The exchange activities of [Fe] hydrogenase (iron–sulfur-cluster-free hydrogenase) from methanogenic archaea in comparison with the exchange activities of [FeFe] and [NiFe] hydrogenases

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Abstract [Fe] hydrogenase (iron–sulfur-cluster-free hydrogenase) catalyzes the reversible reduction of methenyltetrahydromethanopterin (methenyl-H₄MPT⁺) with H₂ to methylene-H₄MPT, a reaction involved in methanogenesis from H₂ and CO₂ in many methanogenic archaea. The enzyme harbors an iron-containing cofactor, in which a low-spin iron is complexed by a pyridone, two CO and a cysteine sulfur. [Fe] hydrogenase is thus similar to [NiFe] and [FeFe] hydrogenases, in which a low-spin iron carbonyl complex, albeit in a dinuclear metal center, is also involved in H₂ activation. Like the [NiFe] and [FeFe] hydrogenases, [Fe] hydrogenase catalyzes an active exchange of H₂ with protons of water; however, this activity is dependent on the presence of the hydride-accepting methenyl-H₄MPT⁺. In its absence the exchange activity is only 0.01% of that in its presence. The residual activity has been attributed to the presence of traces of methenyl-H₄MPT⁺ in the enzyme preparations, but it could also reflect a weak binding of H₂ to the iron in the absence of methenyl-H₄MPT⁺. To test this we reinvestigated the exchange activity with [Fe] hydrogenase reconstituted from apoprotein heterologously produced in Escherichia coli and highly purified iron-containing cofactor and found that in the absence of added methenyl-H₄MPT⁺ the exchange activity was below the detection limit of the tritium method employed (0.1 nmol min⁻¹ mg⁻¹). The finding reiterates that for H₂ activation by [Fe] hydrogenase the presence of the hydride-accepting methenyl-H₄MPT⁺ is essentially required. This differentiates [Fe] hydrogenase from [FeFe] and [NiFe] hydrogenases, which actively catalyze H₂/H₂O exchange in the absence of exogenous electron acceptors.

Keywords Hydrogenase · Exchange reactions · Methanogenic archaea

Abbreviations
H₄MPT Tetrahydromethanopterin
Hmd H₂-forming methylenetetrahydromethopterin dehydrogenase
MV Methyl viologen
D ²H
T ³H

Introduction

Hydrogenases are enzymes that catalyze reversible reactions with H₂ as reactant or product (Eqs. 1a, 1b, 1c):

\[ \text{H}_2 + \text{X} \rightleftharpoons \text{XH}_2, \quad (1a) \]
\[ \text{H}_2 + \text{X} \rightleftharpoons \text{XH}^- + \text{H}^+, \quad (1b) \]
\[ \text{H}_2 + \text{X} \rightleftharpoons \text{X}^- + 2\text{H}^+, \quad (1c) \]

where X is either an enzyme or a coenzyme. This definition is independent of the hydrogenase’s catalytic mechanism which can be “ping-pong” or “ternary complex.” It is therefore broader than the definition used historically that...
hydrogenases catalyze the reversible oxidation of H₂ to two electrons and two protons, which excludes hydrogenases with a ternary complex mechanism [1, 2].

Three types are presently known which are phylogenetically not related [3, 4]: [NiFe] hydrogenases found in archaea and bacteria [5], [FeFe] hydrogenases found in bacteria and eukarya [6–8] and [Fe] hydrogenases only found in methanogenic archaea [9, 10]. [FeFe] hydrogenases are still also referred to as iron-only hydrogenases, which was the name used before it was shown that [Fe] hydrogenase also contains iron [11].

[NiFe] hydrogenases and [FeFe] hydrogenases have many properties in common. They both contain a dinuclear metal center in their active site and at least one iron–sulfur cluster. The iron in both the [NiFe] and the [FeFe] center is complexed by CO, cyanide and sulfur ligands, and the iron in [NiFe] hydrogenase and the distal iron in [FeFe] hydrogenase (distal to the [4Fe–4S] cluster) are in a low-spin Fe(II) oxidation state [12–15]. Both enzymes catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze the reversible heterolytic cleavage of H₂ to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H₂ [1, 16] (Eq. 2). They also catalyze

\[
\begin{align*}
H_2 + 2 \text{MV} & \rightleftharpoons 2 \text{MV}^- + 2\text{H}^+ \\
D_2 + H_2O & \rightarrow HD + DHO \quad \text{(electron acceptor independent)} \quad (2) \\
D_2 + H_2O & \rightarrow H_2 + D_2O \quad \text{(electron acceptor independent)} \quad (3) \\
\text{para-H}_2 & \rightarrow \text{ortho-H}_2 \quad \text{(electron acceptor independent)} \quad (4) \\
\text{para-H}_2 & \rightarrow \text{ortho-H}_2 \quad \text{(electron acceptor independent)} \quad (5)
\end{align*}
\]

The exchange reactions shown in Eqs. 3 and 4 are essentially irreversible because the D⁺ concentration in the bulk H₂O is very low. Mutants of [NiFe] hydrogenase have been made that can catalyze only the reaction shown in Eq. 5 or only the reactions shown in Eqs. 3, 4 and 5, indicating that H₂ binding to the enzyme, exchange of protons with bulk water and electron transfer to the electron acceptor are individual steps [17].

