[FeFe] Hydrogenase Mechanism

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# Identification and Characterization of the "Super-Reduced" State of the H-Cluster in [FeFe] Hydrogenase: A New Building Block for the Catalytic Cycle?**

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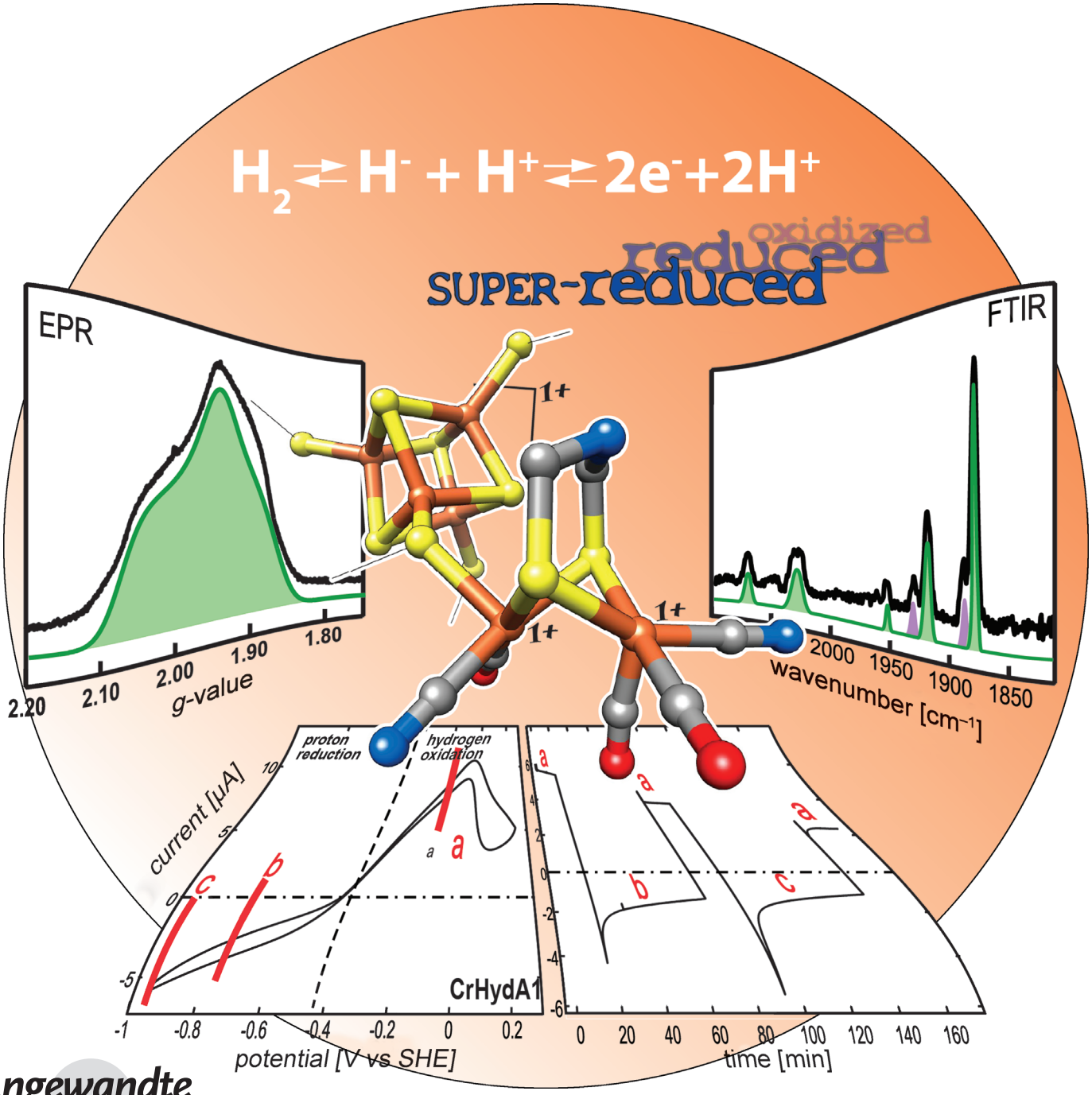

