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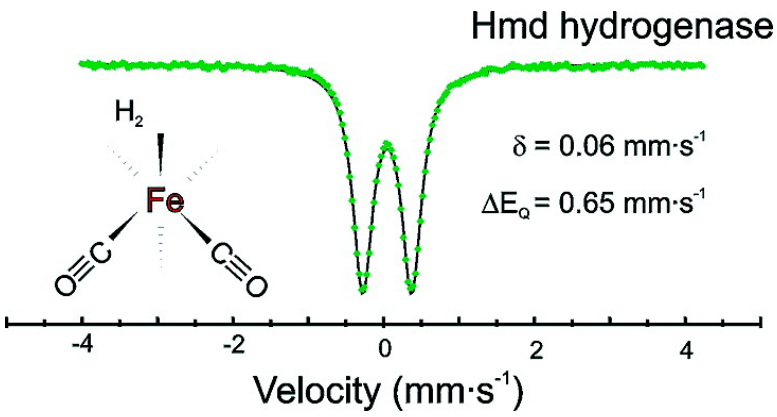
J. Am. Chem. Soc., **2005**, 127 (29), 10430-10435• DOI: 10.1021/ja051895o • Publication Date (Web): 02 July 2005



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Mössbauer Studies of the Iron−Sulfur Cluster-Free Hydrogenase: The Electronic State of the Mononuclear Fe Active Site

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Abstract: The iron−sulfur cluster-free hydrogenase (Hmd) from methanogenic archaea harbors an iron-containing, light-sensitive cofactor of still unknown structure as prosthetic group. The enzyme is reversibly inhibited by CO and cyanide and is EPR silent. We report here on Mössbauer spectra of the ^{57}Fe-labeled enzyme and of the isolated cofactor. The spectrum of the holoenzyme measured at 80 K revealed a doublet peak with an isomer shift $\delta = 0.06$ mm·s^{-1} and a quadrupole splitting of $\Delta E_Q = 0.65$ mm·s^{-1} (at pH 8.0). The signal intensity corresponded to the enzyme concentration assuming 1 Fe per mol active site. Upon addition of CO or cyanide to the enzyme, the isomer shift decreased to -0.03 mm·s^{-1} and $-0.00(1)$ mm·s^{-1}, and the quadrupole splitting increased to 1.38 mm·s^{-1} and 1.75 mm·s^{-1}, respectively. The three spectra could be perfectly simulated assuming the presence of only one type of iron in Hmd. The low isomer shift is characteristic for Fe in a low oxidation state (0, +1, +2). When the spectra of the holoenzyme and of the CO- or cyanide-inhibited enzyme were measured at 4 K in a magnetic field of 4 and 7 T, the spectra obtained could be simulated assuming the presence of only the external magnetic field, which excludes that the iron in the active site of Hmd is Fe(I), high-spin Fe(0), or high-spin Fe(II). Mössbauer spectra of the isolated Hmd cofactor are also reported.

Introduction

Hydrogenases are enzymes that catalyze the reversible oxidation of molecular hydrogen.[1] Their structure and catalytic mechanism are of considerable applied interest as models for the development of efficient chemical catalysts for hydrogen-fueled processes. Despite intensive efforts, however, the understanding of how hydrogenases react with H_2 is only in its infancy.

Three types of hydrogenases are known thus far: the [NiFe] hydrogenases, the [FeFe] hydrogenases, and the H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd),[1] which was previously thought to be "metal-free" but has recently been shown to contain a functional iron after all.[2] The three hydrogenases are phylogenetically not related. Hmd, which is also referred to as iron−sulfur cluster-free hydrogenase, differs from the two other hydrogenases in that this enzyme does not contain iron−sulfur clusters, does not catalyze the reduction of viologen dyes with H_2, and does not per se catalyze the exchange of H_2 with protons of water or the interconversion of *ortho*-H_2 and *para*-H_2.[3] The three hydrogenases are generally inhibited by CO, but only Hmd is inhibited by cyanide.[2]

The [NiFe] hydrogenase, which is found in bacteria and archaea, contains a dinuclear center composed of a nickel and an iron. The [NiFe] center is bound to four cysteine thiol ligands from the protein.[4] Generally, one CO and two CN^- molecules are bound as ligands to the iron.[4,5] Three iron−sulfur clusters are involved in channeling the electrons from the dinuclear center to the final electron acceptor.[4,6,7] In the catalytic reactions of the [NiFe] hydrogenases, H_2 appears to be oxidized at their nickel site, which is also the site of extrinsic CO binding.[8] The nickel site is redox-active, oscillating between Ni(I) and Ni(III) during catalysis.[9] The function of the iron site of the [NiFe] center appears to be merely in the coordination of H_2.[8] This iron is always in a low-spin Fe(II) state in the active and the inactive forms.[10]

The [FeFe] hydrogenases, which are found in bacteria and eucarya, contain a dinuclear center composed of two irons. The