[Fe] hydrogenase differs from [NiFe] and [FeFe] hydrogenases in that it contains a mononuclear metal center rather than a dinuclear metal center in its active site [10] and is devoid of iron–sulfur clusters, which is why the enzyme is also referred to as iron–sulfur-cluster-free hydrogenase [18]. [Fe] hydrogenase catalyzes the reversible reduction of methenyltetrahydromethanopterin (methenyl-H₄MPT⁺) with H₂ to methylenetetrahydromethanopterin (methylene-H₄MPT) (Eq. 6), a reaction involved in CO₂ reduction with H₂ to methane in many methanogenic archaea [9, 19–23]. In the reaction a hydride from H₂ is transferred into the pro-R position of the C(14a) methylene group of the reaction product [24] (Fig. 1). The systematic name for [Fe] hydrogenase is hydrogen-forming methylenetetrahydromethanopterin dehydrogenase, abbreviated Hmd. The Kₘ and Vₘₐₓ of the enzyme for H₂ and D₂ were found to be almost identical, indicating that a step other than the activation of H₂ is rate-determining in the reaction shown in Eq. 6 [25]. [Fe] hydrogenase also catalyzes a stereospecific direct exchange of the pro-R hydrogen of methylene-H₄MPT with protons of water (Eq. 7) [26], a methenyl-H₄MPT⁺-dependent single and double exchange between D₂ and protons of bulk water (Eqs. 8, 9) [27, 28] and a methenyl-H₄MPT⁺-dependent conversion of para-H₂ into ortho-H₂ (Eq. 10) [29]. [Fe] hydrogenase does not catalyze the reduction of viologen dyes (Eq. 2) or other artificial one-electron or two-electron acceptors neither in the absence nor in the presence of methenyl-H₄MPT⁺. The catalytic mechanism of the enzyme is thus clearly “ternary complex,” rather than “ping-pong” and thus quite different from that of [NiFe] and [FeFe] hydrogenases [23].

\[
\begin{align*}
H_2 + \text{methenyl-H}_4\text{MPT}^+ & = \text{methylene-H}_4\text{MPT} + \text{H}^+ \\
\Delta G^\circ & = -5.5 \text{ kJ mol}^{-1} \quad (6) \\
(14aR)-[14a-H]\text{methylene-H}_4\text{MPT} + \text{D}_2\text{O} & \rightarrow (14aR)-[14a-D]\text{methylene-H}_4\text{MPT} + \text{HDO} \quad (7) \\
\text{D}_2 + \text{H}_2\text{O} & \rightarrow \text{HD} + \text{HDO} \quad \text{(methylene-H}_4\text{MPT}^+\text{-dependent)} \quad (8) \\
\text{D}_2 + \text{H}_2\text{O} & \rightarrow \text{H}_2 + \text{D}_2\text{O} \quad \text{(methylene-H}_4\text{MPT}^+\text{-dependent)} \quad (9) \\
\text{para-H}_2 & \rightarrow \text{ortho-H}_2 \quad \text{(methenyl-H}_4\text{MPT}^+\text{-dependent)} \quad (10)
\end{align*}
\]

Of special interest is the dependence of the reactions in Eqs. 8 and 9 on the presence of methenyl-H₄MPT⁺. In its absence the H₂/H⁺ exchange activity was found to be less than 0.01% of the exchange rate in its presence. The residual activity was attributed to small contaminations of the [Fe] hydrogenase preparations with methenyl-H₄MPT⁺ present in high concentrations in the methanogens from which [Fe] hydrogenase was isolated [11].
[Fe] hydrogenase is a cytoplasmic homodimeric enzyme (2 × 38 kDa) harboring an iron-containing cofactor, which can be extracted from the enzyme after denaturation in the presence of mercaptoethanol and with which active hydrogenase can be reconstituted from heterologously produced apoprotein [22]. The iron in the cofactor, both when bound to the enzyme and in the free state, is complexed by a pyridone (possibly functionally equivalent to a cyanide), two CO and a sulfur and is in a low-spin and low-oxidation state [10, 11, 30–32]. [Fe] hydrogenase is thus similar to [NiFe] and [FeFe] hydrogenases in containing a low-spin iron carbonyl complex in its active site, albeit in a mononuclear rather than in a dinuclear metal center. This is remarkable since the three hydrogenases are not phylogenetically related and since iron carbonyl complexes have until now not been found in the active site of any other metalloenzyme. The presence of an iron carbonyl complex in [Fe], [FeFe] and [NiFe] hydrogenases indicates that the low-spin iron could play a crucial role in H₂ activation in all three enzymes.

