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

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ABSTRACT: The [FeFe] hydrogenase HydA1 from Chlamydomonas reinhardtii has been studied using 1H NMR spectroscopy identifying the paramagnetically shifted 1H resonances associated with both the [Fe4S4]H and the [2Fe2]H subclusters of the active site “H-cluster”. The signal pattern of the unmaturated HydA1 containing only [Fe4S4]H is reminiscent of bacterial-type ferredoxins. The spectra of matured HydA1, with a complete H-cluster in the active Hox and the CO-inhibited Hox−CO state, reveal additional upfield and downfield shifted 1H resonances originating from the four methylene protons of the azadithiolate ligand in the [2Fe2]H subsite. The two axial protons are affected by positive spin density, while the two equatorial protons experience negative spin density. These protons can be used as important probes sensing the effects of ligand-binding to the catalytic site of the H-cluster.

Hydrogenases are metalloenzymes that catalyze the reversible conversion of dihydrogen into protons and electrons with the class of [FeFe] hydrogenases being the most active hydrogen producers.† The unique [Fe3] active site of these enzymes, the so-called “H-cluster”, serves as inspiration for the development of inorganic catalysts for production of solar fuels or as part of fuel cells. The H-cluster consists of a [Fe4S4]H cluster connected to the protein via four cysteines, one of which bridges to a unique binuclear Fe subsite [2Fe2]H containing a proximal (Feox) and a distal iron (Fed) (Figure 1). In contrast to most [FeFe] hydrogenase, HydA1 from Chlamydomonas reinhardtii with a molecular weight of about 48 kDa contains no accessory iron sulfur clusters and is thus particularly well suited for spectroscopic investigations of structure and function of the H-cluster. Large quantities of fully active HydA1 can be prepared by the addition of the synthesized inorganic cofactor precursor [Fe2(adt)(CO)4(CN)2] (adt = azadithiolate) to recombinant HydA1 containing only the [Fe4S4]H cluster (apo-HydA1) (Figure 1).2,3 The active site and its highly conserved protein environment are suggested to act synergistically for efficient hydrogen evolution. The electronic structure of the different redox states of the H-cluster has been well characterized by.

Figure 1. Maturation of HydA1 containing only the [Fe4S4]H cluster with the synthetic precursor [Fe2(adt)(CO)4(CN)2] of the binuclear Fe subsite in fully functional HydA1. The image of the [Fe4S4]H cluster and the H-cluster are based on PDB entries 3LX4 and 3C8Y, respectively. The metal clusters and the bridging cysteine are shown as sticks with the following color coding; iron, orange; sulfur, yellow; carbon, cyan; oxygen, red; nitrogen, blue.

EPR and FTIR spectroscopy,4–9 Electronic coupling between the [Fe4S4]H and [2Fe2]H subclusters is of central importance to the electron flow during catalysis.10 The intimate contact between [Fe4S4]H and [2Fe2]H sites translates into magnetic exchange coupling, which has been demonstrated at low temperatures by Mössbauer and EPR/ENDOR spectroscopy.11,12 However, the spin density distribution over the H-cluster and the influence of the protein environment have never been studied in solution at room temperature. Here, the method of choice is solution NMR spectroscopy, which can reveal sign and magnitude of the spin density at each NMR active nucleus. Protons are the most sensitive ones, although other magnetic nuclei, e.g.,13C and 15N, could also be studied easily by NMR techniques.13 For hydrogenases, protons are of particular importance since they are substrate and product of the reversible enzymatic reaction. In principle, NMR allows one in a unique way to directly follow the hydrogen species during the catalytic cycle under physiological conditions.