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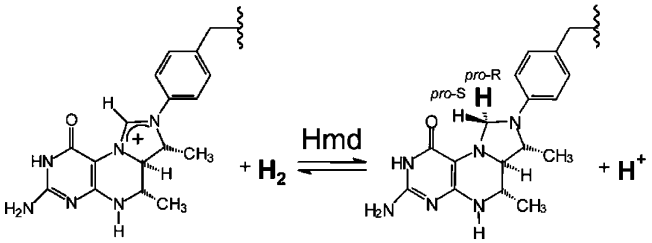

Figure 1. Reaction catalyzed by the iron−sulfur cluster-free hydrogenase (Hmd). Hmd catalyzes the reduction of $N,^5N^{10}$-methenyltetrahydromethanopterin with H_2 to $N,^5N^{10}$-methylenetetrahydromethanopterin and a proton. A hydride is stereospecifically transferred from H_2 into the *pro-R* side of methylenetetrahydromethanopterin. The enzyme also catalyzes a direct exchange of the *pro-R* hydrogen of methylenetetrahydromethanopterin with protons of water.[22,23]

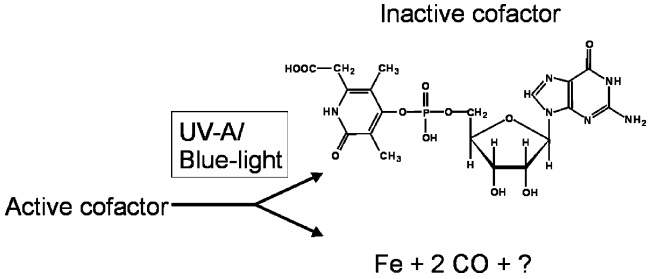

Figure 2. Products formed from the Hmd cofactor upon irradiation with UV−visible light. The structure of the pyridone derivative was elucidated by mass and NMR spectroscopy of the ^{14}N- and ^{15}N-labeled compound.[28] The presence of 2 mol CO per Fe was shown by gas chromatography and IR spectroscopy.[29] The electronic state of the iron in the intact cofactor was shown to be low-spin Fe(II) or low-spin Fe(0) (this work).

[FeFe] center is bound to the protein only through a thiol-sulfur ligand that also connects to a 4Fe−4S cluster.[8,11−13] Three CO and two CN^- ligands are bound to the dinuclear center.[14] Spectroscopic experiments and structural analysis suggested that H_2 oxidation and extrinsic CO binding occur at the distal iron, which is the one in the [FeFe] center furthest from the 4Fe−4S cluster.[8,15] Spectroscopic experiments indicated that a low-spin Fe(II) is involved in the dinuclear iron center of the [FeFe] hydrogenases,[8] but the redox states of the iron center are still in discussion.[16−21]

The iron−sulfur cluster-free hydrogenase Hmd, which is only found in some methanogenic archaea, catalyzes the reversible reduction of methenyltetrahydromethanopterin (methenyl-H_4MPT^+) with H_2 to methylenetetrahydromethanopterin (methylene-H_4MPT) and a proton ($\Delta G^{\circ\prime} = -5.5$ kJ·mol^{-1}).[3,24,25] In the reaction, a hydride is transferred from H_2 into the *pro-R* position of the methylene-carbon of methylene-H_4MPT[22,26] (Figure 1). Hmd harbors an iron-containing cofactor of still unknown structure that is essential for its activity.[27] This iron cofactor is sensitive to UV-A/blue light both in the free state and when bound to the Hmd protein.[2] Upon irradiation, the cofactor breaks down into a pyridone derivative, free Fe, and CO[28,29] (Figure 2). The pyridone could be involved in forming the iron complex via its carboxyl and/or imino group. But it could be more complicated in that in the active cofactor the pyridone ring could be hydrolyzed and the ring is closed only upon light inactivation.

Chemical analysis revealed that active Hmd contains approximately 2 mol CO per iron.[29] From IR spectra it could be deduced that the two CO bind to one iron and that binding is in an angle of 90°. The CO-inhibited enzyme contains 3 mol CO bound to one iron, and the extrinsic CO does not exchange with the intrinsic ones. The IR spectrum of the cyanide-inhibited Hmd shows two CO and one cyanide bound to one iron. Binding of cyanide and of extrinsic CO was found to be mutually exclusive. The isolated cofactor also contains two CO bound to Fe but the free cofactor differs from the enzyme bound one in that it appears not to react with extrinsic CO and that reaction with cyanide is irreversible.[29]

The function and the electronic state of the active-site iron in Hmd still remain elusive. Hmd and its cofactor are EPR silent in both the absence and presence of H_2 and/or methenyl-H_4MPT^+ (unpublished result). Therefore, to get insight into the electronic structure, we measured Mössbauer spectra of the ^{57}Fe-labeled Hmd holoenzyme and the Hmd cofactor in the absence and presence of its substrates and inhibitors.

