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Bridging Hydride at Reduced H-Cluster Species in [FeFe]-Hydrogenases Revealed by Infrared Spectroscopy, Isotope Editing, and Quantum Chemistry

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ABSTRACT: [FeFe]-Hydrogenases contain a H₂-converting cofactor (H-cluster) in which a canonical [4Fe-4S] cluster is linked to a unique diiron site with three carbon monoxide (CO) and two cyanide (CN⁻) ligands (e.g. in the oxidized state, **Hox**). There has been much debate whether reduction and hydrogen binding may result in alternative rotamer structures of the diiron site in a single (**Hred**) or double (**Hsred**) reduced H-cluster species. We employed infrared spectro-electrochemistry and site-selective isotope editing to monitor the CO/CN⁻ stretching vibrations in [FeFe]-hydrogenase HYDA₁ from *Chlamydomonas reinhardtii*. Density functional theory calculations yielded vibrational modes of the diatomic ligands for conceivable H-cluster structures. Correlation analysis of experimental and computational IR spectra has facilitated an assignment of **Hred** and **Hsred** to structures with a bridging hydride at the diiron site. Pronounced ligand rotation during μ H binding seems to exclude **Hred** and **Hsred** as catalytic intermediates. Only states with a conservative H-cluster geometry featuring a μ CO ligand are likely involved in rapid H₂ turnover.

Hydrogenases are the most efficient biological catalysts for hydrogen (H_2) formation and oxidation, making them relevant for renewable fuel technology and catalysis research.¹ Figure 1 shows the active site cofactor (H-cluster) in a crystallized [FeFe]-hydrogenase,^{2,3} comprising a [4Fe-4S] cluster linked via a cysteine to a unique diiron site, [2Fe]. In the “active-ready” oxidized state (**Hox**), the iron atoms in proximal (Fe_p) or distal (Fe_d) position relative to the [4Fe-4S] cluster each carry one terminal CO and CN^- ligand.⁴ Fe_p and Fe_d are interconnected by a bridging carbonyl (μCO) and an aminodithiolate (adt, $(SCH_2)_2NH$).^{5,6} Fe_d shows a vacant coordination site.³ The adjacent adt nitrogen base and a conserved cysteine presumably serve as proton relays in the catalytic cycle.⁷ Putative hydrogen bonds between the protein scaffold and the CN^- ligands involve Ser232 and Lys358.⁸

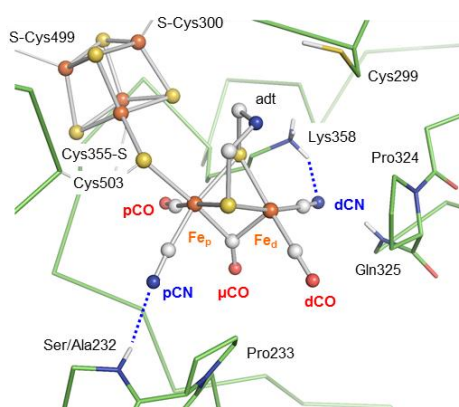


Figure 1. Crystal structure of the H-cluster in bacterial [FeFe]-hydrogenase (CpI, PDB entry 4XDC).² Dashes mark putative H-bonds. Ser232 in CpI is Ala in HYDA₁, Lys358 and Cys299 are conserved residues, the N(adt) proton is omitted.

In the last decades, a variety of intermediate states of the H-cluster has been characterized (see, e.g., refs.^{7,9-23}). However, the chemical nature and the role of many species in the catalytic cycle have remained elusive. In this study, we focus on single or double (“super-”) reduced species,⁷ which carry one (**Hred**, **Hred'**) or two (**Hsred**, **Hhyd**) surplus electrons relative to **Hox**. Recently, **Hred'** was assigned to a structure with a μCO and an apical vacancy,^{9,24} as well as a proton at a cysteine ligand of the [4Fe-4S] cluster.²⁴ For **Hhyd**, a μCO and an apical hydride at Fe_d were assigned.^{10,12,22,25,26} For **Hred** and **Hsred**, several structural isomers have been suggested, differing with respect to the metal-bridging ligand and to the location of protons at the diiron site.^{9,11,13,15-17,27} Three basic model types for **Hred** and **Hsred** can be distinguished: (I) Structures with a μCO ligand and a proton (i.e. a formal hydride) or H_2 bound at Fe_d (Figs. 2a, S5). (II) Structures with an

open metal-bridging site, a vacancy at Fe_d, and additional protonation at the N(adt) (Fig. 2b). (III) Structures with a bridging hydride (μH) or ηH_2 (Figs. 2c, S5).

