Supporting Information

$^1$H NMR Spectroscopy of [FeFe] Hydrogenase: Insight into the Electronic Structure of the Active Site

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SI1. Sample preparation

Apo-HydA1 was expressed and purified as described previously\(^1\). The bacteria suspension was shaken at 37°C aerobically till an OD\(_{600}\) (optical density at a wavelength of 600 nm) of 0.6 was reached. After pH correction to 7.4 and transfer in a glass bottle with Teflon membrane, protein expression was induced with 0.5 M IPTG (isopropyl β-D-thiogalactopyranoside). The suspension was gassed with argon for one hour and expression was continued for 25 h at room temperature. Unless indicated, all samples were handled under strictly anaerobic conditions in a glove box (COY) using a palladium catalyst and forming gas with 1-2.5% hydrogen.

All NMR samples were prepared in NMR buffer 1 (25 mM potassium phosphate pH* 7.4 and 100% D\(_2\)O) or 2 (25 mM potassium phosphate pH* 6.4 and 100% D\(_2\)O).

Preparation of pure states for NMR spectroscopy:

1. The first sample had a concentration of 1.7 mM and contained >95% oxidized apo-HydA1. This sample was purified in the absence of sodium dithionite and the NMR tube was flame sealed.
2. The second sample had a concentration of 4.2 mM and contained >95% reduced apo-HydA1. It was purified in the presence of 2 mM sodium dithionite. The buffer was exchanged directly before the measurement to remove sodium dithionite and the NMR tube was flame sealed.
3. To prepare the third sample, a two-fold excess of a 50 mM \([\text{Fe}_2(\text{adt})(\text{CO})_3(\text{CN})_2]\) solution in DMSO was added to 0.53 ml of 2.65 mM apo-HydA1 in 25 mM Tris/HCl pH 8.0, 25 mM KCl and 2 mM NaDT diluted to 2.5 ml with NMR buffer 1. The maturation and removal of excess \([\text{Fe}_2(\text{adt})(\text{CO})_3(\text{CN})_2]\) was performed as described previously.\(^1^2\) After concentrating the sample to approximately 3.5 mM, it was flashed with argon for 20.5 h. All following steps were performed in a glove box (MBRAUN) filled with N\(_2\) in the absence of hydrogen. Addition of 2.45 mM thionine acetate to remove residual H\(_{\text{red}}\) and H\(_{\text{red}}\) resulted in >95% HydA1 in the H\(_{\text{ox}}\) state (Figure S4, bottom). The sample was measured in a flame sealed NMR tube.
4. The fourth sample was prepared by CO-flushing about 4 mM maturated apo-HydA1 for 1 h and keeping the sample in the CO-filled closed vial for about 1 h. The FTIR spectrum confirmed HydA1 to be present to >95% in the H\(_{\text{ox}}\)-CO state (Figure S7). This sample was measured in a 5 mm medium wall precision quick pressure valve NMR tube.
5. For the NOE-based assignment of the methylene protons of the dithiolate bridge, apo-HydA1 was maturated with Fe\(_2\)(pdt)(CO)\(_3\)(CN)\(_2\) (pdt = propanedithiolate). The resulting HydA1-pdt has been shown to exist only in an oxidized and reduced state and is much more stable than HydA1-adt. Furthermore, the oxidized form of HydA1-pdt has revealed basically the same electronic structure.\(^3\) The sample used for measuring the transient NOE had a concentration of about 3.6 mM, contained about 50% oxidized and 50% reduced HydA1-pdt and was measured in a flame-sealed NMR tube.