Experimental Procedure

Methanothermobacter marburgensis (DSMZ2133) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen. Tetrahydromethanopterin (H_4MPT) and methenyl-H_4MPT^+ were purified from *M. marburgensis*.[30] ^{57}Fe-enriched iron metal (95.85%) was from Chemotrade (Düsseldorf). The metal (100 mg) was dissolved in 1.5 mL of 37% HCl by incubation for 50 h at 60 °C. After the addition of 8.5 mL of H_2O, the 175 mM $^{57}FeCl_2$ solution was stored at 4 °C.

The FPLC system and the chromatographic columns were from Amersham Bioscience. Purifications of Hmd and the cofactor were performed under strictly anoxic conditions using an anaerobic chamber (Coy) filled with 95% N_2/5% H_2 (v/v) containing a palladium catalyst. All manipulations were performed under red light because both Hmd and the isolated cofactor are inactivated in UV/blue light.

Labeling of the Hmd Iron Site with ^{57}Fe. *M. marburgensis* was grown at 65 °C and pH 7.0 on H_2 and CO_2 in a medium containing 40 mM NH_4Cl, 50 mM KH_2PO_4, 24 mM Na_2CO_3, 0.5 mM nitrilotriacetic acid, 0.2 mM $MgCl_2$·$6H_2O$, 1 μM $CoCl_2$·$6H_2O$, 1 μM Na_2MoO_4·$2H_2O$, 20 μM resazurin, 0.65 μM $NiCl_2$, and 50 μM $^{57}FeCl_2$. A 400-mL all-

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glass fermenter was filled with 360 mL of the medium and inoculated with 18 mL of a stock culture of *M. marburgensis* grown on media with a Ni concentration of 5 μM. Before and after inoculation the fermenter was continuously gassed with 80% H_2/20% CO_2/0.1% H_2S at a rate of 100 mL·min^{-1}. After 12 h growth to an OD_{600} of 1.7 a 100-mL aliquot was used to inoculate an 11-L fermenter filled with 10 L of medium containing 50 μM $^{57}FeCl_2$ but no added Ni. The fermenter, which consisted of a glass vessel (11 L) and stainless steel stirring fins, was gassed with 80% H_2/20% CO_2/0.1% H_2S at a rate of 1.5 L·min^{-1}. After 21 h of cultivation, when an $OD_{600\ nm} = 4.8$ was reached, approximately 80 g (wet mass) of cells was harvested anaerobically. Under the nickel-limited growth conditions Hmd is overproduced in *M. marburgensis*.[31] From the ^{57}Fe-labeled cells, approximately 170 mg of Hmd was purified as described previously via ammonium sulfate precipitation, Source 30Q anion-exchange chromatography, and Sephadex G25 gel-filtration chromatography.[2]

Isolation of the Hmd Cofactor. To extract the cofactor from ^{57}Fe-labeled Hmd samples in high yield and to effectively remove the apoprotein from the extract, we modified the previous extraction method.[28] Purified Hmd (200 mg) was dissolved in 30 mL of H_2O containing 60% methanol, 1 mM mercaptoethanol, and 520 mM ammonia (extraction solution). After 20 h incubation in the dark at 4 °C, 0.75 mL of 200 mM $CaCl_2$ was added and the aggregated apoprotein was removed by centrifugation at 15000*g* for 40 min at 4 °C. The precipitate was resuspended in 5 mL of the extraction solution supplemented with 5 mM $CaCl_2$ and then centrifuged at 15000*g* for 20 min at 4 °C. The supernatants (32 mL) were combined and anaerobically evaporated for 30 min at 4 °C in a vacuum system equipped with a cold trap to a final volume of 11 mL. By this short evaporation step most of the methanol in the solution was removed. The concentrated solution was filtrated by centrifugation at 4000*g* for 70 min at 4 °C using 10 kDa cut ultrafilter tubes (Amicon-Ultra-15). The filtrate was dried via anoxic evaporation at 4 °C as described above. The extracted cofactor was dissolved in 2 mL of 250 mM Mes/NaOH pH 6.0 containing 10 mM 2-mercaptoethnol and stored at −80 °C. The cofactor concentration in the 2-mL sample was estimated spectrometrically to be 2.5 mM. HPLC analysis indicated no contamination of the active cofactor with apoprotein or inactivated cofactor.[28]

Preparation of Mössbauer Samples. For the determination of the Mössbauer spectra, 0.7-mL samples (0.7 mM) were transferred within the anaerobic chamber into a 0.7-mL Mössbauer cup each placed in a 250-mL amber-colored bottle, which was subsequently sealed with a rubber stopper held with a screw cap. Where indicated, the Hmd solutions contained 2 mM KCN or 0.7 mM methenyl-H_4MPT^+. Outside the anaerobic chamber the gas phase in the bottles was exchanged via a needle by evacuation and filling with 5% H_2/95% N_2, 100% H_2, 100% N_2, or 100% CO at 1.3 bar. Then the bottles were incubated at 4 °C for 60 min and then frozen at −80 °C in a deep freezer. After 1.5 h of freezing, the caps and the rubber stoppers were removed, and the frozen samples in the Mössbauer cups were quickly transferred into a liquid nitrogen bath. The samples were stored in a dewar filled with liquid nitrogen.