The low-spin iron in [Fe] hydrogenase could in principle function in the activation of H₂ as a Lewis acid or a Lewis base. As a Lewis acid (electrophile) it will bind H₂ side-on [(η²-H₂)Fe] by which the pK of H₂ of 35 is significantly lowered [33–35]. Depending on the decrease in pK, the thus-activated H₂ would exchange more or less rapidly with protons of water and essentially this exchange is not predicted to require the presence of the hydride acceptor methenyl-H₄MPT⁺. When the iron functions as a Lewis base (nucleophile), exchange of the bound H₂ with protons of water is only possible in the presence of the hydride acceptor [36]. These predictions fostered the question whether the residual H₂/H⁺ exchange activity shown by [Fe] hydrogenase preparations might not be independent of methenyl-H₄MPT⁺ after all. Because of the importance of this question for the catalytic mechanism, we took up the problem again and measured the residual exchange activity of [Fe] hydrogenase reconstituted from highly purified recombinant apoenzyme and highly purified iron-containing cofactor.

Materials and methods

Tritium-labeled H₂O (185 MBq mL⁻¹) was from GE Healthcare (Munich, Germany). Lithium wire (99%) of 3.2-mm diameter in mineral oil was from Sigma-Aldrich (Taufkirchen, Germany). Tetrahydromethanopterin (H₄MPT) and methenyl-H₄MPT⁺ were purified from Methanothermobacter marburgensis [37].

Generation of tritium-labeled H₂ from T₂O and elemental lithium

The detection limit of the T₂/H₂O exchange assay employed is strongly dependent on the tritium-labeled H₂ used being as free as possible of T₂O and NT₃, which is why the method of T₂ generation from tritium-labeled water and elemental lithium is described in greater detail.

The reaction of lithium with H₂O had to be done under argon rather than N₂ since lithium in water can slowly reduce N₂ to NH₃, which is tritium-labeled when the reaction is in T₂O. Any tritium-labeled NH₃ in the gas phase will increase the background radioactivity in the exchange assay and thus increase the lower detection limit.

T₂ was produced from T₂O and an excess of metallic lithium under argon [18, 38]. Glass vials with a total volume of 8.5 mL, closed with a rubber stopper, were degassed via vacuum and refilled with N₂. After backfilling three times, they were again placed under vacuum. The lithium wire was then prepared so that it could be added to the vial. Approximately 2.5 cm (0.15 g; approximately 20 µmol) of the wire was removed from the oil in which it was stored and placed on a piece of wax-coated paper. An additional drop of oil was added on top of the wire to ensure that the lithium was protected from N₂, O₂ and H₂O. Under a cover of oil, the lithium wire was dissected into little pieces with a width of approximately 1 mm or less using a scalpel. At this point, the 8.5-mL vial, which was under vacuum, was filled with argon via a needle through the rubber stopper such that the rubber stopper could be lifted slightly under a steady flow of argon and no N₂ or O₂ could enter the vial. Each sliver of lithium was then picked up using tweezers and submerged into a vial containing petroleum ether. Once the oil had been removed the sliver was quickly transferred into the vial, which was being kept under an “argon cover.” After all of the lithium pieces had been transferred in such a manner, the vial was again...
Purification of the [Fe] hydrogenase apoenzyme

The [Fe] hydrogenase apoenzyme from *Methanocaldovorax jannaschii* was heterologously produced in *Escherichia coli* BL21 (DE3) harboring the expression vector pET-24b, which carried the *hmd* gene from *M. jannaschii*. The transformed bacterium was cultured aerobically in 2 L tryptone–phosphate medium [39]. At an optical density at 578 nm of 1.4 *hmd* transcription was induced by the addition of isopropylthiogalactoside (1 mM). Three hours after induction, the 2 L culture was harvested by centrifugation. The cell debris was removed by ultracentrifugation at 116,000 g for 45 min at 4 °C. The supernatant was heated for 20 min at 70 °C, centrifuged again and then applied to a 2.5 cm packed phenyl-Sepharose column (2.6 cm × 20 cm) equilibrated with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH pH 7.0 containing 1 mM dithiothreitol and were then disrupted anaerobically at 4 °C using a French press and a pressure of 130 MPa. Cell debris was removed by ultra-centrifugation at 116,000 g for 45 min at 4 °C. The supernatant was heated for 20 min at 70 °C, centrifuged again for the removal of denatured proteins, and then supplemented with ammonium sulfate to a final concentration of 2 M, centrifuged again and then applied to a phenyl-Sepharose column (2.6 cm × 20 cm) equilibrated with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH pH 7.0 containing 2 M ammonium sulfate and 1 mM dithiothreitol. [Fe] hydrogenase apoenzyme was eluted with a linear decreasing gradient of ammonium sulfate (5-ML fractions). The apoenzyme-containing fractions [1.2–0.8 M (NH4)2SO4] were combined and concentrated to 50 mM tris(hydroxymethyl)aminomethane/HCl pH 7.8 supplemented with 1 mM dithiothreitol and were then disrupted anaerobically at 4 °C using a French press and a pressure of 130 MPa. Cell debris was removed by ultracentrifugation at 116,000 g for 45 min at 4 °C. The supernatant was heated for 20 min at 70 °C, centrifuged again for the removal of denatured proteins, and then supplemented with ammonium sulfate to a final concentration of 2 M, centrifuged again and then applied to a phenyl-Sepharose column (2.6 cm × 20 cm) equilibrated with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH pH 7.0 containing 2 M ammonium sulfate and 1 mM dithiothreitol. [Fe] hydrogenase apoenzyme was eluted with a linear decreasing gradient of ammonium sulfate (5-ML fractions). The apoenzyme-containing fractions [1.2–0.8 M (NH4)2SO4] were combined and subsequently washed with 50 mM MOPS/KOH pH 7.0 containing 1 mM dithiothreitol and then concentrated to 35 mg protein mL–1 using microconcentrators with a 10-kDa cutoff. In one preparation typically 40 mg of [Fe] hydrogenase apoenzyme were obtained.

