 500 mM DT for changing the pH from 8 to the indicated values (pH 3 – pH 7). Note the decrease of the IR signal at pH 3 that follows the population of HoxH (crosses, black trace), reflecting cofactor disintegration, which was absent during the experimental time period for pH values of 4 or larger. (C) Corresponding HoxH formation rates plotted vs. pH. Note the linear relation of the rate constant on a logarithmic scale to the pH (logarithmic proton concentration) over a range of four pH units.
Fig. S5 - Further kinetic studies. Time course of back-convertion of HoxH → Hox for (A) changing the DT concentration from 100 mM to the indicated values at pH 6 or for (B) changing the pH from 4 to the indicated values at 2 mM DT. (C) Time course of HoxH formation at pH 4 and 2 mM DT in HYDA1
\textsuperscript{adt} wild-type (black) and C169D (red) as well as cofactor variant HYDA1
\textsuperscript{pdt} (magenta). About 20-fold slower HoxH formation was observed in the pdt variant (see blue lines for estimation of $t_{1/2}$). (D) Accelerated HoxH formation at 5 mM DT and pH 6 for increasing the concentration of the redox mediator methyl viologen (MV) from 0 to 5 mM (data correspond to relative intensities of the dCO band of HoxH).
Fig. S6 – Influence of protein concentration, hydration level, and temperature. (A) HYDA1 was diluted with buffer from 2 – 0.125 mM protein prior to formation of protein films. For decreasing protein concentrations (P), normalized spectra (amide II, 1540 cm⁻¹) show an increase in hydration level (W, e.g. 3390 cm⁻¹). Protein signals are overlaid by buffer absorption but the decrease of cofactor bands (inset) immediately hints at decreasing protein levels.
(B) The HoxH formation rate was found to be affected by the P/W ratio. The influence is rather small. (C) Variation of crystal plate temperature (T_{cp}) via a custom-made thermostat from 32 – 20°C strongly affected the P/W ratio. For T_{cp} > RT dehydration of sample was observed while T_{cp} < RT promoted an increase of water in the film. (D) With all parameters under control (esp. temperature and mass flow), titration could be performed without significant variation in P/W. A standard titration with 1.5 L min\(^{-1}\) at 24°C from pH 8 to pH 4 (2 mM DT) is shown (same dataset as in Fig. 3AB). The HoxH-minus-Hox difference spectrum comprises a minor decrease in amide I (~1640 cm\(^{-1}\)) and amide II (~1550 cm\(^{-1}\)) band intensities due to film swelling. Accordingly, small difference bands in the asymmetric/ symmetric OH stretching frequencies of liquid water were detected (inset, asymmetric: 3390 cm\(^{-1}\), symmetric.: 3280 cm\(^{-1}\)). The stable P/W ratio facilitated identification of negative bands at 1400 cm\(^{-1}\) and ~1590 cm\(^{-1}\) (asterisks) both which can be assigned to carboxylate vibrations. Around 1730 cm\(^{-1}\), a positive feature indicates an increase of carboxylic acid groups. Together, the IR difference spectrum clearly suggests an acidification of the film. No indications for structural changes (i.e. distinct amide I difference bands) of the hydrogenase protein within a pH range of 4 - 8 were observed. (E) In vitro H\(_2\) release activity as a function of pH. 100 ng native HYDA1 was diluted in 2 mL mixed buffer (50 mM) including 200 mM DT and no MV. The test tube was purged with argon and incubated at 37°C for 30 minutes. Afterwards, 1 mL of the gas phase was analyzed for H\(_2\) by gas chromatography. The H\(_2\) release activity is given relative to the maximal value at pH 7 (100%). For both pH > 7 and pH < 7 a steep decrease of activity was observed.