Mössbauer Spectroscopy. Mössbauer spectra were collected on an alternating constant-acceleration spectrometer. The sample temperature was maintained constant in either an Oxford Variox or an Oxford Mössbauer-Spectromag cryostat. The latter is a split pair superconducting magnet system for applying fields up to 8 T to the samples, which can be kept at temperatures in the range 1.5−250 K. The field at the sample is perpendicular to the γ beam. With the help of reentrant bore tubes, the ^{57}Co/Rh source was positioned at room temperature inside the gap of the magnet system at a distance of about 85 mm from the sample. At this position, the magnetic field is zero. All isomer shifts are quoted relative to iron metal at 300 K. The zero-field spectra were fitted by using Lorentzian line shapes, whereas the applied-field spectra were simulated by using the usual nuclear Hamiltonian.[32]

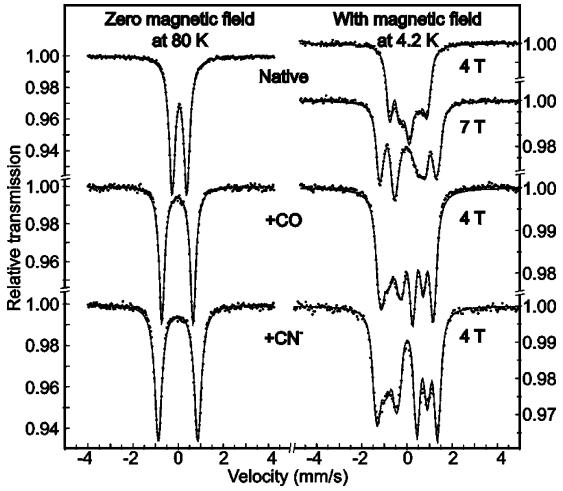

Figure 3. Mössbauer spectra of native Hmd and Hmd in the presence of CO or cyanide. The solid lines in the left panel represent fits with symmetric Lorentzian doublets, whereas for the right panel magnetic simulations with $S = 0$ have been performed. The sample contained 0.7 mM Hmd in 50 mM tricine/NaOH pH 8.0 (pH at 0 °C) and were under a gas phase of 95% N_2/5% H_2 (gas in the anaerobic tent) except in the case of the CO-inhibited enzyme where the gas phase was 100% CO. Where indicated, potassium cyanide was added to a final concentration of 2 mM.

Assay Methods. The protein concentrations were determined by the Bradford method using the Bio-Rad microassay with bovine serum albumin as standard.[33] The activity of Hmd and the cofactor was measured by detecting the formation of methenyl-H_4MPT^+ from 20 μM methylene-H_4MPT ($\epsilon_{336\ nm}$ of methenyl-H_4MPT^+ is 21.6 mM^{-1} cm^{-1}) in 120 mM potassium phosphate pH 6.0 and 1 mM EDTA at 40 °C.[2] The reaction was started by the addition of methylene-H_4MPT. Methylene-H_4MPT was generated by spontaneous reaction from H_4MPT (2 μmol) and formaldehyde (4 μmol) in 1 mL of 120 mM potassium phosphate pH 6.0. The methylene-H_4MPT solution was subsequently completely dried by anoxic evaporation at 4 °C to remove residual formaldehyde, and the residue dissolved in anoxic water. Removal of most of the formaldehyde was necessary since the cofactor is inactivated in the presence of formaldehyde.

Results and Discussion

Hmd from *M. marburgensis* was in vivo enriched with ^{57}Fe (about 95%) and purified for Mössbauer investigations. Zero-field and magnetically perturbed spectra were recorded from the native enzyme, as well as in the presence of the substrates H_2 or methenyl-H_4MPT^+ or in the presence of the inhibitors CO or cyanide. The light-exposed Hmd sample and the extracted Hmd cofactor were also analyzed by this method.

Mössbauer Spectrum of Hmd. The zero-field Mössbauer spectrum of *native* Hmd (0.7mM) at 80 K is depicted in Figure 3, left panel, top. A well-resolved symmetric quadrupole doublet with narrow Lorentzian lines is observed, indicating homogeneous protein conformation and a uniform iron site ($\delta = 0.06$ mm·s^{-1}, $\Delta E_Q = 0.65$ mm·s^{-1}, $\Gamma_{fwhm} = 0.32$ mm·s^{-1}). The spectrum is almost temperature-independent in the range 80−4.2 K without showing paramagnetic line broadening or background signals. Despite the lability of the enzyme there are no indications of nonspecific iron or deteriorated protein.