[Fe] hydrogenase apoenzyme was converted completely into [Fe] hydrogenase holoenzyme by mixing the apoenzyme with a threefold excess of the iron-containing [Fe] hydrogenase cofactor. The reconstituted enzyme was then washed three times with 120 mM potassium phosphate pH 6.0 containing 1 mM EDTA. This step was followed by a concentration of the solution to a final protein concentration of 30 mg mL–1 using microconcentrators with a 10-kDa cutoff. The reconstituted enzyme catalyzed the dehydrogenation of methylene-H4MPT (20 μM) at 25 °C and at pH 6.0 with a specific activity of 150 U mg–1 protein.

Purification of the iron-containing [Fe] hydrogenase cofactor

The [Fe] hydrogenase cofactor was extracted from [Fe] hydrogenase holoenzyme of *M. marburgensis*. The holoenzyme was purified from 80 g cells (wet mass) grown at 65 °C under nickel-limiting conditions, under which the synthesis of [Fe] hydrogenase in *M. marburgensis* is enhanced [40–42]. To extract the cofactor 50 mg purified enzyme was incubated in 10 mL H2O containing 60% methanol, 1 mM mercaptoethanol and 1% ammonia at 4 °C for 20 h, conditions under which the cofactor was completely released. Then the cofactor was separated from the denatured apoprotein by ultrafiltration using Amicon Ultra-15 (10-kDa cutoff). After concentration by anoxic evaporation at 4 °C the cofactor solution was ultrafiltered again. The iron-containing cofactor was further purified at 18 °C by preparative high-performance liquid chromatography (HPLC; Sykam) on HiTrapQFF (GE Healthcare; 0.7 cm × 2.5 cm), which was equilibrated with 10 mM ammonium carbonate pH 9.0 containing 1 mM mercaptoethanol (ACM buffer). The cofactor was eluted using a linear increasing gradient of NaCl from 0 to 1 M in ACM buffer. The cofactor was eluted at approximately 500 mM NaCl. The cofactor-containing fractions were combined and immediately used for reconstitution. All purification steps were done under red light in an anaerobic chamber (Coy) filled with 95% N2/5% H2 and containing a palladium catalyst for the continuous removal of O2.

Determination of [Fe] hydrogenase activities

The rate of methylene-H4MPT dehydrogenation was determined at pH 6.0 and 25 °C by following the formation of methenyl-H4MPT+ from methylene-H4MPT (20 μM) photometrically at 336 nm (ε = 21.6 mM–1 cm–1) [40]. One unit of hydrogenase activity is equivalent to 1 μmol methenyl-H4MPT+ formed per minute.

The rate of T2/H2O exchange was determined at 25 °C in 3.5-mL serum vials closed with a rubber stopper. The 1 mL assay mixtures contained 120 mM potassium phosphate pH 6.0 (the optimum pH of exchange), 1 mM EDTA and methenyl-H4MPT+ and [Fe] hydrogenase in the amounts indicated in the legends to the figures. The 2.5 mL N2 gas phase at 1.2 × 10–5 Pa contained either 14 or 24% tritium-labeled H2 with a specific radioactivity of 2.4 or 3.3 kBq μmol–1. Before the start of the reaction with [Fe] hydrogenase the gas phase was equilibrated with the liquid phase by 15 min of shaking at 1,100 rpm. Then a 0.1 mL sample was taken (for the determination of the background) and subsequently the enzyme was added. Upon further shaking 0.1 mL samples were withdrawn and
placed into empty 20-mL plastic scintillation vials to which 5 mL scintillation cocktail Quicksafe A (Zinsser Analytic) was added. Then the samples were counted for tritium radioactivity in Beckman LS6500 scintillation system. From the specific radioactivity of the tritium-labeled H2 and the radioactivity above the background incorporated into water per 60 min, the exchange activity of the [Fe] hydrogenase added to the assay is calculated. One unit of exchange activity is equivalent to 1 µmol H2 exchanged into water per minute.

It was previously shown that the methenyl-H4MPT+ dependent exchange between D2 and H2O and that between H2 and D2O catalyzed by [Fe] hydrogenase proceed with almost the same specific activity, indicating the absence of major isotope effects [25].