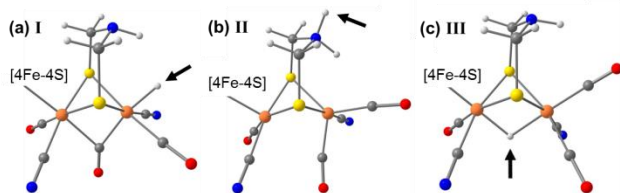


Figure 2. Basic diiron site structures for **Hred** and **Hsred**. Structural variants for DFT were designed following literature proposals^{10-13,15-17} (Fe, orange; S, yellow; O, red; N, blue; C, gray; H, light-gray; [4Fe-4S] cluster abbreviated for clarity). See Fig. S5 for complete and further structures (e.g. with CO/CN⁻ rotation or a H₂ ligand). Arrows mark H⁺ binding.

We have recently demonstrated that the combination of attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) with density functional theory (DFT) is powerful for structural elucidation of the H-cluster.^{14,24,25} The CO/CN⁻ vibrations are specific markers of changes in geometric and electronic configuration in response to redox and protonation reactions, as well as of ligand binding. Selective ¹³CO editing leads to characteristic IR-frequency shifts that serve as benchmarks for DFT.¹⁴ However, modelling of structural and electronic changes in reduced states requires consideration of ‘internal’ isomerism (rotamers), as observed for CO binding at [2Fe] (**Hox-CO**),¹⁴ the influence of hydrogen bonding,^{2,8} variation of protonation sites,^{24,25,28} and effects related to the theory level,^{20,29} which were here addressed to determine the chemical nature of **Hred** and **Hsred**.

Chlamydomonas reinhardtii [FeFe]-hydrogenase HYDA₁ was purified and activated,^{5,30} and ¹³CO editing at [2Fe] was achieved as reported earlier.¹⁴ A novel ATR-FTIR set-up for spectro-electrochemistry (Fig. S1) facilitated selective population of redox states of the H-cluster (Fig. S2). CO/CN⁻ band frequencies and intensities were derived from experimental IR spectra using a global fit approach (Fig. S3). In DFT calculations (Gaussian09), based on the CpI structure,² model variants I-III were generated (Figs. 2, S5) and assignment of anti-ferromagnetic coupling (broken symmetry approach) was followed by geometry optimization. Larger models containing Ala232 or Ala232 and Lys358 (Figs. 1, S5), ¹³CO editing, and DFT functional (BP86 or TPSSh) and dielectric constant (COSMO solvation model) variations were considered. Normal mode analysis yielded the theoretical CO/CN⁻ vibrations.

The experimental IR spectra of **Hox**, **Hred**, **Hred'**, and **Hsred** are compared in Fig. 3. The well-known CO/CN⁻ band pattern of **Hox** agrees with an apical vacancy at Fe_d and a μCO ligand.¹⁴ The similar spectrum of **Hred'** is assigned to a structure with a reduced [4Fe-4S] cluster, a μCO, and apical vacancy as in **Hox**.^{9,24} **Hred** and **Hsred** show IR patterns with pronounced CO/CN⁻ band shifts and relative intensity changes vs. **Hox**. A ~90 cm⁻¹ up-shift of the CO band α, due to μCO in **Hox**, down-shifts of the other CO/CN⁻ bands, and intensity reversal of the CO bands α and β suggested significant structural changes at the diiron site. A doubled frequency difference of the two CN⁻ bands (~40 cm⁻¹ in **Hred** and **Hsred** vs. ~20 cm⁻¹ in **Hox**) was observed.^{11,27} ¹³CO isotope editing and H/D exchange experiments revealed similar CO band frequency shifts for **Hred** and **Hsred** (Fig. S4, Table S1). These results indicated that both states adopt a similar geometry of the H-cluster, which differs from **Hox**. Structural isomerism (ligand rotation) seems not to occur in the **Hred** to **Hsred** reduction, as in the **Hox** to **Hred'** reduction.^{9,24}

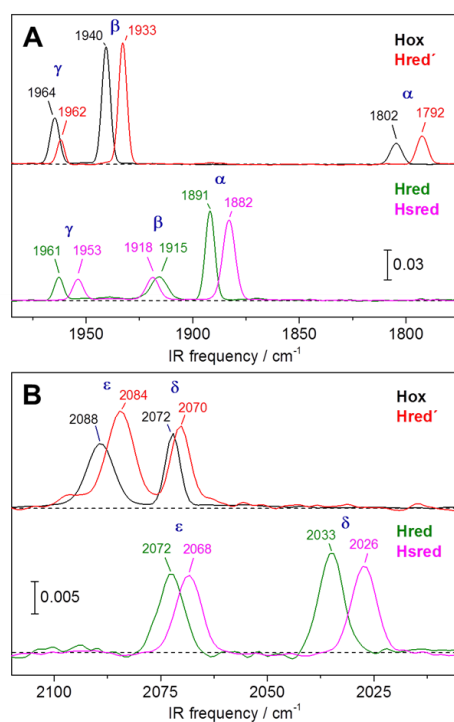


Figure 3. Experimental ATR-FTIR spectra of HYDA₁. (A) CO ligand region. (B) CN⁻ ligand region. Band frequencies (in cm⁻¹) were derived from fit analysis. Spectra were recorded at -200 mV (**Hox**), -400 mV (**Hred**), -750 mV (**Hred'**), or -900 mV (**Hsred**) vs. SHE using a spectro-electrochemical set-up (Fig. S1), background and minor species contributions were subtracted (Fig. S2), and spectra were normalized (Fig. S3).