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The isomer shift of Hmd is remarkably low and clearly indicates the presence of low-spin iron with a low oxidation state, Fe(II), Fe(I), or Fe(0). The value is rather unusual for a non-heme iron protein and even falls under the isomer shifts known for CO derivatives of iron in hemes ($\delta = 0.26-0.30$ mm·s^{-1}, $\Delta E_Q = 0.27-0.95$ mm·s^{-1} at 4.2 K),[34] which are described as low-spin Fe(II) species. Similar Mössbauer parameters from biological systems have been reported only for a subspectrum from the [NiFe] hydrogenase from *Chromatium vinosum*, which represents the iron site in the dinuclear [NiFe] center,[16,35] and for the $[2Fe]_H$ cluster of [FeFe] hydrogenase from *Clostridium pasteurianum* in the reduced state ($\delta = 0.08$ mm·s^{-1}, $\Delta E_Q = 0.87$ mm·s^{-1} at 4.2 K).[16] The $[2Fe]_H$ cluster in the hydrogenase from *Desulfovibrio vulgaris* in different redox states and with CO treatment exhibits a similar range of values ($\delta = 0.13-0.17$ mm·s^{-1}, $\Delta E_Q = 0.65-1.09$ mm·s^{-1}).[17] It has been argued that the low-spin iron in both types of hydrogenase is Fe(II), and upon oxidation one site of the $[2Fe]_H$ cluster adopts the Fe(III) state. However, studies on model compounds point out that Fe(I)−Fe(I) units can mimic metric details of the$[2Fe]_H$ cluster[36] and reflect also similar spectroscopic properties.[18] It is remarkable how close the Mössbauer parameters of iron(0) systems such as the $[Ni(dsdm)(Fe(CO)_3)_2]$ fall to those of Hmd (two sites with $\delta = 0.07/0.03$ mm·s^{-1}, $\Delta E_Q = 0.66/0.57$ mm·s^{-1} at 80 K, similar to other dimeric compounds with the structure $([X(Fe(CO)_3)_2])$.[19] Common iron(0) carbonyl complexes of the type $LFe(CO)_4$, where L is a two-electron donor, also have comparable Mössbauer parameters ($\delta = -0.09$ to $+0.07$ mm·s^{-1}, $\Delta E_Q = 0.4-2$ mm·s^{-1} at 4.2 K),[37] so as tri-carbonyls such as [butadiene $Fe(CO)_3$] ($\delta = +0.03$ mm·s^{-1}, $\Delta E_Q = 1.46$ mm·s^{-1}).[37,38] The similarity of the isomer shifts for these "metal-organic" low-spin iron compounds with CO (and isoelectronic CN^-) ligands in different formal oxidation states has been explained primarily by variations in the back-donation to the empty π^* orbitals of the ligands.[38,39] Consequently, the oxidation state of the iron center in Hmd cannot be readily established by Mössbauer spectroscopy. However, interpretations involving iron(III) or iron(I) are not consistent with the Mössbauer data of Hmd, because these configurations would be paramagnetic whereas the iron complex of Hmd appears to be a monomeric site with spin $S = 0$, as will be shown below.

Mössbauer Spectra of Hmd Inhibited by CO or Cyanide. Inhibition of Hmd by exposure to CO atmosphere before freezing or by the addition of CN^- causes distinct changes of the Mössbauer spectra, which reveal binding of both inhibitors directly to the iron site (Figure 3, left panel, middle and bottom). At pH 8 the spectra of both preparations are nicely symmetric quadrupole doublets that could be perfectly simulated by adopting only one type of iron. The native Hmd sample was completely converted into the CO and cyanide adducts. The corresponding changes in the electronic structure of the iron are seen from the increase of the quadrupole splitting and the decrease of the isomer shifts. Lorentzian line fits of the spectra yield $\delta = -0.03$ mm·s^{-1} and $\Delta E_Q = 1.38$ mm·s^{-1} for the CO-inhibited sample, and $\delta = -0.001$ mm·s^{-1}, $\Delta E_Q = 1.75$ mm·s^{-1} for the CN^- derivative.

Table 1. Mössbauer Parameters of the Iron−Sulfur Cluster-Free Hydrogenase (Hmd) at 80 K and of Its Iron-Containing Cofactor

	δ^d (mm·s^{-1})	$\Delta E_Q{}^e$ (mm·s^{-1})	$\Gamma_{fwhm}{}^f$ (mm·s^{-1})	intensity^g (%)	η^i
		Hmd (pH 8.0)[a]			
95% N_2/5% H_2[b]	+0.06	+0.65[h]	0.32	100	0.3(2)
100% N_2	+0.04	0.66	0.31	100	
100% H_2	+0.06	0.65	0.30	100	
100% CO	−0.03	−1.38[h]	0.29	99	0.5(1)
KCN	−0.001	−1.75[h]	0.38	100	0.6(1)
(2 mM)+5%H_2 methenyl-H_4MPT^+ (0.7 mM)+100% H_2	+0.07	0.67	0.34	100	
		Free Cofactor[c] (pH 6.0)			
95% N_2/5% H_2[b]	+0.03	0.43	0.31	84	
100% CO	+0.04	0.43	0.31	50	
	−0.03	1.19	0.29	38	

