The missing intermediate in the catalytic cycle of [FeFe]-hydrogenases: Diiron site reduced state featuring a bridging CO ligand observed at room temperature

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Abstract

The metalloenzymes hydrogenases are natures redox catalyst for molecular hydrogen (H_2) uptake and production. In [FeFe]-hydrogenases catalysis is facilitated at a unique diiron site. The two iron ions are connected by an azadithiolate bridging ligand ($^-SCH_2NHCH_2S^-$, ADT) and coordinated by a CO and CN^- ligand each. Upon reduction of this diiron site the ligand arrangement of a third CO ligand is heavily debated. Two main geometries that differ by the origin of a bridging ligand in between the iron ions and the occupation of a free binding site are discussed. In a first scenario a hydride is bound in the bridging position and the third CO ligand occupies the open coordination site resulting in a geometry not favourable for catalysis. Experimental data indicative of the latter case has been collected at room temperature. In a second scenario the third CO ligand occupies a position in between the two iron ions resulting in a free coordination site favoured for H_2 catalysis. Here up to date, experimental data indicative of this geometry, in particular the infrared band of the bridging CO (μCO) ligand, has only been observed at cryogenic temperatures questioning its catalytic relevance. In this study, we characterise the diiron site reduced intermediate that features the μCO geometry and preserves the open coordination site via FTIR spectroscopy at room temperature. The observation of this proposed diiron site reduced reaction intermediate with μCO geometry at room temperature makes its involvement in H_2 catalysis more likely.

Introduction

Hydrogenases are reversible and bidirectional redox enzymes catalysing H_2 production and uptake.[1-3] [FeFe]-hydrogenases with their high turnover frequencies are regarded as the biological blueprint for future catalysts leading to a hydrogen economy.[4-5] Their active site cofactor, the H-cluster, is composed of a [4Fe4S] cluster linked via the thiol of a cysteine residue to a unique diiron site ([2Fe]). The two iron ions are coordinated by a CO and CN^- ligand each, and share an azadithiolate bridging ligand ($^-SCH_2NHCH_2S^-$, ADT) and a CO ligand in the bridging position. Hydrogen catalysis occurs at the [2Fe] open coordination site. [FeFe]-hydrogenases have been investigated by e.g. NMR, Mössbauer, EPR and FTIR spectroscopy.[6-11] The latter exploits the CO/CN^- ligands as intrinsic probes directly at the active site, reporting on; protonation; redox state; and cofactor geometry. In particular, the bridging CO ligand can be observed in its characteristic low wavenumber region of the spectrum.[1, 12-13] Based on spectroscopy several redox intermediates of the H-cluster have been characterised around which different catalytic cycles have been put forward.[13-16] Agreement exists on the starting point, the oxidised state (Hox) that features an oxidised [2Fe] subsite (i.e. $Fe^{I}Fe^{II}$) and [4Fe4S] cluster ($[4Fe4S]^{2+}$).[17] Two singly reduced states are reported that differ by the site of reduction.[18-21] Reduction occurs either at the [4Fe4S] cluster (Hred') or at the [2Fe] site (Hred/$HredH^+$). The H-cluster in Hred' retains the

bridging CO ligand (μCO) and its [2Fe] geometry is regarded as conserved compared to Hox. The geometry of the [2Fe] reduced state is debated.[13, 16, 22-23] Further reduction leads to the super-reduced state, Hsred, that is besides the [2Fe] site additionally reduced at the [4Fe4S] cluster.[24] The second formally two electron reduced state is the hydride state (Hhyd), a tautomer of Hsred featuring a terminal hydride on the [2Fe] subsite and proposed as one of the last intermediates in hydrogen turnover. [8-10, 25] The main disagreement is the nature of the [2Fe] site bridging ligand for the [2Fe] reduced state that is proposed either as μCO[11, 26-27] (semi-bridging CO[28-29]) or μH^- geometry. [14, 20, 22, 30] In the μCO geometry the apical coordination site remains open and the ADT amine is proposed to be protonated. In μH^- geometry the former bridging CO ligand rotates into terminal position (tCO), occupying the apical coordination site, and a hydride is bound in bridging position between the two irons. Depending on the [2Fe] geometry the infrared band pattern features either a characteristic μCO band (black spectrum Fig.1) at low wavenumbers or in case of μH^- geometry a terminal CO band (red spectrum Fig.1) at high wavenumbers. Interestingly, the bands of the remaining CO and CN^- ligands seem to be negligibly affected by switching in between these two configurations (Fig.1).[23]