Hydrogenases catalyze the reversible heterolytic formation of $H_2$ from protons and electrons. According to the type of metals in the active site, these enzymes are classified into three groups: [NiFe], [FeFe], and [Fe] hydrogenases.[1,2] All of these enzymes are of high interest in biotechnology, aiming at the generation and conversion of $H_2$ as renewable energy carrier.[1,3] Since [FeFe] hydrogenases are highly efficient in hydrogen production in vivo, elucidation of their catalytic mechanism is of particular relevance for developing artificial hydrogen production systems.[4,5]

The active site of [FeFe] hydrogenase contains the so-called H-cluster (Figure 1) consisting of a di-iron center [2Fe], which is covalently attached via a cysteine thiol bridge to

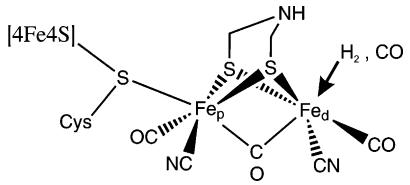


***Figure 1.*** Structure of the active site (H-cluster) in [FeFe] hydrogenases. The arrow points to the open coordination site on the distal iron atom ($Fe_d$).

a cubane-like [4Fe-4S] subcluster.[1,6–8] In the [2Fe] subcluster, both Fe ions are coordinated by CO and CN ligands, keeping the iron centers in low oxidation and spin states.[8–11] The iron atoms in the [2Fe] subcluster are bridged by an azadithiolate group (adt, $(SCH_2)_2NH$).[8,12] The iron distal to the [4Fe4S] subcluster ($Fe_d$) has an open coordination site, which is most likely the $H_2$ binding site (Figure 1).[1,13] It has been proposed that the adt-amine group, which is in a perfect position with respect to the open coordination site, is involved in the proton transfer to and from the catalytic site.[8,12]

Until now, two redox states of the H-cluster were identified that are believed to take part in the catalytic cycle: that is, the active "oxidized" state $H_{ox}$ which is paramagnetic and characterized by a mixed-valence ($Fe^IFe^{II}$) binuclear part, and the active "reduced" state $H_{red}$, which adopts the ($Fe^IFe^I$) configuration.[1]

These active forms can be inhibited by CO, resulting in a single oxidized state, $H_{ox}$-CO.[1,6,9] The $[4Fe4S]_H$ subcluster in these states is oxidized (+2) and formally diamagnetic ($S_{4Fe4S}=0$).[14,15] The redox transition $H_{ox}$ to $H_{red}$ has its midpoint potential around −400 mV (pH 8.0) and has been studied in the [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (*Dd*H) using FTIR spectroelectrochemistry.[11] The same study also identified a subsequent irreversible reduction step at $E_m=-540$ mV to the so-called "super-reduced state" $H_{sred}$. An analogous study of the [FeFe] hydrogenase of *Chlamydomonas reinhardtii* (*Cr*HydA1) showed the same redox transitions.[16] However, in *Cr*HydA1 the super-reduced species $H_{sred}$ occurs at a much higher potential $E_m=-460$ mV (pH 8.0) and the $H_{red}$ to $H_{sred}$ transition is fully reversible.[16] The redox potentials of both $H_{red}$ and $H_{sred}$ in *Cr*HydA1 are close to that of its (isolated) natural redox partner PetF (that is, −400 mV at pH 7.0).[17] Moreover, it has been shown that the PetF redox potential can be reduced substantially upon interaction with its redox partners, such as ferredoxin-$NADP^+$ reductase (FNR).[18] Therefore, in *Cr*HydA1, $H_{sred}$ seems to be a resting state of the enzyme, similar to $H_{ox}$ and $H_{red}$. There are two possibilities for the electronic structure of the H-cluster in the $H_{sred}$ state: either the binuclear part is further reduced to [$Fe^0$-$Fe^I$] or the $[4Fe\text{-}4S]^{2+}$ subcluster is reduced to $[4Fe\text{-}4S]^+$. Similar to $H_{ox}$, $H_{sred}$ is expected to be paramagnetic, exhibiting an $S=1/2$ ground state.