SI2. FTIR measurements of H\(_{\text{ox}}\) and H\(_{\text{ox}}\)-CO

An aliquot of 8 μl of the NMR samples (3) and (4) was employed to assess the purity of the H\(_{\text{ox}}\) and H\(_{\text{ox}}\)-CO redox states using FTIR spectroscopy.\(^4\) FTIR measurements were carried out on a Bruker IFS 66v/S or a Bruker VERTEX 80v spectrometer with a resolution of 2 cm\(^{-1}\) in forward-backward measuring mode for 1000 scans at room temperature. Baseline correction was performed using a self-written routine in MATLAB.
SI3. NMR experiments

1D $^1$H NMR spectra shown in Figure 2 were acquired at 298 K on a Bruker AVANCE 600 spectrometer equipped with a cryogenic TCI probehead using the normal one pulse experiments with $^1$H$_2$O presaturation or using the super-WEFT pulse sequence. Relaxation delay times were 200 ms. In case of HydA1 in the $H_{ox}$ and $H_{ox}$-CO state, methylene protons of the [2Fe]$_{II}$ site were identified by comparison of the $^1$H 1D spectra of samples maturated with deuterated and non-deuterated [Fe$_2$(adt)(CO)$_4$(CN)$_2$].

1D NOE (Figure S6)

The 1D NOE experiments of HydA1-pdt were acquired at 298 K on a Bruker AVANCE 600 equipped with a cryogenic TCI probehead. For the measurement of the NOE, a modified super-WEFT sequence including CW irradiation off resonance from the carrier position was used. To minimize the artifacts the following irradiation scheme was used as follows: the spectrum with CW irradiation on resonance with the target signal is acquired twice and co-added, then the spectra with the CW irradiation symmetrically off-resonance with respect to the target signals are acquired and subtracted. The used on and off resonance frequencies are summarized in the following table.

Table S1. Summary of the used on and off resonance frequencies to measure the NOE at 600 MHz

| Signal             | Off resonance (downfield) frequency (Hz) subtracted | On resonance frequency (Hz) acquired twice | Off resonance (upfield) frequency (Hz) subtracted |
|--------------------|-----------------------------------------------------|------------------------------------------|-----------------------------------------------|
| oxidized HydA1-pdt |                                                     |                                          |                                               |
| 1+2                | 6999.64                                             | 6711.60                                  | 6430.56                                       |
| 3                  | -12932.45                                           | -13208.90                                | -15329.66                                     |
| 4                  | -15329.66                                           | -15800.77                                | -15871.88                                     |
Temperature-dependence of the hyperfine shifted resonances of oxidized and reduced apo-HydA1

**Figure S1.** Plot of the observed chemical shifts versus the reciprocal temperature for the assigned contact shifted Cys resonances from oxidized (a) and reduced (b) HydA1 containing only the [4Fe-4S]₄ cluster. The peaks are labeled as in Figure 2.
Temperature-dependence of the hyperfine shifted resonances of H_{ox}

**Figure S2.** Plot of the observed chemical shifts versus the reciprocal temperature for the contact shifted resonances from HydA1 in the H_{ox} state. The peaks are labeled as in Figure 2. The downfield shifted resonances are displayed in a) and the upfield shifted resonances are shown in b).
Temperature-dependence of the hyperfine shifted resonances of $\text{H}_{\text{ox}}$-CO

Figure S3. Plot of the observed chemical shifts versus the reciprocal temperature for the contact shifted resonances from HydA1 in the $\text{H}_{\text{ox}}$-CO state. The peaks are labeled as in Figure 2. The downfield shifted resonances are displayed in a) and the upfield shifted resonances are shown in b).
Table S2. $^1$H NMR spectral parameters for the hyperfine shifted resonances for different forms of HydA1.