Fig. S7 – Formation of HoxH-CO. (A) The IR spectrum of HYDA1 at pH 8 (dark-yellow) is compared to the IR spectrum that was obtained at ~60 min after changing the pH to 4 in the presence of 1% CO and 2 mM DT in the aerosol (dark-cyan). The latter state is denoted HoxH-CO. The black line shows the pH 4 – 8 difference spectrum. (B) Modified reaction cycle that explains the formation of HoxH-CO as a function of pH and DT. Reduction of Hox-CO weakens the iron-carbonyl bond of the externally added CO ligand (so that the CO is transiently detached). Oxidation of Hred′ via H2 release restores an oxidized species (i.e., HoxH), which is re-converted into a CO-inhibited species (HoxH-CO) in the presence of CO gas.
**Fig. S8 – Sulfur/ Selenium exchange.** (A) $^{13}$CO isotope editing of Hox-CO in wild-type HYDA1*adt (dCO and μCO) due to exposure to $^{13}$CO gas (red), blue light irradiation (blue), and recovery of Hox under N$_2$ (magenta). The CO region is shown. Protocol after Senger et al., PNAS 2016. (B) Co-deposition of $^{13}$CO-edited HYDA1*adt (magenta) and a HYDA1*adt with a [4Fe-4Se] cluster (green) and no $^{13}$CO ligands. The film shows a 50:50 mixture of Hox bands from both samples (black). Dashed lines mark respective Hox band positions. Co-deposition was chosen to facilitate pH titration under identical conditions for unambiguous band assignment and kinetics determination. (C) IR difference spectra collected on the mixed film during a change from pH 8 to 4 in the presence of 2 mM DT. Indicated frequencies are assigned to the respective Hox (negative bands) or HoxH species (positive bands) in the two variants (magenta/ green as above). (D) Time course of the HoxH yield for [4Fe-4S] and [4Fe-4Se] variants as determined from acidification of the mixed protein film. Both variants show a similar halftime of HoxH formation of ~2.7 min, meaning that an effect of Se substitution on HoxH yield or formation rate was not discernible.
Fig. S9 - H/D exchange, KIE, and cofactor integrity. (A) Quantitative H$_2$O $\rightarrow$ D$_2$O exchange in the protein film on the ATR cell occurred when the aerosol was enriched with D$_2$O by passing the gas stream through a respective buffer as monitored by the decrease of the H$_2$O band around $\sim$3400 cm$^{-1}$ and concomitant increase of the D$_2$O band at $\sim$2500 cm$^{-1}$. AII – III: amide I – III vibrations of the protein backbone (All’ and AllI’ denote the respective vibrations in D$_2$O buffer). (B) HYDA1$^{ad}$ was maturated and isolated in D$_2$O buffer. Infrared spectra were collected with D$_2$O in the aerosol. Upon acidification with 0.5% D$_2$SO$_4$ in presence of 2 mM DT, Hox converts into HoxH (“HoxD”) and respective band frequencies are practically identical to Hox in H$_2$O buffer. The negative band at 1891 cm$^{-1}$ is attributed to residual Hred. (C) Formation rates of HoxH (black) and “HoxD” (red) do not differ significantly. This tentatively disfavors protonation as rate-limiting step in the formation of HoxH. A reliable resolution of kinetic isotope effects (KIE) is difficult in our setup due to the uncertainty in the determined rate constants (see Fig. S2) and because diffusion steps of reactants into the film may contribute to the observed kinetics. (D) Cofactor integrity in presence of 0.5% H$_2$SO$_4$ (pH $\sim$1.5) and 2 mM DT. Within ten hours, the decrease of respective IR band intensity indicates approximately 50% cofactor degradation (red) while the protein concentration according to the amide band intensities was stable (green).