In this work, we aim to identify and characterize the $H_{sred}$ state from *Cr*HydA1 in detail using EPR and FTIR spectroscopy. To verify the possible catalytic role of the $H_{sred}$ state, we performed protein film electrochemistry (PFE) on *Cr*HydA1 as well as *Dd*H, and the [FeFe] hydrogenase from *Clostridium acetobutylicum* (*Ca*HydA).

*Cr*HydA1 overexpressed in *C. acetobutylicum* was subjected to different reductive and oxidative treatments. Figure 2 presents a selection of the corresponding FTIR- and FID-detected (Q-band) EPR spectra for each sample going from oxidative (A) to reductive conditions (D). The characteristic IR bands and *g* values for each H-cluster species are indicated at the top of the spectra. The FTIR frequencies were taken from the literature[16] and presented in more detail in the Supporting Information, Figure S1. It is clear that the as isolated samples with sodium dithionite (NaDT) in the buffer show a mixture of $H_{ox}$, $H_{red}$, and $H_{sred}$. Treatment with $H_2$ or with additional equivalents of NaDT shifts the equilibrium towards the reduced species, while thionine treatment induces the formation of oxidized species.[19]

The EPR spectra in Figure 2 clearly show the appearance of a broad contribution with low *g* values ($2.076\pm0.002$, $1.943\pm0.003$, $1.868\pm0.001$) in the reduced samples. The spectral parameters of this species resemble typical reduced [4Fe-4S] cluster signals.[20] The contribution of $H_{ox}$ and $H_{ox}$-CO to each EPR spectrum was also evaluated using spectral simulations as indicated in Figure 2. As it is evident from the comparison of the EPR and FTIR data, the increase of the [4Fe4S]-like EPR signal coincides with the appearance of the $H_{sred}$ signal in the FTIR measurements. We therefore assign this EPR signal to the $H_{sred}$ state. This shows that $H_{sred}$ is characterized by a $[Fe^IFe^I][4Fe4S]^+$ configuration rather than for example, $[Fe^IFe^0][4Fe4S]^{2+}$. According to the FTIR and EPR spectra, most preparations contain a mixture of the