[a] Hmd (0.7 mM) samples were dissolved in 50 mM tricine/NaOH pH 8.0 (at 0 °C). The samples were incubated under different conditions (gas phase, absence and presence of methenyl-H_4MPT^+) and then the spectrum was recorded at 80 K in the absence of an external magnetic field. [b] Gas mixture in the anaerobic tent, in which the samples were manipulated. [c] The free cofactor (2.6 mM) was dissolved in 250 mM Mes/NaOH pH 6.0 supplemented with 10 mM mercaptoethanol. It contained 16% (under 95% N_2/5% H_2) and 12% (under 100% CO) adventitiously bound iron in a high spin ferrous state. [d] Isomer shift (typical error ±0.01 mms^{-1}). [e] Quadrupole splitting (typical error ±0.02 mms^{-1}). [f] Line width (fwhm). [g] Relative intensity. [h] Sign of the main component of the electric field gradient at 4.2 K taken from applied field measurement. [i] Asymmetry parameter of the electric field gradient at 4.2 K. [j] The Mössbauer parameters of Hmd at pH 8.0 (50 mM tricine/NaOH pH 8.0) and pH 6.0 (50 mM Mes/NaOH pH 6.0) were not significantly different.

Mössbauer Spectra of Hmd at Different pH and in the Presence of H_2 and/or Methenyl-H_4MPT. The Mössbauer spectra of the native enzyme and of the CO- or cyanide-inhibited enzyme did not change significantly when the pH was lowered from 8 to 6. The spectra of Hmd in the presence and absence of its substrates H_2 and/or methenyl-H_4MPT^+ were almost identical (Table 1). There are slight variations in the isomer shift and quadrupole splitting, but these are too small to be considered significant. This finding indicates that, when the enzyme interacts with its substrates (H_2 and methenyl-H_4MPT^+) or products (H^+ and methylene-H_4MPT), the electronic state of the iron site in Hmd is not affected sufficiently to be picked up by the Mössbauer nucleus. In this respect, it is of interest that the IR bands of Hmd shift somewhat when the enzyme is incubated in the presence of H_2 and methenyl-H_4MPT^+,[29] indicating that the two substrates bind in the vicinity of the iron site, but not as direct ligands. We suggest that the coupling modes of the carbonyl groups and the resulting vibrational spectrum are affected by the influence of next nearest neighbors, for instance via changes in the hydrogen-bonding pattern or steric effects.

Indications for a Mononuclear Iron Center in the Active Site of Hmd. The Mössbauer spectra of Hmd in the presence of CO, cyanide, or methenyl-H_4MPT^+ (Table 1) never exhibited any other heterogeneity than that due to remains of unperturbed native enzymes with 10−30% relative intensity at most.

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In this respect, Hmd is significantly different from [FeFe] hydrogenases, in which the two iron sites of the dinuclear metal center show significantly different zero-field Mössbauer parameters at least for the CO-bound state.[17] We take this as a compelling indication that the active center of Hmd, in contrast to the other hydrogenases, contains unique, single iron sites. The conclusion strongly supports our previous interpretation of the IR data from Hmd and its CO and CN^- derivatives.[29]

To further bolster the argument, we performed quantifications of the Mössbauer intensities of the spectra shown in Figure 3 to determine the total iron concentration of the protein. To this end, a solution of 15 mM $K_4Fe(CN)_6$ has been measured for reference at the same conditions of buffer, temperature, and spectrometer geometry (2.17% natural abundance of ^{57}Fe, single-line spectrum with $\delta = 0.03$ mm·s^{-1}, $\Gamma_{fwhm} = 0.37$ mm·s^{-1}). The γ-spectrum of the ^{57}Co source was monitored during collection of the Mössbauer data for both spectra and fitted with Gaussians to obtain the contribution of the 14 keV Mössbauer line in the discriminated energy window of the counting system and to determine the nonresonant background radiation. After correction for the differences, the total iron concentrations of the Hmd samples could be estimated. We obtained a mean value from three spectra of 1.14 ± 0.25 iron per protein monomer (the enrichment of Hmd with ^{57}Fe was adopted to be 95%, the error range is determined by the signal-to-noise ratio of the spectra, and the error range does not contain the uncertainty in protein concentration, which was determined to be ±5% when we used bovine serum albumin as standard). The result clearly supports the conclusion that the active site of Hmd has a single monomeric iron center.