DFT calculations were carried out on model types **I-III** of **Hred** and **Hsred** (Figs. 2, S5). The theoretical IR spectra revealed that the coupling of the CO stretching vibrations strongly differs between the model types, meaning that the CO bands (α , β , γ) were attributable to different vibrational modes (see SI and Fig. S6 for a detailed analysis). Comparable effects were observed for rotational isomers (e.g. equatorial vs. apical CN⁻ at Fe_d) and ¹³CO isotopomers. Linear fits to data in plots of calculated vs. experimental IR frequencies (F) for **Hred** and **Hsred** structures revealed variable CO/CN⁻ F-offsets and deviations from an ideal slope of unity, depending on the DFT functional and dielectric constant, redox state, and structure type/size (Fig. S7). Alignment of calculated and experimental IR spectra using a common offset/slope correction, which works for species with a similar [2Fe] geometry (i.e. **Hox**, **Hred'**),^{14,24,25} thus was inadequate for comparing **Hred** and **Hsred** with **Hox**. We used individual F-offset corrections (i.e. the same F-shift for all CO/CN⁻ bands, neglecting slope deviations) as a least biased evaluation approach, which facilitated a statistical correlation of experimental and calculated CO/CN⁻ frequencies of **Hred** and **Hsred** using the root-mean-square deviation (Eq. 1; n = 5 CO/CN⁻ ligands, F^{cor} = F^{cal} - F^{offset}):

$$(1) \quad rmsd = \sqrt{\sum (F_i^{exp} - F_i^{cor})^2 / n} .$$

Tables S2-S6 summarize calculated IR frequencies and intensities, F-shifts, and rmsd values for all calculated models. Linear fits to calculated vs. experimental CO/CN⁻ frequencies and frequency differences yielded R²-factors as a further quality criterion (Tables S7, S8; Fig. S9).

Figure 4 shows a correlation plot of calculated frequencies for **Hred** and **Hsred** model types **I-III** vs. experimental frequencies. The frequencies for type **III** models (μ H) are in best agreement with the experimental data (see also Figs. S7, S8). The small rmsd for models lacking amino acids was in the range of 13-25 cm⁻¹ for all six type **III** rotamers, whereas an at least 2-fold larger rmsd (34-67 cm⁻¹ or 25-57 cm⁻¹) was obtained for models **I** or **II** (Tables S2, S3). Inclusion of Ala232 or Ala232/Lys358 improved the rmsd of all models, so that type **III** remained superior. Protonation at a cysteine ligand of the [4Fe-4S] cluster^{24,25} in model **III** of **Hsred** (μ H) shifted the CO/CN⁻ bands by <3 cm⁻¹ on average. The linear regression parameters supported that models with a μ H represent the best description of **Hred** and **Hsred** (Tables S7, S8). Variation of the DFT functional or dielectric constant had a considerable effect on the rmsd values, but models with a μ H were favored at all theory levels (Table S5).

Discrimination of the actual rotamer structure of **Hred** and **Hsred** required in-depth analysis of the DFT data. Lowest rmsd values were obtained for type **III** structures with an apical CO or CN⁻ at Fe_d. Type **III** rotamers with p/dCO/CN⁻ ligand inversion or pCO/CN⁻ inversion and an apical CN⁻ at Fe_d were disfavored by their larger rmsd (Tables S2, S3, S6). Trans-axial effects between bridging and apical ligands and hydrogen bonding of N(adt) also affect the rmsd. For example in models **II** of **Hred** and **Hsred**, the 2-fold diminished rmsd for apical CN⁻ vs. CO is due to the dCN⁻-HN(adt) interaction, resulting in rotation of the semi-bridging CO (Fig. S5) and in p/dCN⁻ vibrational decoupling (as in model **III**).