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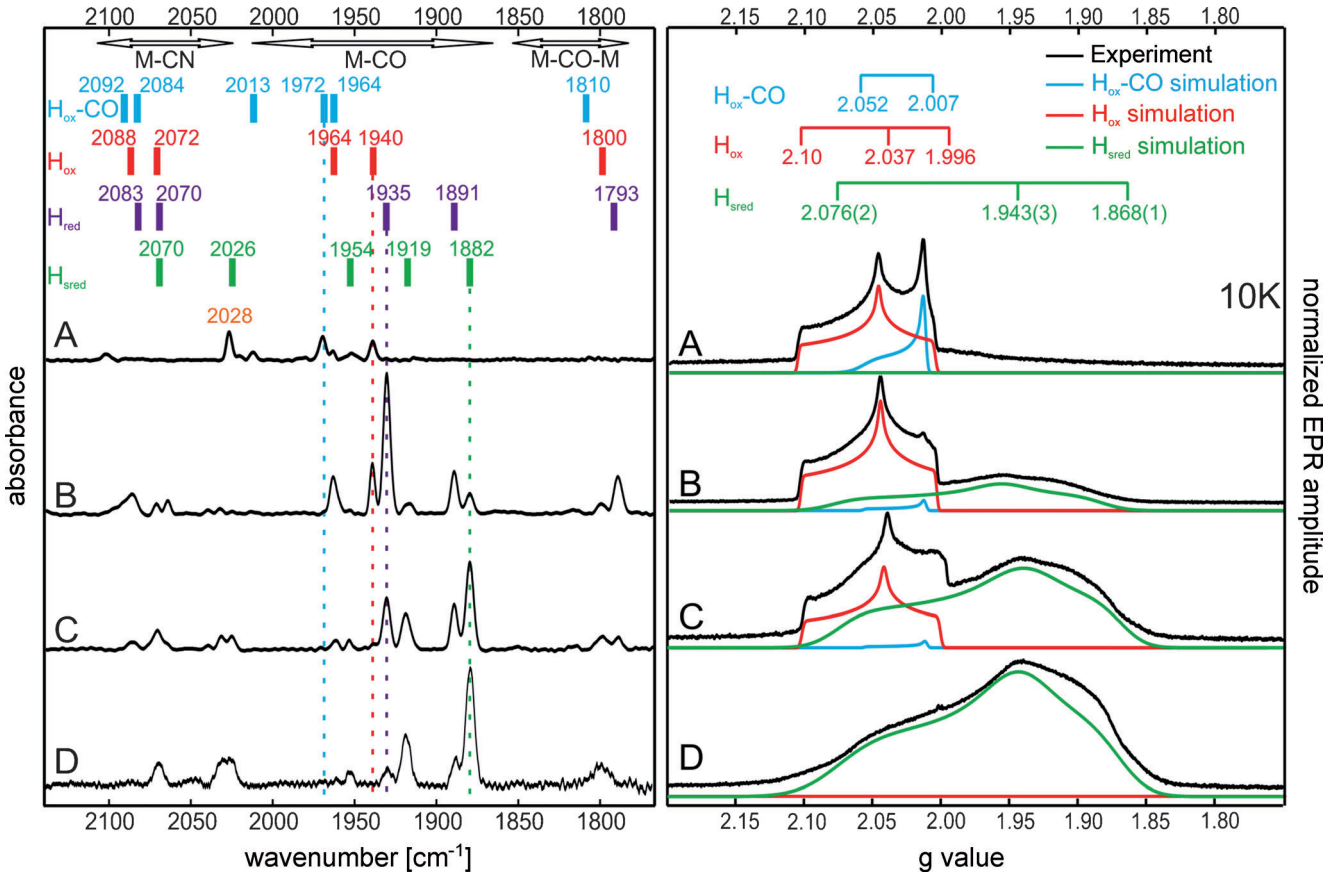


***Figure 2.*** Left: FTIR spectra normalized to 500 μM enzyme concentration and recorded at 100 K. Right: Normalized Q-band FID-detected EPR spectra measured at 10 K. Sample compositions: A) 700 μM *Cr*HydA1 and 1.5 mM thionine; B) 700 μM *Cr*HydA1; C) 700 μM *Cr*HydA1, 12 mM Na-dithionite; D) 280 μM *Cr*HydA1, flushed with 1 bar $H_2$. All samples initially contained 2 mM NaDT to avoid oxygen damage to the protein. Information about FTIR and EPR signal positions for each redox state is presented at the top of the figure. Further details of sample preparation are collected in the Supporting Information.

three states, $H_{ox}$, $H_{red}$, and in particular $H_{sred}$. Our experiments show that even samples that are predominantly in the $H_{sred}$ state could be completely oxidized with thionine. This shows that all redox states are in equilibrium with each other. Importantly, all samples, including those with high concentrations of $H_{sred}$, show a high hydrogenase activity (typically 560 nmol $H_2$ per minute and μg protein), suggesting that $H_{sred}$ is an active state.