| Peak label | Assignment | Chem. shift (ppm) | Line-width (Hz) | Temp. dep. | Relative area | Peak label | Assignment | Chem. shift (ppm) | Line-width (Hz) | Temp. dep. | Relative area |
|------------|------------|-------------------|-----------------|------------|---------------|------------|------------|-------------------|-----------------|------------|---------------|
|            |            |                   |                 |            |               |            |            |                   |                 |            |               |
| oxidized apo-HydA1 |            |                   |                 |            |               | reduced apo-HydA1 |            |                   |                 |            |               |
| a          | β-CH$_2$   | 21.38             | 300             | aC         | 1             | A          | β-CH$_2$   | 55.59             | 1500            | C          | 2             |
| b          | β-CH$_2$   | 17.23             | 200             | aC         | 1             | B          | β-CH$_2$   | 53.54             | 1500            | aC         | 2             |
| c          | β-CH$_2$   | 11.97             | 300             | aC         | 1             | C          | β-CH$_2$   | 44.38             | 1800            | aC         | 2             |
| d          | β-CH$_2$   | 10.4              | 200             | aC         | 1             | D          | β-CH$_2$   | 33.98             | 1600            | C          | 2             |
| e          | β-CH$_2$   | 7.11              | 200             | n. d.      | n. d.         | E          | α-CH$_2$   | 11.74             | 400             | aC         | 1             |
| H$_{ox}$ state |            |                   |                 |            |               | H$_{ox-CO}$ state |            |                   |                 |            |               |
| a          | β-CH$_2$   | 32.22             | 300             | C          | 1             | A          | β-CH$_2$   | 75.66             | 4000            | C          | n. d.         |
| b          | β-CH$_2$   | 30.87             | 300             | C          | 1             | 1          | adt-CH$_2$ | 60.09             | 4000            | C          | n. d.         |
| 1          | adt-CH$_2$ | 28.63             | 600             | C          | 1             | 2          | adt-CH$_2$ | 45.48             | 1800            | C          | n. d.         |
| 2          | adt-CH$_2$ | 27.95             | 400             | C          | 1             | B          | β-CH$_2$   | 38.71             | 1400            | C          | n. d.         |
| c          | β-CH$_2$   | 17.22             | 200             | aC         | 1             | C          | α-CH$_2$   | 25.39             | 300             | C          | n. d.         |
| d          | β-CH$_2$   | 16.47             | 200             | aC         | 1             | D          | α-CH$_2$   | 13.36             | 300             | C          | n. d.         |
| 3          | adt-CH$_2$ | -10.13            | 200             | pC         | 1             | E          | α-CH$_2$   | 11.69             | 300             | aC         | n. d.         |
| 4          | adt-CH$_2$ | -21.07            | 200             | pC         | 1             | F          | α-CH$_2$   | -2.92             | 300             | pC         | 1             |
| e          | α-CH$_2$   | 11.7              | 200             | aC         | 1             | 3+4        | adt-CH$_2$ | -7.25             | 300             | pC         | 2             |
| f          | α-CH$_2$   | 11.4              | 100             | aC         | 1             | G          | β-CH$_2$   | -8.48             | 400             | pC         | 1             |
| H          | β-CH$_2$   | -27.78            | 800             | pC         | 1             | H          | β-CH$_2$   | -27.78            | 800             | pC         | 1             |

[a] full-width at half-maximum, n. d. = not determined, C = Curie, aC = anti-Curie, pC = pseudo-Curie
FTIR spectra of HydA1 in the $H_{ox}$ state

Due to the appearance of the active site (with a free ligand site at Fe$_d$), there are two terminal –CO vibrations and one bridging –CO as well as two –CN’ vibrations expected in FTIR spectrum. For the [FeFe] hydrogenase HydA1 from *Chlamydomonas reinhardtii* in the $H_{ox}$ state, bands at 2088/2070 cm$^{-1}$ corresponding to the –CN’ vibrations and bands at 1964/ 1939/ 1803 cm$^{-1}$ for the –CO vibrations are described.$^1$ With small deviations these vibrations can be detected for the used NMR samples for the $H_{ox}$ state (Figure S4). The deuteration of methylene protons of adt does not affect the band positions in the $H_{ox}$ state. Impurities, mainly $H_{ox}$-CO state (see asterisk Figure S4 and compare Figure S7), are slightly more pronounced in the sample maturated with $^2$H-adt. Based on the FTIR spectra the purity of both freshly prepared NMR samples in the $H_{ox}$ state has been estimated as >95%.