Fig. S10 - Accumulation of reduced states in HYDA1<sup>adt</sup> in presence of H<sub>2</sub>. (A) Contour plots depict the yield of reduced states (z, population in %, see color code on the right) as a function of time (y) and pH (x). At t<sub>0</sub>, N<sub>2</sub> was exchanged with H<sub>2</sub> to start the reaction. Left panel,
**Hred**; right panel, **Hred**’. From pH 5 – 8, predominant formation of **Hred** was observed ($t > 5$ s). **Hred**’ shows a bias towards more alkaline pH values (predominant formation between pH 7 - 10 for $t > 5$ s). Notably, pH values < 7 completely suppressed population of **Hred**’. **(B)** Comparison of the **Hred**/ **Hred**’ yield at $t = 10$ s (cyan line in (A)). The increase from pH 10 – 7 suggests pH dependent formation rates for both **Hred** and **Hred**’. The dashed area in the background represents the H$_2$ release activity as a function of pH (same data as in Fig. S6E). Note the decrease of **Hred**’ for pH < 7. **(C)** Component spectra for Fig. 6B in the main manuscript (black: pH 8, blue: pH 6). **(D)** Spectral deconvolution of IR spectra of HYDA1$_{adt}$ in presence of H$_2$ for different concentrations of DT. Respective species as indicated (only the dCO frequencies are given). Not the presence of Hhyd (grey). Black line: exp. data, red line: fit envelope. The right panel shows the relative populations of **Hox**/ **HoxH** and **Hred**’/ **Hred**’H plotted vs. DT concentration. For increasing amounts of DT, the protonated species **HoxH** and **Hred**’H accumulate in increasingly higher amounts.
Fig. S11 – Inter-molecular electron transfer in hydrogenase films. (A) Time course of Hred' formation under H2 for decreasing percentages of HYDA1pdt protein in the film due to “dilution” with bovine serum albumin protein (BSA). (B) Time course of HoxH formation under N2 and 2 mM DT after acidification of the aerosol from pH 8 to pH 4 for decreasing percentages of HYDA1pdt. (C) Relative rates of Hred' and HoxH formation as a function of the relative amount of HYDA1pdt in the film. The decrease of the Hred' formation rate for decreasing enzyme amounts suggests that population of Hred' under H2 involves inter-molecular electron transfer between hydrogenase proteins. No clear systematic effect of enzyme dilution is observed for the rate of HoxH formation, suggesting that this process does not require inter-molecular electron transfer. (D) Changes in the relative population of H-cluster species in HYDA1pdt after changing the aerosol from H2 to N2 at pH 8. Note that the two-electron reduced Hsred state is converted first to the one-electron reduced Hred' and Hred states (yellow area) which are converted to the oxidized Hox state afterwards. This behavior suggests inter-molecular electron transfer between “super-reduced” and oxidized hydrogenases to form one-electron reduced species like Hred and Hred'.
Fig. S12 - Model structures for DFT. The smallest structures (based on the CPI crystal structure of Hox, PDB entry 4XDC) used for DFT comprised the complete H-cluster (cysteine ligands were truncated to methyl groups). In some cases, amino acids (Ala, Lys, Cys) and water molecules (W1-3) as visible in CPI crystal structures were included in the calculations (backbone atoms and O-atoms of water molecules were fixed to their crystallographic coordinates). For all structures, the Hox state was calculated as a reference for comparison with reduced and/ or protonated structures (HoxH, Hred’, and Hred’H). Protons were added to the structures at the sulfur atoms of the H-cluster (same numbering as in Fig. 1) or at water molecules. For DFT results on larger model structures and for release of restraints see Fig. S13.
Fig. S13A – Calculation results for model and theory level variation. Left panels: IR frequencies as calculated for Hox, HoxH, and Hred⁻ structures plotted vs. the experimental frequencies (top to bottom). Right panels: IR frequencies as calculated after correction for systematic frequency offset and correlation line slope deviations from unity shown in Fig. S13B using Eqs. 1-3 in Materials and Methods (frequencies for Hox were corrected with the individual model data and frequencies for HoxH and Hred⁻ were corrected using the offset and slope values for the corresponding Hox model) plotted vs. the experimental frequencies. Lines represent linear fits to the data. Note that similar relative IR frequencies and qualities of correlation with the experimental data were derived for all approaches for the three H-cluster species after correction for systematic effects.