To confirm the catalytic activity of the enzyme at the low potentials at which $H_{sred}$ is formed, protein film electrochemistry (PFE) was performed on *Cr*HydA1, which was adsorbed on a carbon rotating-disk electrode.[21] For comparison, the same electrochemical experiments were performed for *Dd*H and *Ca*HydA, which were covalently attached to the rotating-disk electrode (see the Experimental Section and the Supporting Information). The cyclic voltammograms of the three studied enzymes (Figure 3, top) show that $H^+$ reduction activity increases with negative potential. No inactivation processes are observed at very low potentials during the experiments. The persistence and stability of the observed catalytic currents was verified using chronoamperometry (Figure 3, lower part). In these experiments, the potential is kept constant initially at the reference potential of −209 mV at which anaerobic oxidation is not dominant and a steady $H_2$ oxidation current is observed. Subsequently, two negative potentials (−960 and −760 mV) were applied for one hour each. While the experiments on *Dd*H and *Ca*HydA showed persistent negative $H^+$ reduction catalytic currents at each potential step, the experiment on *Cr*HydA1 showed some current loss, which we assign to film loss owing to the noncovalent immobilization of the enzyme. As shown above, reductive treatment favors formation of the $H_{sred}$ state. If this would be a dead-end state, these conditions would allow accumulation of this state and we should see a substantial reduction of the catalytic current when applying more negative potentials. As this is clearly not the case, these experiments indicate that $H_{sred}$ is a part of the catalytic cycle.

$H_{sred}$ seems to be better stabilized in algal [FeFe] hydrogenases than for example in *Dd*H. It is tempting to infer that this must be related to the lack of accessory [4Fe-4S] clusters in these enzymes (the so-called "F-clusters"). It seems likely that the redox potential of the $H_{sred}/H_{red}$ transition is changing depending on the presence of the F-clusters owing to the so-called redox cooperativity effect commonly observed in systems with multiple redox centers.[22] In prokaryotic [FeFe] hydrogenases, $H_{sred}$ may only occur as transient species, as the cubane part is quickly reoxidized during the reaction cycle by the F-clusters, thus generating the $H_{red}$ state. Therefore, in these enzymes under turnover conditions the

