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ORIGINAL PAPER

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-H_4MPT$^+$) with H_2 to methylene-H_4MPT, a reaction involved in methanogenesis from H_2 and CO_2 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 H_2 activation. Like the [NiFe] and [FeFe] hydrogenases, [Fe] hydrogenase catalyzes an active exchange of H_2 with protons of water; however, this activity is dependent on the presence of the hydride-accepting methenyl-H_4MPT$^+$. 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-H_4MPT$^+$ in the enzyme preparations, but it could also reflect a weak binding of H_2 to the iron in the absence of methenyl-H_4MPT$^+$. 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-H_4MPT$^+$ 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 H_2 activation by [Fe] hydrogenase the presence of the hydride-accepting methenyl-H_4MPT$^+$ is essentially required. This differentiates [Fe] hydrogenase from [FeFe] and [NiFe] hydrogenases, which actively catalyze H_2/H_2O exchange in the absence of exogenous electron acceptors.

Keywords Hydrogenase · Exchange reactions · Methanogenic archaea

Abbreviations

H_4MPT	Tetrahydromethanopterin
Hmd	H_2-forming methylenetetrahydromethopterin dehydrogenase
MV	Methyl viologen
D	^{2}H
T	^{3}H

Introduction

Hydrogenases are enzymes that catalyze reversible reactions with H_2 as reactant or product (Eqs. 1a, 1b, 1c):

$$H_2 + X \rightleftharpoons XH_2, \quad (1a)$$

$$H_2 + X \rightleftharpoons XH^- + H^+, \quad (1b)$$

$$H_2 + X \rightleftharpoons X^{2-} + 2H^+, \quad (1c)$$

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

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hydrogenases catalyze the reversible oxidation of H_2 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 H_2 to two protons and two electrons as evidenced by the reversible reduction of the artificial one-electron acceptor methyl viologen (MV) with H_2 [1, 16] (Eq. 2). They also catalyze a single and a double exchange of D_2 with protons of bulk water (Eqs. 3, 4) and the exergonic conversion of *para*-H_2 to *ortho*-H_2 (Eq. 5) in the absence of exogenous electron acceptors, indicating that [NiFe] and [FeFe] hydrogenases have a ping-pong catalytic mechanism [2].

$$H_2 + 2\ MV \rightleftharpoons 2\ MV^- + 2H^+ \quad (2)$$

$$D_2 + H_2O \rightarrow HD + DHO \quad \text{(electron acceptor independent)} \quad (3)$$

$$D_2 + H_2O \rightarrow H_2 + D_2O \quad \text{(electron acceptor independent)} \quad (4)$$

$$\textit{para}\text{-}H_2 \rightarrow \textit{ortho}\text{-}H_2 \ \text{(electron acceptor independent)} \quad (5)$$

The exchange reactions shown in Eqs. 3 and 4 are essentially irreversible because the D^+ concentration in the bulk H_2O 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 H_2 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-H_4MPT$^+$) with H_2 to methylenetetrahydromethanopterin (methylene-H_4MPT) (Eq. 6), a reaction involved in CO_2 reduction with H_2 to methane in many methanogenic archaea [9, 19–23]. In the reaction a hydride from H_2 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 K_m and V_{max} of the enzyme for H_2 and D_2 were found to be almost identical, indicating that a step other than the activation of H_2 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-H_4MPT with protons of water (Eq. 7) [26], a methenyl-H_4MPT$^+$-dependent single and double exchange between D_2 and protons of bulk water (Eqs. 8, 9) [27, 28] and a methenyl-H_4MPT$^+$-dependent conversion of *para*-H_2 into *ortho*-H_2 (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-H_4MPT$^+$. 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].