Magnetic Properties of the Active-Site Iron in Hmd. Applied-field Mössbauer spectra were measured at 4.2 K to determine the electronic spin ground state of the metal ion center in Hmd and to probe the sign and the asymmetry parameter η of the efg tensor at the ^{57}Fe nucleus. Corresponding spectra of native Hmd and the CO- and CN^--inhibited enzyme are presented in the right panel of Figure 3 for 4 and 7 T fields applied perpendicularly to the γ-beam. The patterns result from mixed hyperfine interaction with weak overall magnetic splitting. They could be readily simulated by nuclear Zeeman splitting due to the applied fields, which reveals complete absence of internal fields. Thus, the three enzyme preparations are diamagnetic with spin $S = 0$ for the iron. Since the diamagnetism cannot be achieved by antiferromagnetic interaction, due to the mononuclear character of the metal center, the spin is consistent with putative Fe(II) or Fe(0) valence states, but not with paramagnetic Fe(I) or Fe(III) low-spin. Moreover, the relatively small quadrupole splitting of Hmd also appears to be in contrast to the large valence contribution expected for electric field gradients (efg) of the corresponding $3d(t_{2g})^5$ or $3d\ (t_{2g})^6e_g^1$ electronic configurations.

Inhibition of Hmd by CO or cyanide induces remarkable changes in the efg of the iron site (Table 1). Not only the size of the quadrupole splitting increases, as already shown above, but the magnetic Mössbauer spectra also reveal a change in the sign of the main component V_{zz} of the efg and an increase of the asymmetry parameter η with respect to the native state. This corroborates the conclusion of significant rearrangement in the electronic structure of iron and the direct binding of the small inhibitor molecules.

Mössbauer Spectrum of Hmd after Light Inactivation. We have shown previously that Hmd can be inactivated by white light.[2] To study the related molecular changes in the active center of the enzyme, a sample of ^{57}Fe-enriched native Hmd (0.8 mM) at 4 °C was exposed to white light in a quartz cuvette under 100% N_2 atmosphere as described previously.[2] After 20 h of exposure time, the enzymatic activity had decreased to approximately 25% of the original value. The sample was then transferred to a Mössbauer cup and frozen under 95% N_2/5% H_2 and stored in liquid nitrogen for the measurements.

The zero-field Mössbauer spectrum of the inactivated sample is a complex superposition of several lines, which are distinctly different from that of native Hmd but could be deconvoluted as being derived from four different symmetric quadrupole doublets (Supporting Information, Figure S1, top), which are (i) remaining native (active) Hmd (native, relative intensity < 5%), (ii) CO-inhibited Hmd (+CO, approximately 20%), (iii) a high-spin ferrous component "Fe(II)" (approximately 15%), and (iv) a major component "X" of less clear nature (approximately 60%).

The formation of CO-inhibited Hmd upon light exposure was already reported in previous IR studies.[29] Via light, the intrinsic CO molecules are flashed off the Hmd iron site, and a part of the released CO may subsequently rebind to other Hmd molecules not yet light-inactivated. Moreover, CO-inhibited Hmd was found previously to be much less susceptible to light inactivation than native Hmd. This finding can explain why at the high Hmd concentration used in the light inactivation experiment with the Mössbauer sample (0.8 mM) the inactivation of the *native* enzyme was relatively slow (only 75% in 20 h): the intrinsic CO released from part of the Hmd molecules protected the rest from light inactivation by forming CO-inhibited Hmd, which made up almost 20% of the sample after 20 h of illumination as deduced from the Mössbauer spectrum (Supporting Information, Figure S1).

It is remarkable that the light-treated Mössbauer sample showed about 25% of its original enzymatic activity, although <5% of the total iron remained in the native, active state. This can be explained by the fact that the 20% CO-inhibited enzyme becomes active again upon dilution because the CO concentration in the enzyme assay is decreased.

Mössbauer Spectrum of the Iron-Containing Cofactor. The Mössbauer spectrum of the free active cofactor shows a superposition of two subspectra due to the presence of two distinct iron species in the preparation (Figure 4 top; for composition of the cofactor see Figure 2). The minor component (labeled "Fe(II)", 16% relative intensity) has large isomer shift $\delta = 1.40$ mm·s^{-1} and quadrupole splitting $\Delta E_Q = 3.12$ mm·s^{-1}, similar to the nonspecifically bound high-spin ferrous ions found in light-inactivated Hmd samples. Since we know from biochemical experiments that the free cofactor is much more sensitive to light and heat than the holoenzyme and that the inactivated cofactor readily loses its iron,[2,28] we assign the ferrous Mössbauer subspectrum to the deteriorated cofactor complex, which has completely lost the CO ligands. The major Mössbauer subspectrum, in contrast, closely resembles that of the holoenzyme in having the same low isomer shift (0.03 mm·s^{-1}, 84% intensity), but a somewhat different quadrupole splitting ($\delta = 0.43$ mm·s^{-1} versus 0.65 mm·s^{-1} natively, Table 1). Like in the holoenzyme, the iron is clearly low-valent and