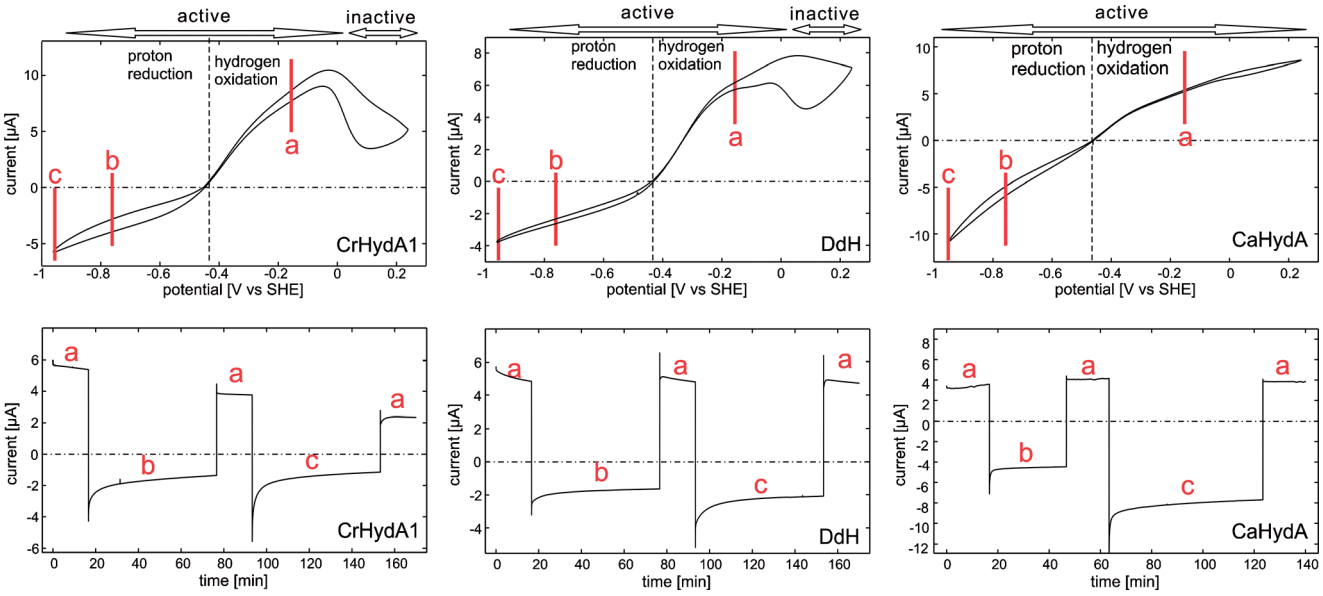


***Figure 3.*** From left to right: cyclic voltammetry (upper part) and chronoamperometric experiments (lower part) on *Cr*HydA1 adsorbed on and *Dd*H and *Ca*HydA covalently attached to a pyrolytic graphite edge (PGE) electrode. Perpendicular markings and letters on the cyclic voltammograms indicate the potentials used in the chronoamperometric measurements ($a = -209$ mV, $b = -759$ mV, $c = -959$ mV). The experiments were carried out at pH 7.0, 10 °C, 1 bar $H_2$ and 2500 rpm electrode rotation rate.

low potential resting state is $H_{red}$, while in eukaryotic enzymes (lacking the F-clusters), $H_{sred}$ accumulates as the resting state.

We will now consider the possible role of the $H_{sred}$ state in the catalytic cycle. The H-cluster in *Cr*HydA1 has to perform a turnover involving two protons and two electrons in the absence of accessory [4Fe4S] clusters. The hydrogen oxidation cycle, which presumably starts with $H_{ox}$ (as "ready state") will result in an exit state where two electrons (reduction equivalents) are left on the H-cluster. As a stable $Fe^0Fe^I$ configuration has never been observed for the H-cluster, the most likely candidate for such "exit" state is $H_{sred}$. It is therefore not surprising that particularly large amounts of $H_{sred}$ are formed in HydA1 under $H_2$ without a redox partner (electron acceptor) being present in the buffer. $H_{sred}$ is, however, also formed under reductive conditions without $H_2$. Under these conditions, high turnover activities ($H_2$ production) are measured in vitro. Our PFE experiments also show high catalytic activity at negative potentials at which $H_{sred}$ is shown to be present. Thus, it seems very likely that the $H_{sred}$ state is a part of the catalytic cycle. Moreover, as the structure of the H-cluster is highly conserved in all [FeFe] hydrogenases and the same behavior at negative potentials was found for *Cr*HydA1, *Dd*H, and *Ca*HydA hydrogenases in our PFE experiments, it can be assumed that its catalytic mechanism also is conserved. We therefore propose that $H_{sred}$ is the starting point for proton reduction in the reaction cycle of all [FeFe] hydrogenases.

The implication of these considerations is reflected in Figure 4, which shows our proposed reaction cycle for *Cr*HydA1, including $H_{sred}$. Although certainly some speculation is involved, most of these intermediates were postulated earlier; in particular, the hydride form of $H_{sred}$ has already been discussed.[10] It is clear that a few questions are still left to be answered: We propose $H_{sred}$ to be the active species reacting with the bound proton to form the (terminal) hydride, which in turn reacts with the proton available at the ADT amine, ultimately producing molecular hydrogen. It is not clear at which stage this "hydride-to-be" proton is bound to the H-cluster. Interestingly, all DFT-calculated protonated or unprotonated variants of the $H_{sred}$ species are characterized by a reduced [4Fe4S] cluster.[23] According to these DFT calculations, only the second protonation (in the ADT bridge) triggers the electron transfer from the $[4Fe4S]_H$ cluster to the 2Fe subcluster and the formation of the hydride bound to the mixed-valence 2Fe core.[24,25] How the first proton is stabilized at the reduced states of the H-cluster ($H_{red}$ and/or $H_{sred}$) is still not clear. Experimental evidence for the existence of hydrides in $H_{sred}$ and/or $H_{red}$ as well as detailed QM calculations of the reaction mechanism including the role of the cubane subcluster might help to solve these open questions.