To end the confusion in [2Fe] reduced state nomenclature of [FeFe]-hydrogenases we propose to use the Hred nomenclature exclusively for the state showing a tCO ligand, and the $HredH^+$ nomenclature exclusively for state featuring a μCO band. Figure 1 summarises the nomenclature, respective band patterns, and proposed [2Fe] geometry for the two different [2Fe] site reduced states, Hred and $HredH^+$.

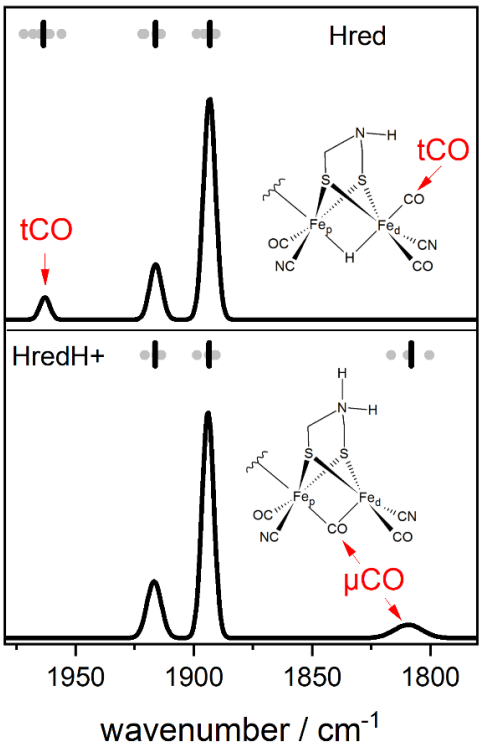

Figure 1 Diiron site reduced state geometry and simulated spectra of Hred and HredH+. The band positions of the simulated Hred (top) and HredH+ (bottom) spectra were obtained as the mean values (black bars) of 15 different band patterns for Hred (grey dots) [10-12, 18-20, 22, 28, 31-36] and 4 different band patterns for HredH+ (observed at cryogenic temperatures, grey dots). [11, 26-27] The individual band patterns and host organisms can be found in Table S1. The Hred spectrum (top) highlights the tCO band at 1963 cm^{-1} (indicated red) while the HredH+ spectrum (bottom) exhibits a μCO band at 1809 cm^{-1} (indicated red). Note that the mean band positions of the remaining CO and CN^- ligands vary only by 1 cm^{-1} between Hred and HredH+.

The tCO band for the [2Fe] reduced state has been observed in at least 15 studies and in 6 different [FeFe]-hydrogenases[10-12, 18-20, 22, 28, 31-36] (Fig.1) while at room temperature the only isolated and incontrovertible μCO band was observed for the putative sensory [FeFe]-hydrogenase of *Thermotoga maritima* (TmHydS).[37] Only at cryogenic temperatures have μCO bands attributable to [2Fe] site reduced state HredH+ (HredLT) been observed in prototypical [FeFe] hydrogenases from *Chlamydomonas reinhardtii* (HydA1), *Desulfovibrio desulfuricans* (DdH) and *Clostridium acetobutylicum* (CaI) (Fig.1). [11, 26-27] At elevated temperatures the μCO band has so far escaped detection, and the μCO geometry has been proposed to convert back into μH^- geometry.[23]
All currently discussed catalytic cycles favour a conserved cofactor geometry and redox states, such as Hred with μH^- geometry, which would force ligands to rotate multiple times during catalysis, are in contradiction to fast turnover.[38-39] The [2Fe] reduced state with conserved geometry (μCO) is often postulated as one of the missing intermediates in H_2 catalysis of [FeFe]-hydrogenase.[1, 13, 16] Accordingly, for more than two decades intense research efforts have aimed for characterisation of a reduced [2Fe] subsite state with conserved cofactor geometry at ambient conditions, more specifically the detection of their μCO band.[11, 18-19, 22, 24, 26-28, 30, 40-41]
Here we show the accumulation of a [2Fe] reduced state featuring a μCO ligand band [FeFe] hydrogenases from *Chlamydomonas reinhardtii* (HydA1) at room temperature. ATR-FTIR difference spectroscopy allows us to correlate the population of the μCO band and the remaining CO/CN^- ligand bands of the [2Fe] reduced state further strengthening this assignment. Changes of carboxylic acid residues in the enzymatic proton transfer pathway indicate that a rearrangement of the hydrogen bonding network is associated with population of $HredH^+$.