![FTIR spectra of HydA1 in the $H_{ox}$ state](image)

*Figure S4.* FTIR spectra of HydA1 in the $H_{ox}$ state at room temperature. The spectrum of the unlabeled sample is shown in blue and the sample with the deuterated [2Fe]$_a$ site ($^2$H-adt) is shown in green. The marker band of the $H_{ox}$-CO state at 2013 cm$^{-1}$ is indicated by *. 
Assignment of the adt methylene protons in the H\textsubscript{ox}-CO state

In order to distinguish the methylene protons of the [4Fe-4S]\textsubscript{II} cluster-coordinating cysteines and of the adt in the [2Fe]\textsubscript{II} cluster of the H\textsubscript{ox} state, apo-HydA1 was maturated using \textsuperscript{2}H-adt. By comparison of the \textsuperscript{1}H spectra of unlabeled H\textsubscript{ox} with H\textsubscript{ox} containing \textsuperscript{2}H-adt, signals labelled 1 to 4 in Figure 2c have been unambiguously assigned to the four methylene protons of [2Fe]\textsubscript{II}. The amine proton of adt exchanges with water and is thus not present in the \textsuperscript{1}H NMR spectra when 100\% D\textsubscript{2}O is used as the solvent.

\textbf{Figure S5.} 1D \textsuperscript{1}H NMR spectra (600 MHz) at 298 K of unlabeled oxidized HydA1 (blue, H\textsubscript{ox}) and oxidized HydA1 maturated with deuterated adt (green, H\textsubscript{ox}-\textsuperscript{2}H-adt). a) Downfield region from 35 to 11 ppm and b) upfield region from -3 to -33 ppm. Peaks that belong to other HydA1 states investigated here are labelled with *. Resonances marked with ** originate probably from reduced HydA1 states present in the sample. The contaminations constitute about 5\% of the total sample for unlabeled H\textsubscript{ox} and about 20\% for H\textsubscript{ox}-\textsuperscript{2}H-adt. As the FTIR spectra for H\textsubscript{ox} and H\textsubscript{ox}-\textsuperscript{2}H-adt are almost identical (Figure S4), the sample maturated with \textsuperscript{2}H-adt appears to be less stable than the sample maturated with adt.
Assignment of the axial and equatorial methylene protons of adt

a) Distance and linewidth considerations

In the X-ray structure with protons added, the axial protons are closer to [4Fe-4S]₄ as well as to the proximal and distal Fe sites (Feₚ and Feₜ) compared to the equatorial protons (Figure 1 (right) and Table S2). Since the paramagnetic relaxation enhancement also depends on the inverse sixth power of the distance between metal ion and nuclear spin, in general the closer a proton is to an Fe, the larger is its linewidth of the corresponding ¹H signal.

Table S3. Distances of the methylene protons of adt to Feₚ and Feₜ

|     | distance to Feₚ (Å) | distance to Feₜ (Å) |
|-----|---------------------|---------------------|
| H1  | 3.54                | 3.98                |
| H2  | 3.44                | 4.07                |
| H3  | 4.40                | 4.19                |
| H4  | 4.37                | 4.12                |

b) Experimentally observed characteristic NOE patterns for axial and equatorial protons