In conclusion, our combined electrochemical, FTIR, and EPR investigations on HydA1 identified the superreduced state $H_{sred}$ as paramagnetic and catalytically active. We therefore propose that $H_{sred}$ is involved in the reaction cycle of all [FeFe] hydrogenases, but it is stabilized as resting state only in algal enzymes owing to the lack of F-clusters, which immediately reoxidize $H_{sred}$ to $H_{red}$ in bacterial enzymes. The reaction cycles of prokaryotic and eukaryotic enzymes are identical but because of the presence or absence of F-clusters in the protein, different resting states of the active site are stabilized at low potential.

The involvement of $H_{sred}$ in the catalytic cycle is mechanistically attractive because it enables heterolytic splitting of $H_2$ bound to $H_{ox}$ without the need for any intermediate electron-transfer step.[10] Also it would explain why the cubane sub-cluster is an essential part of the active site of [FeFe] hydrogenases in contrast to [NiFe] hydrogenase, where such a redox-active "ligand" to the binuclear site is not required.

  

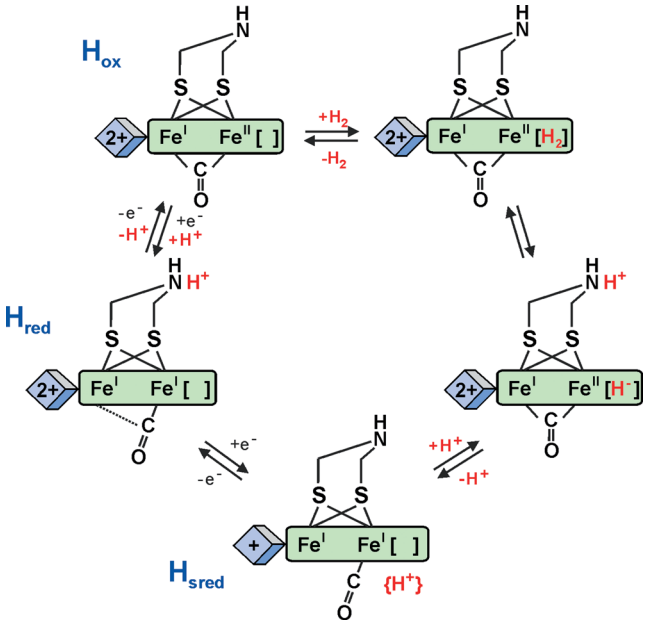


***Figure 4.*** Proposed catalytic cycle for [FeFe] hydrogenase including the $H_{sred}$ state. $\{H^+\}$ in $H_{sred}$ indicates that the proton is believed to be associated with the H-cluster and possibly bound to a nearby amino acid residue.

## *Experimental Section*

*Cr*HydA1 was obtained from *Clostridium acetobutylicum* (overexpressed) as reported previously.[26,27] All sample preparation steps, including treatment with thionine and exposure to $H_2$, were performed under strictly anaerobic conditions in a glovebox under a nitrogen atmosphere with 1.5–2% $H_2$. FTIR measurements were performed on a BRUKER IFS 66 v/s FTIR spectrometer equipped with a Bruker MCT (mercury cadmium telluride) detector. Q-band EPR spectra were recorded using free induction decay (FID)-detected EPR with a 1 µs microwave pulse. All pulse experiments were performed on a Bruker ELEXYS E580 Q-band spectrometer with a SuperQ-FT microwave bridge and home-built resonator described earlier.[28] Protein-film electrochemistry experiments were carried out inside an anaerobic glovebox (MBraun) using a gas-tight three-electrode setup connected to an electrode rotator (Princeton Applied Research model 636A). Electrochemical experiments were controlled by a VersaStat 4 potentiostat (Princeton Applied Research). Detailed experimental procedures are described in the Supporting Information.

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