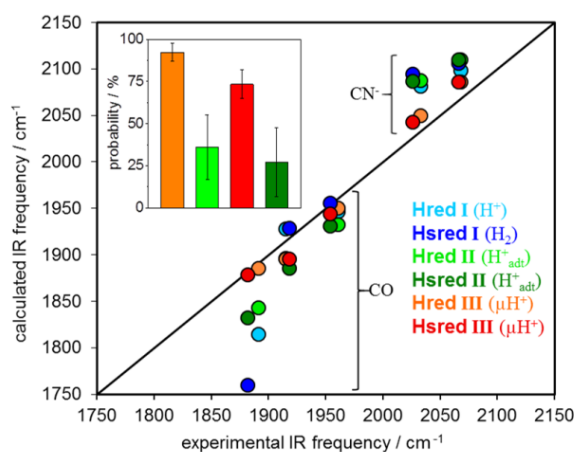


Figure 4. Comparison of calculated and experimental CO/CN⁻ frequencies. DFT data (TPSSh, $\epsilon = 4$) refers to indicated structures (containing Ala232; Figs. 2, S5). Computational and experimental frequencies were aligned (Tables S2, S3). Inset: “probability” of selected structures (see Table S11).

¹³CO editing provided further insight into the rotamer structure (Tables S1, S6, Fig. S9). Pronounced shifts of CO band β in models **II** (protonation at N(adt), open bridge) with an apical CO at Fe_d for **Hred** and **Hsred** disfavored such structures. More equal shifts of all three CO bands in models **II** and **III** with an apical CN⁻ at Fe_d for all ¹³CO labeling patterns also disagreed with experimental data. Only models **III** (μ H) with an apical CO at Fe_d of **Hred** and **Hsred** reproduced the experimental ¹³CO band shifts reasonably well, which suggested equatorial CO and CN⁻ ligands at Fe_d as in **Hox**. Two further features of the experimental **Hred** and **Hsred** IR spectra were well described only by models **III** (μ H) with an apical CO (Fig. S8): (i) The large frequency gap of the CN⁻ ligands (29-44 cm⁻¹), in contrast to an about 2-fold smaller gap for an apical CN⁻. (ii) The large intensity increase of the CO band α , which remains weak for an apical CN⁻. An analysis of the electronic

configuration of the **Hred** and **Hsred** structures with a bridging hydride is provided in the SI. For **Hox** and **Hred'**, H-bonding amino acids in the DFT structures have no decisive effect on the IR bands.^{14,24,25} For **Hred** and **Hsred** models **III**, inclusion of Ala232 or Ala232/Lys358 yielded significant CN⁻ frequency down-shifts (≤ 10 cm⁻¹), leading to improved agreement with the experimental data (Table S4). H-bonding seemingly has a more significant effect on the electron density distribution in the H-cluster with a reduced rather than oxidized diiron site.

In this study, the combination of ATR-FTIR, ¹³CO editing, and DFT has yielded a structural assignment of the **Hred** and **Hsred** states of the H-cluster. Our analysis favors a one- or two-electron reduced structure for **Hred** and **Hsred**, both carrying a bridging hydride and an apical CO ligand at the diiron site (Table S11). **Hox** has been assigned to a (formal) [4Fe-4S]²⁺-[Fe^{II}Fe^I] complex^{16,31} and **Hred'** represents a [H⁺4Fe-4S]¹⁺-[Fe^{II}Fe^I] complex,^{9,24} both with a μ CO ligand and an apical vacancy.² Here, we assign **Hred** to a (formal) [4Fe-4S]²⁺-Fe^{II}(μ H⁻)Fe^{II}] complex and **Hsred** to a [4Fe-4S]¹⁺-Fe^{II}(μ H⁻)Fe^{II}] complex.¹⁶ Protonation at the [4Fe-4S] cluster besides of the μ H in **Hsred** is not excluded. Reversible **Hred** and **Hsred** formation³² seems to be compatible with a μ H because ligand rotation at the H-cluster upon hydride binding/removal may readily occur on the minutes timescale of typical spectroscopic experiments on enzymes in solution. Pronounced ligand rotation at the diiron site during μ H binding implies that **Hred** and **Hsred** are unlikely catalytic intermediates during sustained H₂ conversion.^{33,34}

HYDA₁ is located in the stroma of the algal chloroplast.³⁵ However, **Hred** and **Hsred** seem to accumulate preferentially at acidic pH.^{11,22,24} Such a pH may favor faster protonation at the diiron site than at the [4Fe-4S] cluster, which biases the first surplus electron to the diiron site and facilitates μ H binding.^{24,25} More rapid [4Fe-4S] cluster protonation, i.e. at physiological alkaline pH, locates the first electron at the cubane and likely prevents ligand rotation and μ H binding. These considerations suggest that only states with a conservative diiron site geometry (i.e. **Hox**, **Hred'**, **Hhyd**) are involved in the rapid H₂ turnover of [FeFe]-hydrogenases.

ASSOCIATED CONTENT

Supporting Information

ATR-FTIR spectro-electrochemistry methods, computational structures and IR data evaluation, electronic structure analysis (file type PDF). The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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