Results

The H2/H+ exchange activity of [Fe] hydrogenase was measured by following the incorporation of tritium from tritium-labeled H2 (2.4 kBq µmol–1) into 1 mL H2O. This method is much more sensitive than following the formation of HD and D2 from H2 in D2O or of HD and H2 from D2 in H2O by mass spectrometry. At the times indicated, 0.1 mL samples were withdrawn and counted by liquid scintillation counting. Via this method an increase in radioactivity in the 0.1 mL sample by 10 Bq (4.1 nmol tritium-labeled H2 exchanged into 0.1 mL H2O) within 60 min could easily be seen (Fig. 2, triangles). Also an increase by 5 Bq (2.05 nmol) in 60 min was still well observable (Fig. 2, circles). An increase of less than 1.2 Bq (0.5 nmol) in 60 min is the lower detection limit, which is equivalent to an exchange activity of the [Fe] hydrogenase in the 1 mL assay of 0.1 mU. This indicates that at a [Fe] hydrogenase concentration of 1 mg mL–1 assay, the specific exchange activity would have to be higher than 0.1 mU mg–1 to be observable.

The following experiments were performed with [Fe] hydrogenase reconstituted from recombinant [Fe] hydrogenase apoenzyme from M. jannaschii and iron-containing [Fe] hydrogenase cofactor of Methanothermobacter marburgensis. The apoenzyme from M. jannaschii rather than that from M. marburgensis was chosen because it did not form inclusion bodies when heterologously overproduced in E. coli. The apoenzyme was purified via heat denaturation of most of the E. coli proteins (M. jannaschii is a hyperthermophile) followed by chromatography on phenyl-Sepharose. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the preparations contain only one polypeptide of apparent molecular mass of approximately 40 kDa (not shown). The iron-containing cofactor was extracted from [Fe] hydrogenase purified from M. marburgensis.

Fig. 2 Detection limit of the method employed for the determination of the T2/H2O exchange activity of [Fe] hydrogenase. The assays were performed in 3.5-mL vials closed with a rubber stopper. The vials contained 1 mL standard assay mixture: 120 mM potassium phosphate pH 6.0, 1 mM EDTA and either no enzyme (open squares) or 0.5 mg (circles), 1.0 mg (triangles) or 10 mg (filled squares) purified [Fe] hydrogenase from Methanothermobacter marburgensis. The 2.5 mL gas phase consisted of 24% tritium-labeled H2 (2.4 kBq µmol–1) and 76% N2 at 1.2 × 105 Pa. At the times indicated, 0.1 mL liquid samples were withdrawn and analyzed for tritium radioactivity. From the specific radioactivity of tritium-labeled H2 and the radioactivity above the background incorporated into water per 60 min, the exchange activity of the [Fe] hydrogenase in the 1 mL assay is calculated to be 0.35 mU (circles), 0.7 mU (triangles) and 8 mU (filled squares). The results show that the lower detection limit is near 0.1 mU. One unit is equivalent to 1 µmol H2 exchanged into water per minute

Reconstitution of the enzyme of M. jannaschii with this cofactor was possible since the [Fe] hydrogenases from different methanogens all appear to have the same iron-containing cofactor. Under the alkaline extraction conditions employed (1% NH3 in H2O; pH ~ 11) any contaminating methenyl-H4MPT+ would have been destroyed. Other contaminants and the destroyed methenyl-H4MPT+ were separated from the iron-containing cofactor by HPLC. [Fe] hydrogenase holoenzyme was reconstituted by the addition of 3 times the molar amount of the iron-containing cofactor to the apoprotein and subsequently excess cofactor was removed by ultrafiltration. The T2/H2O exchange activity of the reconstituted [Fe] hydrogenase was determined at room temperature since at this temperature the enzyme was active in the exchange assay for more than 60 min (Fig. 2). At 65 °C the reconstituted enzyme was rapidly inactivated despite the fact that the optimum temperature for growth of M. jannaschii is 85 °C.

The reconstituted enzyme catalyzed the conversion of methylene-H4MPT (20 µM) to methenyl-H4MPT+ and H2 with a specific activity of 150 U mg–1 at pH 6.0 and room
temperature. The apparent \( K_m \) for methylene-H\(_4\)MPT was 10 \( \mu \)M and the apparent \( V_{\text{max}} \) was 220 U mg\(^{-1}\). At 65 °C the specific activity was above 1,000 U mg\(^{-1}\) (results not shown).

Reconstituted [Fe] hydrogenase catalyzed the T\(_2/H_2O\) exchange reaction in the presence of methenyl-H\(_4\)MPT\(^+\) with a specific activity of 10 U mg\(^{-1}\) under our standard assay conditions (5 \( \mu \)M methenyl-H\(_4\)MPT\(^+\), 24% \( H_2 \) in the gas phase equilibrated with the liquid phase by shaking at 1,100 rpm, pH 6.0, room temperature). The reaction proceeded linearly with time for more than 20 min (Fig. 3a) and the rate was proportional to the protein concentration up to 40 \( \mu \)g mL\(^{-1}\) (Fig. 3b). At higher protein concentrations the specific exchange activity decreased because the rate of \( H_2 \) diffusion from the gas phase into the liquid phase became rate limiting as indicated by the finding that at shaking rates lower than 1,100 rpm the specific exchange activity decreased at lower protein concentrations (not shown). When methenyl-H\(_4\)MPT\(^+\) was omitted from the assays, no T\(_2/H_2O\) exchange was observable (Fig. 3).