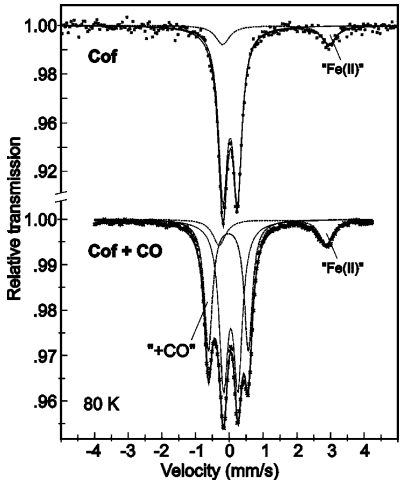

Figure 4. Mössbauer spectra of the free Hmd cofactor (Cof) in the presence and absence of CO. The samples contained 2.5 mM of extracted cofactor in 250 mM Mes/NaOH pH 6.0 (pH at 0 °C) supplemented with 10 mM mercaptoethanol. (Cof) under 95% N_2/5% H_2. (Cof + CO) under 100% CO. The solid and dashed lines represent fits with Lorentzians, "Fe(II)" is a high-spin ferrous component, and "+CO" is the iron-CO adduct of the free cofactor, as described in the text.

low-spin and most probably has the same number and kind of ligands as in native state. Only the coordination geometry appears to be slightly different, as reflected in the quadrupole splitting.

After the Mössbauer measurement, the [57]Fe-enriched cofactor sample was thawed again and incubated at 4 °C in 100% CO atmosphere (1.3 bar) for 1 h. The sample was refrozen in the presence of CO and stored in liquid nitrogen for Mössbauer measurements. The 80 K spectrum of the treated sample was similar to the previous one (Figure 4, bottom and top), except that a new quadrupole doublet had emerged with lower isomer shift $\delta = -0.03$ mm·s^{-1} and larger quadrupole splitting, $\Delta E_Q = 1.19$ mm·s^{-1}, similar to that of CO-inhibited holo-Hmd (Table 1) but with only 38% relative amount of the total intensity. The result indicates that external CO can bind to the free cofactor, but the affinity of the iron complex for additional CO binding is lower than that of the holoenzyme, which showed nearly 100% occupancy of the external CO site under the same condition (Table 1). The finding seems contradictory to the observation from IR spectroscopy indicating that the free cofactor does not bind external CO.[29] The discrepancy might be related to the differences in experimental temperatures and handling: IR samples were incubated under 100% CO at 20 °C and then transferred into an open IR cuvette under 95% N_2/5% H_2 and measured at 20 °C,[29] whereas the Mössbauer samples were incubated under 100% CO at 4 °C in Mössbauer cups, which were subsequently frozen at −80 °C under 100% CO. We therefore repeated the IR measurements with a sample incubated at 4 °C under 100% CO. The sample was transferred into an IR cuvette filled with 100% CO-saturated buffer, which was displaced by the sample, and the cuvette was then closed. The IR spectrum obtained showed an additional IR band at 2080 cm^{-1}, indicating that the iron-containing cofactor reacted with the external CO (unpublished result). However, the intensity of the third band was only approximately 10% of that of the two other bands, and the spectrum was too noisy to allow an exact identification and quantification.

In the presence of cyanide the free Hmd cofactor is highly unstable, which is why a Mössbauer spectrum of the cofactor cyanide adduct could not be obtained.

Conclusion

The Mössbauer spectra of native Hmd, the CO- or CN^--inhibited Hmd, and the free cofactor indicate that Hmd contains per active site one iron in a low oxidation and spin state, either low-spin Fe(0) or low-spin Fe(II), which is redox inactive. The very low isomer shift appears to favor low-spin Fe(0). The finding that Hmd reversibly binds cyanide is more in favor of low-spin Fe(II) since Fe(0) cyanide complexes are rare, although compounds such as $[Fe(CO)_4(CN)]^-$ and $[Fe(CO)_3(CN)^2]^{2-}$do exist.[40,41] It is of interest that a redox-inactive low-spin Fe coordinated by CO is also present in the dinuclear metal centers of the [NiFe] hydrogenases and of the [FeFe] hydrogenases.[8,35] Considering that the three types of hydrogenases are phylogenetically not related and therefore must have evolved by convergent evolution, this correspondence is quite astonishing. The elucidation of the ligand structure and of the catalytic properties of this special iron is therefore probably the key to understanding how hydrogenases function. In addition to X-ray structure analysis, [57]Fe NMR is one method to obtain further insight into the structure of the iron complex, which upon irradiation with UV-A/blue light breaks down into a pyridone derivative, free iron, two CO, and at least one other product (Figure 2).

Acknowledgment. This work was supported by the Max Planck Society and by the Fonds der Chemischen Industrie. We thank Caroline Fichtner and Dr. Helmut Görner from the Max Planck Institute for Bioinorganic Chemistry for help in recording IR spectra.

Supporting Information Available: Mössbauer spectrum of Hmd after 20 h of light inactivation, recorded at 80 and 4.2 K. This material is available free of charge via the Internet at http://pubs.acs.org.

JA051895O

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