Results and discussion

Absolute ATR-FTIR spectra display a bridging CO band for [2Fe] reduced state ($HredH^+$) at room temperature. A HydA1 enzyme film (1 μl, 1 mM) was deposited on the surface of the ATR crystal, dried and rehydrated as reported earlier, and in the following exposed to a humidified stream of different gases at room temperature.[42-43] Under a N_2 aerosol HydA1 auto oxidises[19, 42] and the observed redox state composition consists of mainly Hox with small contributions of the CO inhibited state Hox-CO and [2Fe] reduced state (Fig.2 top). Only the bands at 1915 and 1891 cm^{-1} are detected for the [2Fe] reduced state precluding a discrimination of Hred/$HredH^+$. Reduction via H_2 gas shifts the redox state equilibrium to mainly Hred/$HredH^+$ with contributions of Hhyd and Hox (Fig.2 bottom). An unknown band at 1823 cm^{-1} is detected in the region of bridging iron carbonyls (1870-1750 cm^{-1}) in [FeFe]-hydrogenases while all other bands observed have been assigned to individual redox states erlier.[10, 12, 40] At cryogenic temperatures, a μCO band at 1817 cm^{-1} has been assigned to HredLT in HydA1 and for the cryogenic super reduced state (HsredLT) an upshift of the μCO band position by 4 cm^{-1} was reported when the temperature was elevated from 40 to 200 K.[26-27] Combining this information it is tempting to assign the new band as a bridging CO ligand present in [2Fe] reduced state at room temperature. Comparing the spectrum to the two simulated spectra in Figure 1, the new μCO band at 1823 cm^{-1} indicates μCO geometry and in the following the [2Fe] site reduced state observed in this study will be referred to as $HredH^+$.