In Fig. 4 the dependence of the T\(_2/H_2O\) exchange rate on the methenyl-H\(_4\)MPT\(^+\) concentration is shown at two different \( H_2 \) concentrations. Reciprocal plots of the rates versus the methenyl-H\(_4\)MPT\(^+\) concentration yielded two straight lines intersecting on the abscissa to the left of the ordinate at a \( K_A \) of 4 \( \mu \)M. At zero methenyl-H\(_4\)MPT\(^+\) concentration the rate was essentially zero. At infinite methenyl-H\(_4\)MPT\(^+\) concentration the apparent \( V_{\text{max}} \) was 7 U mg\(^{-1}\) for 14% tritium-labeled \( H_2 \) and 12 U mg\(^{-1}\) for 24% tritium-labeled \( H_2 \) in the gas phase (Fig. 4). When the \( H_2 \) concentration in the gas phase was increased above 24% the exchange activity increased hyperbolically with the \( H_2 \) concentration, half-maximal activity being reached at a \( H_2 \) concentration of approximately 60% in the gas phase (approximately 0.5 mM \( H_2 \) in the liquid phase at 25 °C). The extrapolated maximal specific activity at infinitely high \( H_2 \) and methenyl-H\(_4\)MPT\(^+\) concentrations was 40 U mg\(^{-1}\) (\( V_{\text{max}} \)) (results not shown). \( V_{\text{max}} \) for the exchange reaction (40 U mg\(^{-1}\)) is thus 18% of \( V_{\text{max}} \) of indicated microgram amounts of reconstituted [Fe] hydrogenase from Methanocaldococcus jannaschii (jHmd). Where indicated, the assays contained 5 \( \mu \)M methenyl-H\(_4\)MPT\(^+\). One unit is equivalent to 1 \( \mu \)mol \( H_2 \) exchanged into water per minute.
methylene-H₄MPT dehydrogenation to methenyl-H₄MPT⁺ and H₂ (220 U mg⁻¹) under the same experimental conditions. The kinetic properties of the reconstituted [Fe]−hydrogenase from *M. jannaschii* are very similar to those of the holoenzyme purified from *M. marburgensis* [23].

To substantiate the finding that the T₂/H₂O exchange activity of [Fe] hydrogenase is absolutely dependent on methenyl-H₄MPT⁺, the [Fe] hydrogenase concentration was increased to 1 mg mL⁻¹ (0.025 mM) (Fig. 3b). Even at this high concentration, there was no measurable T₂/H₂O exchange in the absence of added methenyl-H₄MPT⁺. The radioactivity measured for the 0.1 mL samples was 1.3 ± 0.4 Bq throughout the incubation time of more than 2 h. The same radioactivity was found when bovine serum albumin (1 mg mL⁻¹) instead of [Fe] hydrogenase was present in the assays (not shown). The relatively high but constant background radioactivity of 1.3 ± 0.4 Bq determined by scintillation counting is mainly due to traces of T₂O in the T₂ gas, which was generated from T₂O and Li(0).

**Discussion**

The results show that [Fe] hydrogenase has a relatively high affinity for methenyl-H₄MPT⁺ (Kₐ = 4 μM; Kₐ = 1/2Kₛ = 0.25 × 10⁶ M⁻¹) (Fig. 4) and that the enzyme catalyzes T₂/H₂O exchange only in the presence of methenyl-H₄MPT⁺ even when determined at very high [Fe] hydrogenase concentrations (Fig. 3b). The high affinity of the enzyme for methenyl-H₄MPT⁺ indicates that some methenyl-H₄MPT⁺, if present, will partially copurify with [Fe] hydrogenase. This can explain why [Fe] hydrogenase holoenzyme purified from methanogenic archaea, which contain high H₂MPT and methenyl-H₂MPT⁺ concentrations (more than 1 mM) [43], exhibit some exchange activity in the absence of added methenyl-H₂MPT⁺ (approximately 1 compared with 10 U mg⁻¹ in the presence of methenyl-H₂MPT⁺). We therefore heterologously produced the apoenzyme in *E. coli* which does not contain H₂MPT and reconstituted the holoenzyme from the apoenzyme and highly purified iron-containing cofactor. In the absence of added methenyl-H₂MPT⁺ the reconstituted enzyme did not catalyze the exchange reaction within the detection limit of the method employed, which was near 0.1 mU mg⁻¹ (Fig. 2). The apoprotein and iron-containing [Fe] hydrogenase cofactor, when tested alone in the absence or presence of methenyl-H₂MPT⁺, did not catalyze the exchange reaction, indicating that for T₂/H₂O exchange the apoprotein, the iron-containing cofactor and methenyl-H₂MPT⁺ are required.