Model annotations: T or B = TPSSh of BP86 DFT functional (and TZVP basis set), D or O = DFT or DFT plus ONIOM (QM/MM) calculations (for the ONIOM QM/MM approach as implemented in the Gaussian program we used our previously reported large model structure constructed on basis of the CPI crystal structure of Hox, see Senger et al., Proc. Natl. Acad. Sci. USA 113, 8454-8459, 2016), U = unrestraint calculation (all coordinates variable), S = small model (H-cluster only), M = medium-size model including amino acids in contact with the cofactor, E = extended model including amino acids in contact with the cofactor and further backbone residues (see Fig. S12 and Senger et al. for drawings of the model structures), A and L = models including alanine and lysine residues in contact with the cofactor (see Fig. S12), 3W = inclusion of three water molecules (1-3 in Fig. S12) close to the cofactor in the models, 4 or 9, = protonation in HoxH and Hred’ at sulfur atoms S4 (µS atom) or S9 (cysteine sulfur) (Fig. S12). Note that calculated IR frequencies for restraint and unrestraint models varied by a mean of <2 cm\(^{-1}\).

Reference:
Fig. S13B - Calculation results for model and theory level variation. Frequency offset (left), correlation line slope (middle), and fit quality (R) factors (right) from fits of data in Fig. S13A (left panels) for Hox, HoxH, and Hred' (top to bottom). Note that R values ranged between about 96-99 % for all models, indicating good correlation to the experimental data.
Fig. S13C - Calculation results for model and theory level variation. Top: IR frequency differences between HoxH and Hox for protonation at S4 or S9 in HoxH for each three model/theory level variations (see Fig. S13A) compared to the experimental data (exp) for the individual CO and CN⁻ bands of the H-cluster. Note that similar trends for band shifts were observed in the different approaches, that the TPSSh approach using a small H-cluster model resulted in good agreement with the experimental data, and that protonation at S9 (terminal cysteine at the [4Fe-4S] cluster) overall was favored over protonation at S4 (µS atom of the [4Fe-4S] cluster). Bottom: IR frequency differences between Hox, HoxH, and Hred for protonation at S4 or S9 for each two model/theory level variations compared to the experimental data. Note that similar trends for IR band shifts were observed upon calculation approach variation and protonation at S9 resulted in good agreement with the experimental data for all three H-cluster species. For a conservative H-cluster geometry, use of small model structures in DFT is sufficient to model even small band shifts in response to protonation.
Fig. S14AB - DFT on Hox and HoxH. IR band differences from DFT. Protons were added at the atoms specified on the x-axes in the HoxH structures (see Figs. 1 and S12). HoxH – Hox frequency (A) and intensity (B) differences for the three CO and two CN bands were calculated and compared to the experimental data (exp). DS9* denotes difference data for addition of a deuteron instead of a proton at S9 in HoxH.
Figure S14CD - DFT on Hox and HoxH. Correlation of experimental and calculated IR data. The x-axes denote calculations for Hox or for HoxH with a proton added at the indicated positions (compare Figs. 1 and S12). Root-mean-square deviations (rmsd) were derived after correction of calculated IR frequencies of CO/CN- ligands for systematic effects using Eqs. 1-3 (see Experimental Section) and using the respective Hox structure (with or without inclusion of water molecules) as a reference (rmsd values for different structures were normalized to the rmsd of Hox for the model comprising only the H-cluster). (A) rmsd for IR band frequencies. (B) rmsd for normalized IR band intensities.
Fig. S15 - DFT on Hox-CO and HoxH-CO. (A) Correlation between experimental and calculated IR frequencies and intensities (inset) of CO/CN bands for Hox, Hox-CO, and HoxH-CO. DFT (TPSSh/TZVP) involved model structures comprising the H-cluster, an apical CN⁻ ligand at Fe₆ in Hox-CO and HoxH-CO, and protonation at S₉ (see Figs. 1 and S12) in HoxH-CO. Lines show linear fits to the Hox data. (B) Comparison of experimental (left; experimental data, thin black lines; fit curves, thick lines) and calculated (right) IR spectra. Calculated IR frequencies were corrected for systematic effects using Eqs. 1-3 and stick spectra were broadened with the band widths derived from the fits to the experimental spectra. Notably, rmsd values for frequencies of calculated Hox-CO and HoxH-CO spectra were lower by a factor of ~2 for an apical CN⁻ instead of an apical CO at Fe₆ (Hox-CO: ~11 cm⁻¹ vs. ~21 cm⁻¹, HoxH-CO: ~8 cm⁻¹ vs. ~19 cm⁻¹), which supports our previous assignment of the ligand orientation in the CO-inhibited state of the H-cluster.