Here, we present the first NMR spectroscopic investigation of a hydrogenase enzyme, the [FeFe] hydrogenase HydA1. Similar to other iron sulfur proteins, magnetic coupling among iron centers reduces the NMR line widths and renders the spectroscopic investigation feasible.14 The β-CH2 protons of four cysteine coordinating [Fe4S4]H as well as the protons within the [2Fe2]H site are contact shifted out of the diamagnetic envelope (-1 to 11 ppm). Size and sign of the

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contact shift depend on (i) the spin state of the Fe in the cluster to which the cysteine is attached, (ii) the spin density at the nucleus, which largely depends on the Fe-S-Cβ−βCH2 dihedral angle θ, and (iii) temperature. Although the hyperfine shifted signals are significantly broadened due to the interaction of the unpaired electron(s) with the resonating nucleus, they provide a distinctive fingerprint of the cluster environment and protons inherent in the H-cluster. To our knowledge, the only other iron sulfur proteins of high molecular weight studied by NMR are the homodimeric nitrogenase Fe-protein and the hemeprotein subunit of sulfi reductase. The results presented here provide unique insight into structure and function of [FeFe] hydrogenases in solution at room temperature including an exclusive view of the catalytically active Hox state. These data open new prospects to unravel intimate details about geometric and electronic structure of the H-cluster and the influence of the surrounding amino acids.

The amenability of HydA1 to a high-resolution NMR study is demonstrated on oxidized apo-HydA1. The measured 1H NMR spectra reveal three contact shifted resonances downfield of 11 ppm with line width up to 300 Hz (Figure 2a and Table S2). Their pattern resembles bacterial-type ferredoxins in the oxidized [4Fe-4S]2+ form, which can be viewed as two antiferromagnetically coupled Fe(II)Fe(III) pairs that form a diamagnetic ground state with a total spin state $S = 0$. Paramagnetism arises at room temperature due to population of low-lying excited states with $S = 1, 2$, etc. Consistent with an oxidized [4Fe-4S]H2+, all contact shifted resonances exhibit anti-Curie temperature dependence (Table S2 and Figure S1a). Reduction of the [4Fe-4S]H2+ cluster to the [4Fe-4S]H+ form is accompanied by about four-fold increased contact shifts and line widths, which is in agreement with a paramagnetic $S = 1/2$ ground state (Figure 2b). For reduced apo-HydA1, the downfield shifted resonances A and D exhibit Curie, whereas B and C show anti-Curie temperature dependence (Figure S1b). Based on their chemical shifts and line widths, signals A to D belong most likely to $\beta$-CH2 protons of cysteinyl ligands. Signal E was assigned as a cysteine $\alpha$-CH proton as its line width is smaller when compared to signals A to D (Figure 2b and Table S2).

Maturation of apo-HydA1 with [Fe2(adt)(CO)4(CN)2] yields HydA1 with a fully functional H-cluster (see Supporting Information S11). In this [6Fe] system, spin coupling is in effect. For $H_{ox}$ and CO-inhibited $H_{ox}$ state, the H-cluster contains the cubane in the oxidized 2+ state. According to a theoretical model, [4Fe-4S]H2+ is composed of two valence-delocalized Fe pairs, [2Fe]$_A$ and [2Fe]$_B$, which are antiferromagnetically coupled to each other via the strong intracluster coupling $J_{\text{cube}}$ ($\approx 200$ cm$^{-1}$). In addition, [4Fe-4S]H+ is coupled through [2Fe]$_B$ to [2Fe]$_A$ in the $[\text{Fe}^4\text{Fe}^4]$
redox configuration via the intercluster exchange coupling \( j \) (Figure 3).

![Figure 3. Schematic representation of the active site H-cluster of [FeFe] hydrogenases in the \( H_{\text{ox}} \) state.](image)