It has been shown that C(14a) of methenyl-H₂MPT⁺, when bound to [Fe] hydrogenase, has carbocation character [44–46]. Carbocations can bind H₂ either side-on or end-on, which is followed by a heterolytic cleavage of H₂, the hydride reacting with the carbocation and the proton with a base [36, 47, 48, 49]. For methenyl-H₂MPT⁺ the activation barrier for the reduction reaction has been calculated to be reasonable only when in the transition state H₂ binds end-on to the carbocation and a base is positioned relative to H₂ such that it can directly accept the proton [50–52]. The base was assumed to be the amine group of a lysine or the carboxyl group of an aspartate or a glutamate and the protonated base to be in proton exchange with bulk water [36, 47]. At that time iron was not considered as a base since the enzyme was then still thought to be “metal free” [23, 36] (for metal-free hydrogen activation see [53]). But the supposed base could principally also be an iron(0) complex or an iron(II) complex forming an iron(II) hydride or an iron(IV) hydride [54–56], respectively, upon protonation. The proposed mechanism predicts that any H₂/H⁺ exchange catalyzed by [Fe] hydrogenase should be absolutely dependent on the presence of methenyl-H₂MPT⁺, which is what we found.

The mechanism described above fails to explain why the base has to be an iron and cannot be a proton-accepting group of the protein. It is also not favored by the finding that the exchange reaction is inhibited by CO and by cyanide. CO inhibition is competitive to H₂, which strongly suggests that H₂ binds to an open coordination site rather than to a free electron pair [30]. Iron carbonyl complexes with an open coordination site have been shown to form side-on (η²-H₂)Fe complexes that are in proton exchange with bulk water [57–59]. Since in the absence of methenyl-H₂MPT⁺ [Fe] hydrogenase does not catalyze such an exchange, it has to be assumed that in the absence of methenyl-H₂MPT⁺ H₂ binds to the iron in [Fe] hydrogenase only weakly, resulting in an exchange activity too low to be detected by the method employed. The requirement of methenyl-H₂MPT⁺ for H₂ binding to the enzyme is also indicated by the finding that the IR spectrum [30] and the circular dichroism spectrum (unpublished result) of [Fe] hydrogenase change significantly only when both H₂ and methenyl-H₂MPT⁺ are present.

If the iron in [Fe] hydrogenase functions as Lewis acid, as suggested in the paragraph above, then how can the methenyl-H₂MPT⁺ dependency of the H₂/H₂O exchange be explained? There are three possibilities which come to mind:

1. Methenyl-H₂MPT⁺ induces a conformational change within the active site of the enzyme leading to an activation of the iron which catalyzes the H₂/H₂O exchange without the carbocation center of methenyl-H₂MPT⁺ being involved.
2. Methenyl-H₄MPT⁺ binds to the enzyme such that its carbocationic C(14a) is juxtapositioned to the iron such as to allow the interaction of H₂ with both the iron and the carbocation. This interaction would lead to a polarization of H₂ to an extent in which a bridging hydride and a bridging proton are formed between the two nucleophiles (shown for D₂ binding in Fig. 5) (the carbocation is assumed to resemble a second transition metal center as do carbenes [49]). The cationic (µ-H)₂ complex thus formed is predicted to be a relatively strong acid and therefore to be in proton exchange with bulk water.

3. Both the methenyl-H₄MPT⁺-induced conformational change and the juxta position of the carbocationic C(14a) of methenyl-H₄MPT⁺ are required for H₂ activation, which is a combination of possibilities 1 and 2.

A larger conformational change of [Fe] hydrogenase upon methenyl-H₄MPT⁺ binding is indicated by the finding that [Fe] hydrogenase crystals crack upon soaking with methenyl-H₄MPT⁺. Also, it was found that in the presence of methenyl-H₄MPT⁺, [Fe] hydrogenase does not crystallize under the conditions under which the enzyme crystallizes in the absence of methenyl-H₄MPT⁺ (unpublished results).

In Fig. 6a, the kinetics of exchange between D₂ and H₂O as catalyzed by [Fe] hydrogenase in the presence of methenyl-H₄MPT⁺ (Fig. 6a). The carbocationic C(14a) of methenyl-H₄MPT⁺ bound to the enzyme is shown as C'. The carbocation is assumed to resemble a second transition metal center as do carbenes [49]. The catalytic cycle starts with the formation of the (η²-D₂)Fe complex, which is in electronic equilibrium with the cationic (µ-D)₂ complex. This is followed by an exchange of the (µ-D)₂ complex with protons of bulk water and either by a rotation of HD in the (η²-HD)Fe complex followed by a second exchange or by the dissociation of the complex with the release of HD. The parallel formation of HD and H₂ at equal rates (Fig. 6a) can be explained assuming that \( k_{off} = k_{exchange} \) and that all other steps in the catalytic cycle are not rate limiting.