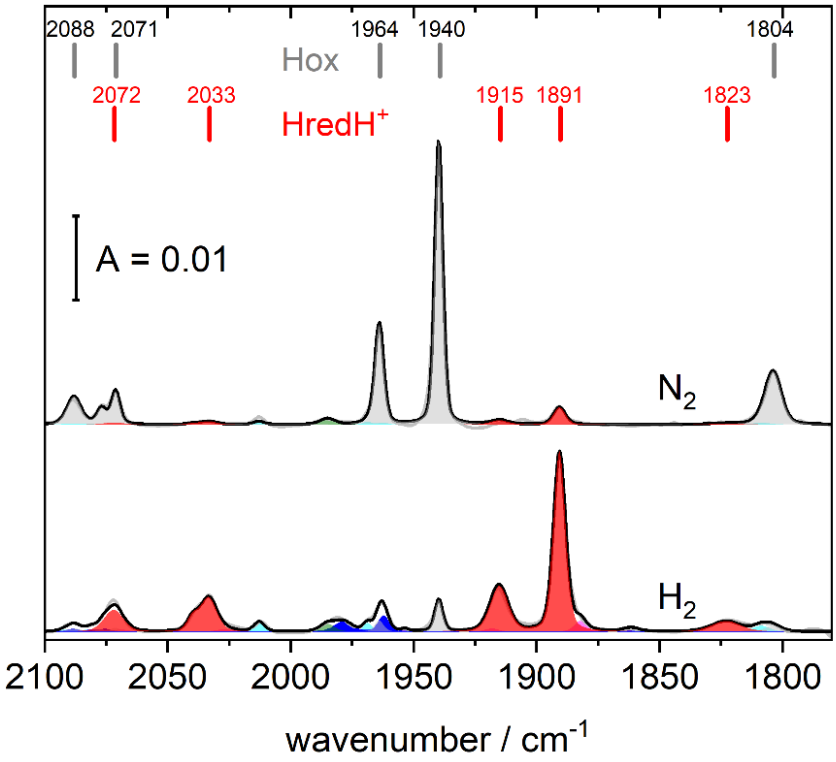

Figure 2 Absolute ATR-FTIR spectra of [FeFe]-hydrogenase HydA1 under N_2 and H_2 atmosphere at room temperature. (top) Mainly Hox (grey bands) is enriched under auto-oxidising conditions (N_2). Traces of the CO inhibited state Hox-CO (cyan) and bands of the [2Fe] reduced state (red, 1915 and 1891 cm^{-1}) are observed. (bottom) Under H_2 atmosphere the [2Fe] reduced state (red bands) gets populated while the Hox population gets diminished. In the µCO wavenumber region a clear band at 1823 cm^{-1} is detected and allows to assign $HredH^+$ as the [2Fe] reduced state. Contributions of Hox-CO (cyan), Hhyd (blue bands) and Hsred (magenta band) are detected. The green band at 1985 cm^{-1} present in both spectra most likely reports on degraded cofactor. Band positions for Hox and $HredH^+$ are indicated by grey and red bars respectively.

Difference ATR-FTIR spectra correlate µCO band to $HredH^+$. We further analysed the new band µCO band (1823 cm^{-1}) during the reaction of HydA1 with H_2 gas by difference spectroscopy (Fig.3). The spectra reveal differences in intensity in the wavenumber region of the cofactor CN^- ligands (2100-2020 cm^{-1}), CO ligands (2020-1870 cm^{-1}) and bridging CO ligands bands (1870-1750 cm^{-1}). Small differences are detected also in the band region of carboxylic acids of amino acid residues (1750-1660 cm^{-1}).

The differences in the cofactor CO/CN^- ligand region report on the reduction of the H-cluster (Fig.3A). Bands assigned to Hox get depopulated (negative bands) in favour of bands associated with $HredH^+$ and Hhyd (positive bands). The collective behaviour of the five cofactor ligand bands (2CN, 3CO) for each redox state allows to define relative redox state populations in each spectrum.[44-45] Figure 3B displays the obtained differences in relative redox state populations after exposure to H_2 over time. The Hhyd population rises within 20 seconds to its maximum population and subsequently decreases. The $HredH^+$ population increases exponentially while the Hox population decreases with the combined characteristics of the Hhyd and the $HredH^+$ population kinetics. Apparently as a consequence of H_2 uptake the two electron reduced Hhyd state is formed as a first detectable intermediate, which then transforms into the $HredH^+$ population that slowly accumulates. Here, the $HredH^+$ redox state population is defined as only the $2CN^-$ and 2CO bands excluding a tCO or µCO band.

The shared kinetics of the cofactor band populations belonging to one redox state enable correlation of unassigned bands to incomplete sets of cofactor ligand bands for a given H-cluster state. Figure 3C shows a part of the μCO region, namely the positive, broad 1823 cm^{-1} band and the negative μCO band of Hox at 1804 cm^{-1}. The kinetics of the 1823 cm^{-1} band follow the kinetics of $HredH^+$ while the 1804 cm^{-1} band is clearly associated with Hox (Figure 3D). The concomitant increase in intensity of the $HredH^+$ bands and the new μCO band at 1823 cm^{-1} allow for a clear assignment of the latter to $HredH^+$. The same correlation is observed for absorbance changes in the carboxylic acid residue region (1715/1701 cm^{-1}) that were reported to originate from hydrogen bonding changes in the Proton Transfer Network (PTP) during the Hox/Hred transition (Fig.3 E and F).[33]