This coupling, previously investigated by ENDOR and Mössbauer spectroscopy, has been found to be about 25 cm\(^{-1}\) for \( H_{\text{ox}} \) and 95 cm\(^{-1}\) for \( H_{\text{ox}}-\text{CO} \).\(^{11,13,22}\) The 4-fold increased \( j \) in the \( H_{\text{ox}}-\text{CO} \) state causes the spin density to be strongly localized on \( Fe_{\text{ox}} \) while it is more evenly distributed over \( Fe_{\text{ox}} \) and \( Fe_{\text{red}} \) in the \( H_{\text{ox}} \) state.\(^{11}\) Nevertheless, both \( j \) values are small compared to \( J_{\text{cube}} \) \( J_{\text{cube}} \) leads to an orientation of \( S_A \) antiparallel to \( S_B \) and \( S_B \) antiparallel to \( S_C \). Hence, \( S_{\text{Hox}} \) is oriented parallel to \( S_A \) (Figure 3). Magnitude and sign of the spin density are reflected by the observed proton hyperfine shifts that depend mainly on the \( Fe-S-C-H \) dihedral angle \( \theta \). The angular dependence of the chemical shift \( \Delta \) follows the general Karplus relationship \( \Delta = a \cos^2 \theta + b \cos \theta + c \) with \( b \) and \( c \) being small and often neglected.\(^{15}\) As the remaining angular term \( \cos^2 \theta \) is always positive, solely the sign of the spin density on the coordinated \( Fe \) determines the direction of the paramagnetic shift.