![Fig. 5](image)

**Fig. 5** Mechanism that can explain the parallel formation of HD and H₂ from D₂ and H₂O as catalyzed by [Fe] hydrogenase in the presence of methenyl-H₄MPT⁺ (Fig. 6a). The carbocationic C(14a) of methenyl-H₄MPT⁺ bound to the enzyme is shown as C'. The carbocation is assumed to resemble a second transition metal center as do carbenes [49]. The catalytic cycle starts with the formation of the (η²-D₂)Fe complex, which is in electronic equilibrium with the cationic (µ-D)₂ complex. This is followed by an exchange of the (µ-D)₂ complex with protons of bulk water and either by a rotation of HD in the (η²-HD)Fe complex followed by a second exchange or by the dissociation of the complex with the release of HD. The parallel formation of HD and H₂ at equal rates (Fig. 6a) can be explained assuming that \( k_{off} = k_{exchange} \) and that all other steps in the catalytic cycle are not rate limiting.

![Fig. 6](image)

**Fig. 6** Kinetics of H₂ and HD formation from D₂ and H₂O catalyzed a by [Fe] hydrogenase from *M. marburgensis* and b by the H₂-signaling [NiFe] hydrogenase from *Rhodobacter capsulatus*. a The 7 mL assay mixtures at 40 °C contained 120 mM potassium phosphate pH 6.0, 7 µM methenyl-H₄MPT⁺ and 150 µM D₂ (20% saturation). The reactions were started by the addition of 0.13 U purified [Fe] hydrogenase from *M. marburgensis*. Dihydrogen isotopes were determined on line by mass spectrometry. b The 1.5 mL assay mixtures at 30 °C contained 50 mM citrate–phosphate pH 7.0 and cells of *R. capsulatus* mutants containing only the H₂-signaling hydrogenase. The suspension was gassed with 100% D₂ before the vessel was closed and the formation of HD and that of H₂ were monitored by mass spectrometry. (a The data were taken from [27] with permission; b the data were taken from [67] with permission)
outlined in Fig. 5 in which it is assumed that the iron and the C(14a) of methenyl-H₄MPT⁺ have to be juxtapositioned such that the H₂ can interact with both the iron and the C(14a) (possibilities 2 or 3 discussed above). The mechanism implies that upon binding of D₂ side-on to [Fe] hydrogenase in the presence of methenyl-H₄MPT⁺ an (η²-D₂)Fe complex is formed which is in rapid equilibrium with a cationic (μ-D)₂ complex. Only the bridging D⁺, the one not covalently bound to C(14a), exchanges with a proton of water, yielding a (μ-H)(μ-D) complex in equilibrium with a (η²-HD)Fe complex. From the (η²-HD)Fe complex the HD can dissociate off. Alternatively, after rotation of HD in the (η²-HD)Fe complex by 180° [60] a (μ-H)(μ-D) complex is re-formed in which the bridging H is covalently bound to C(14a) of methenyl-H₄MPT⁺ and in which the bridging D is acidic and therefore exchanges with protons of bulk water. For the observed double exchange the rotation is required since the bridging hydride ends up only in the pro-R position of the methylene group of methylene-H₄MPT. The second exchange yields a (μ-H)₂ complex in equilibrium with a (η²-H₂)Fe complex, from which H₂ can dissociate off (Fig. 5). With respect to the kinetics it is assumed that the two proton exchange rates (kₜₜₜ) are equal and that also the two off-dissociation rates (kₜₜₜ) (include diffusion rates out of the enzyme’s active-site pocket) are equal and that both the exchange rates and the off-dissociation rates are slow relative to the rates of all other steps, including the rotation step involved in the proposed catalytic cycle. With these assumptions the exchange mechanism proposed in Fig. 5 predicts that HD and H₂ are parallelly formed from D₂ and H₂O at equal rates when kₜₜₜ ≅ kₜₜₜ. If kₜₜₜ < kₜₜₜ then more HD than H₂ is formed and if kₜₜₜ > kₜₜₜ then more H₂ than HD is generated.

The cationic (μ-H)₂ complex proposed to be formed upon binding of methenyl-H₄MPT⁺ and H₂ to [Fe] hydrogenase (shown for D₂ binding in Fig. 5) is most likely also formed upon binding of methylene-H₄MPT to the enzyme, which can explain the direct exchange of the pro-R hydrogen of methylene-H₄MPT with protons of bulk water (Eq. 7).

In Fig. 6b the kinetics of single and double exchange of D₂ with H₂O catalyzed by H₂-signaling [NiFe] hydrogenase from Rhodobacter capsulatus are shown. They are very similar to those shown in Fig. 6a for the [Fe] hydrogenase from M. marburgensis but are also similar to the exchange kinetics for reactions catalyzed by other [NiFe] and [FeFe] hydrogenases, the only differences being that the relative rates of the single-exchange and the double-exchange reaction vary from enzyme to enzyme (Table 1). This finding substantiates the notion that the low-spin iron carbonyl in the active sites of the three types of hydrogenases could have the same function, namely, to help polarize the H₂ such that a hydride can be transferred to an acceptor, which is the C(14a) of methenyl-H₄MPT⁺ in the case of [Fe] hydrogenase, the proximal iron of the diiron center in the case of [FeFe] hydrogenases and the nickel in the NiFe center in case of [NiFe] hydrogenases.

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