Fig. S16AB - DFT on Hred' and Hred'H. IR band differences from DFT. (A) Frequency differences (Hred' - Hox) of the CO/CN' bands for the indicated protonation positions in Hred' (see Figs. 1 and S12) from DFT compared to the experimental differences (exp). (B) Frequency differences (Hred'H – Hox) for the indicated protonation positions in Hred'H from DFT compared to the experimental differences (exp).
**Fig. S16C - DFT on Hred’ and Hred’H.** IR band differences from DFT. (C) Frequency differences ($Hred’H \text{ - } Hred’$) for the indicated protonation positions in $Hred’$ (first position in the x-axis annotation) and for the additional protonation site in $Hred’H$ (second position in the x-axis annotation) from DFT are compared to the experimental differences ($\text{exp}$). The unprotonated $Hred’$ structure is denoted “noH”, “H(api)” denotes protonation (i.e. formal hydride binding) at the apical position at Fe$_d$, “N(adt)” denotes additional protonation at the NH(adt) group.
Fig. S16DE - DFT on Hred’ and Hred’H. Correlation of experimental and calculated IR data. The x-axes denote calculations for Hred’ or for Hred’H with one or two protons added at the indicated positions (compare Figs. 1 and S12). Root-mean-square deviations (rmsd) were derived after correction of calculated IR frequencies of CO/CN' ligands for systematic effects using Eqs. 1-3 (see Materials and Methods). (D) rmsd for IR band frequencies of Hred’. (E) rmsd for IR band frequencies of Hred’H.
Fig. S17A - Vibrational modes, spin densities, and charges from DFT. (A) Calculated vibrational normal modes of CO/CN⁻ ligands in geometry-optimized structures of the indicated H-cluster species (protons were placed at S9 in HoxH and Hred⁻ and at S8 and S9 in Hred'H, see Fig. S12). Arrows show relative amplitudes and directions of vibrations (not drawn to scale). Black numbers give exp. vibrational frequencies (in cm⁻¹) corresponding to the shown modes of the ligands at Feₚ (p), Feₜ (d), or in bridging position (µ).
Fig. S17BC - Vibrational modes, spin densities, and charges from DFT. (B) Spin density distribution in HoxH (top) and Hred'H (bottom) structures (similar spin density distributions were observed for Hox and Hred'). (C) Charge (e) distribution in the four H-cluster species (2Fe = Fe_p + Fe_d, adt = (SCH_2)_2NH group, 4Fe4S = [4Fe4S] cluster core excluding the cysteine ligands, Cys = (truncated) cysteine ligands). Inset: mean experimental IR frequencies of the three CO ligands plotted vs. the summed charge on Fe_d plus Fe_p (the line shows a linear fit to the data).
Fig. S18A – Putative proton path to the [4Fe-4S] cluster in CPI. Molecular detail (left) and protein surface (right). The PDB code is given for each structure. The water molecule marked “B” could represent the putative base included in the reaction scheme shown in Fig. S7, “S9” is the sulfur atom in C417 (HYDA1) or C499 (CPI). The H-cluster and the most proximal, accessory [4Fe-4S] cluster are shown. Note that HYDA1 lacks the accessory domain. Structures of the following enzymes are shown (PDB codes in parenthesis): CPI holo-enzyme (4XDC) and apo-protein (4XDD), CPI holo-enzyme (3CBX and 1FEH). In the DDH holo-enzyme (1HFE) the water channel apparently has no contact with bulk water, which may be due to the lack of crystallographically visible (ordered) water molecules in the structure.