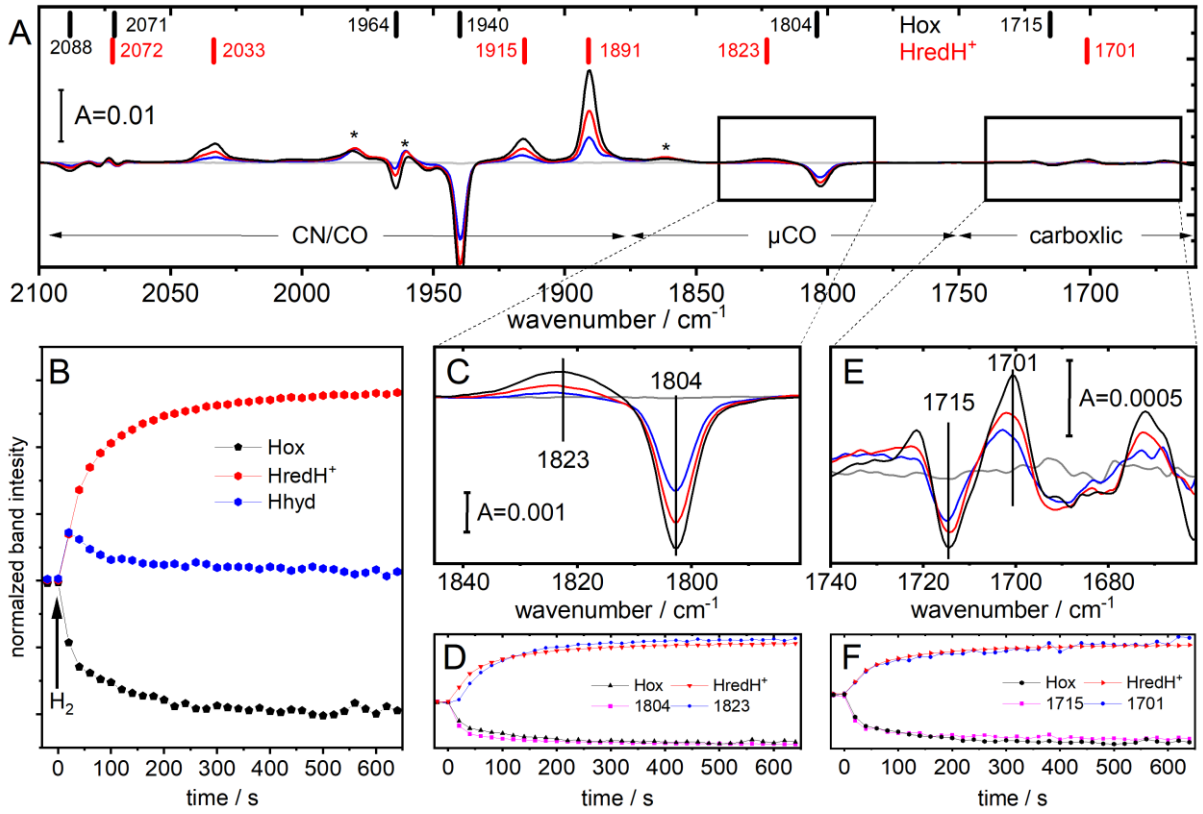

Figure 3: Difference ATR-FTIR spectra and kinetics following the reaction of HydA1 with H_2 gas. (A) Difference spectrum following the population of HredH+ (positive bands) and the depopulation of Hox (negative bands) upon exposure of HydA1 to H_2. Bands indicated by an asterisk belong to a small contribution of Hhyd. Besides the bands in the CO/CN^- and μCO region (inset Fig.3C) differences are detected in the carboxylic acid region (inset Fig.3E) of the spectrum. (B) Kinetics of redox state (de)-population upon exposure to H_2 gas (indicated by an arrow). Every point relates to a difference spectrum recorded every 22 seconds. Ito the expense of Hox, $HredH^+$ and Hhyd get populated. Hhyd rises fast and decays to a constant level while $HredH^+$ and Hox still interconvert. (C) Zoom in of the μCO region of the spectrum reveals a positive broad band at 1823 cm^{-1} and a negative band at 1804 cm^{-1}. The μCO band at 1860 cm^{-1} is not shown for clarity. (D) The kinetics of these bands follow the (de)-population of $HredH^+$ and Hox, and allow to assign the μCO bands to $HredH^+$ and Hox, respectively. Here, the redox state population comprises only $2CN^-$ and 2CO bands excluding a μCO band (E) Zoom in of the carboxylic acid region of the spectrum reveals a negative band at 1715 cm^{-1} and a positive band at 1701 cm^{-1}. These bands have been assigned to originate from changes of a glutamic acid located in the PTP of [FeFe]-hydrogenases.[33] (F) The kinetics of the carboxylic acid bands allow for a clear assignment to $HredH^+$ and Hox respectively.