After preparation of HydA1 in the \( H_{\text{ox}} \) state (see Supporting Information S1 and Figure S4), six downfield shifted resonances are observed in the \( ^1H \) NMR spectrum between 11 and 33 ppm and also two upfield shifted resonances at −10 and −21 ppm (Figure 2c). The downfield shifted signals \( a, b, 1, \) and \( 2 \) exhibit Curie and signals \( c \) to \( f \) anti-Curie temperature dependence (Table S2 and Figure S2). The two upfield shifted resonances show pseudo-Curie temperature dependence. No hyperfine-shifted signals were detected at positions observed in the spectrum for apo-HydA1. This demonstrates the influence of the \([2Fe]_B\) on the \([4Fe-4S]_{\text{Hox}}\) cluster via exchange coupling in solution. In order to distinguish the methylene proton resonances originating from the cysteines coordinating \([4Fe-4S]_{\text{Hox}}\) from those of \( adt \), \( H_{\text{ox}} \) was also prepared using a deuterated \([2Fe]_{\text{Hox}}\) site (\( ^3\text{H}-\text{adt} \)). Thus, the downfield shifted signals \( 1 \) and \( 2 \) and the upfield shifted signals \( 3 \) and \( 4 \) (Figures 2c) have been unambiguously assigned to the four methylene protons of \([2Fe]_{\text{Hox}}\) (Figure S5). They can be attributed to two pairs of geometrically and electronically similar protons. Based on their distances to \( Fe_a \) and \( Fe_b \) line widths, and observed \( ^1H \) NOE connectivities, signals 1 and 2 are assigned to the axial and signals 3 and 4 to the equatorial protons (Figure 1 and Table S2). Further details are provided in the Supporting Information (S14, Figure S6 and Table S1). By flushing active HydA1 with \( CO \), pure \( H_{\text{ox}}-\text{CO} \) state is prepared (see Supporting Information S1 and Figure S7). This \( CO \)-inhibited state is an important source of information reporting about the redistribution of spin density in the H-cluster upon binding of an electron donating external ligand to the open coordination site at \( Fe_{\text{Cox}} \). In its \( ^1H \) NMR spectrum, seven downfield and four upfield shifted resonances are observed in the range 11 to 85 ppm and −2 to −30 ppm, respectively (Figure 2d). The downfield shifted signals \( A, 1, 2, B, C, \) and \( D \) show Curie and \( E \) weak anti-Curie temperature dependence. As for the \( H_{\text{ox}} \) state, all upfield shifted resonances show pseudo Curie temperature dependence (Figure S4). In agreement with an increased \( j \) due to coordination of the external \( CO \) ligand at \( Fe_{\text{Cox}} \) \( ^1H \) resonances were broader (as much as 4 kHz) and more dispersed than those for all other HydA1 states investigated here (Figure 2 and Table S2). In contrast, the line widths of the contact shifted proton signals of \( H_{\text{ox}} \) are \( \sim 300 \) Hz similar to oxidized apo-HydA1. For the \( H_{\text{ox}}-\text{CO} \) state, signals 1 to 4 have been assigned analogous to the \( H_{\text{ox}} \) state to axial and equatorial protons (Figure S8). Although the temperature dependence of signals 1 to 4 is weak in the \( H_{\text{ox}}-\text{CO} \) state, their temperature-dependence in the \( H_{\text{ox}} \) state is the strongest of all observed hyperfine shifted resonances in that state (Figures S2 and S3). This large temperature dependence of the adt methylene protons in the \( H_{\text{ox}} \) state indicates that the energies of the populated excited states of \([2Fe]_{\text{Hox}}\) are closer than those of \([4Fe-4S]_{\text{Hox}}\) and agrees well with \( j \), determined to be small for this state. One possible explanation for the relatively small temperature dependence of the adt methylene protons in the \( H_{\text{ox}}-\text{CO} \) state is that binding of the external \( CO \) ligand increases not only \( j \) but also the energies of the levels populated at room temperature. The assignment of the methylene protons of the adt bridge provides insight into the spin density at four additional positions of the \([2Fe]_{\text{Hox}}\) site. Large negative hyperfine shifts are observed for signals 3 and 4 in the \( H_{\text{ox}} \) and large positive hyperfine shifts are detected for signals 1 and 2 in the \( H_{\text{ox}}-\text{CO} \) state (Figure 2c). These hyperfine shifts reflect the larger \([2Fe]_{\text{Hox}}\) spin density for \( H_{\text{ox}} \) when compared to \( H_{\text{ox}}-\text{CO} \), resulting from the different ratio of \( j \) and \( J_{\text{cube}} \). The NMR spectra observed for the \( H_{\text{ox}} \) and \( H_{\text{ox}}-\text{CO} \) states can be interpreted based on the spin coupling model described above (Figure 3). Spin-polarization mechanisms will transmit positive and negative spin density to the \(^1H\) atoms of the cysteines coordinating \([2Fe]_{\alpha} \) and \([2Fe]_{\beta} \) respectively. Thus, ligation of \([2Fe]_{\alpha} \) results in downfield and ligation of \([2Fe]_{\beta} \) in upfield shifted \(^1H\) resonances. However, the bridging cysteine experiences not only positive spin density from \([2Fe]_{\beta} \) but also negative spin density from \([2Fe]_{\alpha} \). Taking into account the larger spin density at \([4Fe-4S]_{\text{Hox}} \) and the lower spin density at \([2Fe]_{\alpha} \) in the \( H_{\text{ox}}-\text{CO} \) as compared to the \( H_{\text{ox}} \) state, a net upfield shift is expected for the \( \beta-\text{CH}_2 \) protons of the bridging cysteine. Accordingly, peaks \( F, G, \) and \( H \) can be assigned to the \( \beta-\text{CH}_2 \) protons of the bridging and nonbridging cysteine coordinating \([2Fe]_{\alpha} \) (Figure 2d). For the \( H_{\text{ox}} \) state, only two upfield shifted resonances are observed because of the smaller spin density at \([4Fe-4S]_{\text{Hox}} \) resulting from \( j \), which is four-fold smaller than for the \( H_{\text{ox}}-\text{CO} \) state (Figure 2c).
We show here that paramagnetic NMR can be applied to the important class of [FeFe] hydrogenases. The derived assignments of the axial and equatorial protons of the unique [2Fe]₄H complex interface as well as dynamics related to complex formation as recently reported for cytochrome P450 and b₅. Furthermore, NMR spectroscopy allows for the investigation of HydA1 states with a diamagnetic ground state like Hred and HoxH⁺ that are EPR silent. Most importantly, the terminal hydride intermediate, which already has been discussed based on FTIR, Mössbauer, and NRVS spectroscopy, can be accessed directly at ambient temperatures using solution NMR spectroscopy.

**REFERENCES**


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