Fig. S18B - Putative proton path to the [4Fe-4S] cluster in CPI. The structure is for CPI holo-enzyme (4XDC). Catalytic domain drawn in green cartoon, the accessory domain is shown in grey. On the right side, relay sites of the adt proton pathway are highlighted (magenta). The sulfur atoms of the ligating cysteines in the catalytic domain are labelled S7 – S10 (compare Fig. S12). Four oxygen atoms (of putative water molecules) are located in the cleft between catalytic and accessory domains. The most proximal atom may correspond to the base, B, shown in Fig. S7). According to our experimental and theoretical data, sulfur atom S9 is protonated in HoxH most likely. Distances are given in Å.
Fig. S19 – Accumulation of HoxH and basic reaction cycle. The oxidized state Hox is converted into Hred’ by one-electron reduction and protonation at the [4Fe-4S] cluster, i.e. via an adjacent base (B, presumably a water molecule). A second reduction step and delivery of two protons, most likely via the ‘catalytic’ pathway, induces H₂ release and re-oxidation of the H-cluster to form HoxH. Recovery of Hox proceeds via deprotonation of the [4Fe-4S] cluster and re-protonation of the base (BH⁺), thereby closing the cycle. HoxH accumulates when the base is protonated at low pH (i.e. via an adjacent chain of water molecules, see Fig. S18) and regain of Hox is impaired.
Fig. S20 – Numerical simulations on HoxH formation. Simulations were performed with the CAIN ordinary differential equation solver (freeware, developed by S. Mauch at CalTech, USA; available, e.g., at https://sourceforge.net/projects/cain/). (A) Reaction scheme involving three H-cluster species (Hox, Hred’, HoxH), a one-electron reductant (DT) in reduced (r) or oxidized (o) forms, protons (H+), an (apparent) protein-reductant complex (HoxDTTr), a proton-accepting base (for example an amino acid residue or a water species) in its unprotonated (B) or protonated (BH+) forms, and six rate constants (k1-6, in arbitrary units). We note that HoxDTTr formation formally accounts for the time lag observed in the experimental HoxH formation traces and is not meant as binding of the reductant to the protein in the narrow sense, but presumably reflects a delay due to diffusion of the reductant from the aerosol into the protein film on the ATR cell. Back-reactions were neglected for simplicity (except for BH+ deprotonation).
We emphasize that quantitative simulation of the experimental kinetics from our IR data was not attempted, but simulation parameters (in particular the rate constants) were chosen in a way that the main features of the kinetic data were qualitatively reproduced. More complicated reaction sequences (i.e. including further intermediates and steps) are certainly conceivable, but apparently were not required to simulate the main features of HoxH formation. (B) For the indicated starting populations (“concentrations” in arbitrary units) of Hox, BH, H⁺, and DTr (all other populations were initially zero) and for the given relative magnitudes of the rate constants k₁,₃,₄,₅,₆ = 0.01 and a 100-fold larger k₂ = 1, quantitative Hox to HoxH conversion without significant accumulation of Hred' is observed. The dashed line shows the sum of Hox and HoxDTr. (C) Increasing the DTr concentration at a high H⁺ concentration (same starting populations of species and k₁-₆ values as in B) yields quantitative HoxH formation for super-stoichiometric DTr. (D) The HoxH population yield increases with increasing DTr and quantitative HoxH formation is observed at excess DTr. (E) The rate of HoxH formation (defined as 1/t for t corresponding to half-maximal HoxH population) increases with increasing DTr and saturates at excess DTr concentrations. (F) Increasing the H⁺ concentration (decreasing the –log H⁺ = “pH” value) at a high DTr concentration yields quantitative HoxH formation at low “pH”. (G) The yield of HoxH formation shows an apparent titration curve on the “pH” scale. (H) The logarithmic rate of HoxH formation increases (approximately linearly) and by less than one order of magnitude for decreasing the “pH” by one unit.