The new band appears at an unexpectedly high μCO band position assuming a conserved [2Fe] geometry for $HredH^+$. For comparison an additional oxidation of Hox at the [2Fe] as seen for Hhyd or the inactivated state Hinact leads to 20-60 cm^{-1} up-shift of the μCO band, albeit binding a terminal ligand.[10, 46-48] A down-shift in a similar order of magnitude would be expected for the μCO band of $HredH^+$ with its reduced [2Fe], like as observed for TmHydS.[37] For $HredH^+$ the [2Fe] is reduced by one electron compared to Hox and the μCO band should be found at lower wavenumbers, however it is even up-shifted to higher wavenumbers. The up-shift of the μCO band for $HredH^+$ observed here could originate from a structural alteration compared to the conserved Hox geometry. Early crystal structures of [FeFe]-hydrogenase exposed to H_2 gas proposed a semi bridging geometry for the cofactor in the [2Fe] reduced state.[28-29] However recently, both, Stripp *et al*. and Birrell *et al*., excluded this option by correlation of experimental data for HredLT and HsredLT with Density Functional Theory calculations favouring a strict μCO (conserved) geometry and a protonated ADT ligand.[23, 27] Studies of model complexes show that protonation of the ADT amine should compensate the effect of the increased electron density on the μCO band position only by a 16-20 cm^{-1} upshift.[49-50] In the case of the H-cluster inside the enzyme a 10 cm^{-1} up-shift has been observed for the hydride state following the protonation of the ADT amine ($HhydH^+$).[51] Still, the here observed up-shift of the μCO band of HredH+, even assuming a protonated ADT amine, seems too large for a strictly conserved geometry.
Induced by H_2 uptake we observe similar changes of carboxylic acid bands in the PTP that have been associated with proton uptake upon reduction before.[33] These findings imply that the PTP rearranges similarly when the [2Fe] reduced state is induced via the (photo)reductive or the H_2 oxidative path. Whether this rearrangement is a result of additional protonation of the ADT ligand is not clear. However, studies on [FeFe]-hydrogenase cofactormimics and recent theoretical work support the notion of the ADT amine being protonated in $HredH^+$.[52-54]

A [2Fe] reduced state with conserved cofactor geometry (μCO ligand) at room temperature represents one of the missing intermediates in H_2 catalysis of [FeFe]-hydrogenase often postulated.[11, 26-27] HredH+ characterised in this study features clearly a μCO ligand at room temperature as shown by the correlation of redox state bands. The here found state is an important step towards understanding hydrogen catalysis, however a broader investigation beyond HydA1 and prototypical "Group A" [FeFe]-hydrogenases should arguably be prioritised.[2, 34, 55] As an example, to date only an oxidised (Hox), a [2Fe] reduced (Hred) and a CO inhibited state (Hox-CO) have been observed for Group D [FeFe]-hydrogenases which lack the PTP conserved in Group A.[32, 34] The universal reaction mechanism of [FeFe]-hydrogenase continues to be elusive.

Hred, featuring the tCO band implying μH^- geometry, was suggested to be involved in H_2 sensing mechanisms.[15] For the Group D putative sensory [FeFe]-hydrogenase from *Thermoanaerobacter mathranii* (TamHydS) no μCO band was detected for the [2Fe] reduced state (Hred).[32, 34] In contrast, the [2Fe] reduced state in putative sensory hydrogenase TmHydS features a μCO ligand, as evidenced from a distinct signal in the μCO region of the FTIR spectrum.[37] However, the implications of [2Fe] reduced cofactor geometry on possible H_2 and, in general, the function of Hred (μH^-) observed in HydA1 and multiple other [FeFe]-hydrogenases still remain unclear.[10, 12, 18, 22-23, 34, 36]

In summary, the data presented here provides convincing support for the formation of a [2Fe] reduced state featuring a μCO ligand, and a conserved cofactor geometry compared to Hox, at room temperature. This redox intermediate, denoted $HredH^+$, decades ago and has been discussed as a key intermediate in H_2 catalysis of [FeFe]-hydrogenases. The conserved geometry of the [FeFe]-hydrogenase cofactor during catalysis is regarded essential for fast and efficient hydrogen turnover, and thus has been verified for Hox, Hred, Hhyd and finally $HredH^+$.

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