In addition to [Fe₂(adt)(CO)₄(CN)₂] other synthesized inorganic cofactors can be incorporated into recombinant HydA1 containing only the [4Fe-4S]₄ cluster. The resulting analogues of HydA1 have a reduced or no activity but may have other advantages over fully active HydA1.¹ Using the propanedithiolate (pdt) analogue Fe₂(pdt)(CO)₄(CN)₂ for maturation to prepare the “so-called” HydA1-pdt provides the advantage of high sample stability concomitant with only an oxidized and a reduced state. Importantly, the oxidized form of HydA1-pdt has revealed basically the same electronic structure as fully active HydA1 in the Hᵒ state.¹ Hence the Hᵒ state of HydA1-pdt has been used to obtain ¹H NOE connectivities between the methylene protons of the dithiolate bridge of [2Fe]₄. These ¹H NOE connectivities present another strong evidence for signals 1+2 to correspond to the axial and for signals 3+4 to correspond to the equatorial protons. The measured ¹H NOE spectra revealed NOEs between signals 3 and 1+2 as well as between signals 4 and 1+2. In addition, no NOE was observed between signal 3 and 4 (Figure S6). Accordingly, the downfield shifted signals 1+2 with a line width of about 500 Hz have been tentatively assigned to the axial protons H1 and H2 and signals 3+4 with a line width of about 200 Hz have been assigned to the equatorial protons H3 and H4 (Figure 1 and Table 1). The NOE experiments were carried out with the mixture of 50% oxidized and 50% reduced HydA1-pdt to observe any possible saturation transfer from one oxidation state to the other.
Figure S6. a) Overlay of the NOE spectra obtained upon irradiating the hyperfine shifted resonances and the full spectrum of an about 1:1 mixture of oxidized and reduced HydA1-pdt (black). The trace in light blue indicates a x10 multiplication of the intensity. Upon irradiating signal 3 (green trace) or signal 4 (red trace), a signal at about 11.2 ppm responds (signal 1+2). Signal 1+2 (violet trace) is coupled to signal 3 and also signal 4. The arrows indicate the irradiation frequencies. Irradiation is performed to minimize the artifacts as shown in Figure S1. The resonances originating from the reduced portion of the sample are indicated by *. b) The 1D $^1$H NMR spectrum of H$_{ox}$ as also shown in Figure 1c. The black lines connect the corresponding resonances of H$_{ox}$ in b) and oxidized HydA1-pdt in a). The strong chemical shift differences of signals 1-4 observed when comparing the $^1$H 1D NMR spectra of HydA1-adt and HydA1-pdt in the H$_{ox}$ state are caused by the presence of a methylene group instead of a secondary amine in the bridgehead position of the dithiolate ligand connecting the two irons of [2Fe]$_2$. 
FTIR spectra of HydA1 in the H\textsubscript{ox}-CO state

In the H\textsubscript{ox}-CO state an additional –CO ligand is bound to the open coordination site at Fe\textsubscript{d}. Thus, four instead of three –CO vibrations occur. In the literature band positions at 2092/2084/2013/1970/1964 and 1810 cm\textsuperscript{-1} are described\textsuperscript{9} and the detected signals for the used NMR samples in H\textsubscript{ox}-CO state are almost identical. The rather broad contributions in the HydA1-\textsuperscript{2}H-adt H\textsubscript{ox}-CO spectrum (Figure S7, green line) are impurities (\textsuperscript{\textdagger}) and do not belong to a second redox state. For the unlabelled sample, a tiny amount of H\textsubscript{ox} (1939 cm\textsuperscript{-1}) and H\textsubscript{red}H\textsuperscript{+} (1891 cm\textsuperscript{-1}) estimated as less than 5 % of the total sample were detected (Figure S7).

\textit{Figure S7.} FTIR spectra of HydA1 in the H\textsubscript{ox}-CO state at room temperature. The spectrum of the unlabeled sample is shown in red and the sample with the deuterated [2Fe] site (\textsuperscript{2}H-adt) is shown in green. Impurities are indicated by \textsuperscript{\textdagger} and other redox states are indicated by \textsuperscript{*}. 
Assignment of the adt methylene protons in the H_{ox}-CO state

Three of the hyperfine shifted signals observed for unlabeled HydA1 in the H_{ox}-CO state are not present when deuterated [Fe_{2}(adt)(CO)_{4}(CN)_{2}] has been used for maturation. Given that the integral of the disappearing upfield peak at -7.25 ppm suggests that it consists of two proton signals (3+4), all four methylene protons of [2Fe]_{4} could thus also be identified for H_{ox}-CO (Figure 2c).

**Figure S8.** 1D $^1$H NMR spectra (600 MHz) at 298 K of unlabeled HydA1 in the H_{ox}-CO state (red) and H_{ox}-CO after maturation with deuterated ADT (green). a) Downfield region from 84 to 15 ppm and b) upfield region from -2 to -32 ppm. Peaks that belong to other HydA1 states investigated here are labelled with *. The contaminations constitute about 5 % of the total sample.
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