$$H_2 + \text{methenyl-}H_4\text{MPT}^+ \rightleftharpoons \text{methylene-}H_4\text{MPT} + H^+ \quad \Delta G^{\circ\prime} = -5.5\ \text{kJ mol}^{-1} \quad (6)$$

$$(14a\textit{RS})\text{-}[14a\text{-}H]\text{methylene-}H_4\text{MPT} + D_2O \rightarrow (14a\textit{R})\text{-}[14a\text{-}D]\text{methylene-}H_4\text{MPT} + HDO \quad (7)$$

$$D_2 + H_2O \rightarrow HD + HDO \ (\text{methenyl-}H_4\text{MPT}^+\text{-dependent}) \quad (8)$$

$$D_2 + H_2O \rightarrow H_2 + D_2O \ (\text{methenyl-}H_4\text{MPT}^+\text{-dependent}) \quad (9)$$

$$\textit{para}\text{-}H_2 \rightarrow \textit{ortho}\text{-}H_2 \ (\text{methenyl-}H_4\text{MPT}^+\text{-dependent}) \quad (10)$$

Of special interest is the dependence of the reactions in Eqs. 8 and 9 on the presence of methenyl-H_4MPT$^+$. In its absence the H_2/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-H_4MPT$^+$ present in high concentrations in the methanogens from which [Fe] hydrogenase was isolated [11].

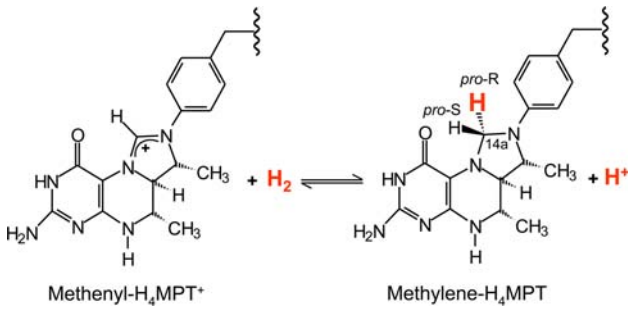

Fig. 1 Reaction catalyzed by [Fe] hydrogenase. N^5,N^{10}-Methenyltetrahydromethanopterin (*methenyl-H_4MPT$^+$*) reduction with H_2 to N^5,N^{10}-methylenetetrahydromethanopterin (*methylene-H_4MPT*) and a proton, whereby a hydride is stereospecifically transferred from H_2 into the *pro-R* side of methylene-H_4MPT [24]

[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 H_2 activation in all three enzymes.

The low-spin iron in [Fe] hydrogenase could in principle function in the activation of H_2 as a Lewis acid or a Lewis base. As a Lewis acid (electrophile) it will bind H_2 side-on [(η^2-H_2)Fe] by which the pK of H_2 of 35 is significantly lowered [33–35]. Depending on the decrease in pK, the thus-activated H_2 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-H_4MPT$^+$. When the iron functions as a Lewis base (nucleophile), exchange of the bound H_2 with protons of water is only possible in the presence of the hydride acceptor [36]. These predictions fostered the question whether the residual H_2/H^+ exchange activity shown by [Fe] hydrogenase preparations might not be independent of methenyl-H_4MPT$^+$ 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 H_2O (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 (H_4MPT) and methenyl-H_4MPT$^+$ were purified from *Methanothermobacter marburgensis* [37].

Generation of tritium-labeled H_2 from T_2O and elemental lithium

The detection limit of the T_2/H_2O exchange assay employed is strongly dependent on the tritium-labeled H_2 used being as free as possible of T_2O and NT_3, which is why the method of T_2 generation from tritium-labeled water and elemental lithium is described in greater detail.

The reaction of lithium with H_2O had to be done under argon rather than N_2 since lithium in water can slowly reduce N_2 to NH_3, which is tritium-labeled when the reaction is in T_2O. Any tritium-labeled NH_3 in the gas phase will increase the background radioactivity in the exchange assay and thus increase the lower detection limit.

T_2 was produced from T_2O 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 N_2. 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 N_2, O_2 and H_2O. 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 N_2 or O_2 could enter the vial. Each sliver of lithium was then picked up using tweezers and submersed in 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

closed and the stopper fastened in place with an aluminum cap. A vacuum was drawn on the vial and then to the evacuated vial 50 μL of tritiated H_2O (2.8 μmol) was added. The vial was placed in a sonic bath for 2 h and then left to stand at room temperature at least 7 days to allow the excess lithium to react with any residual T_2O.

Purification of the [Fe] hydrogenase apoenzyme

The [Fe] hydrogenase apoenzyme from *Methanocaldococcus 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 *E. coli* cells (4–5 g wet mass) were anaerobically resuspended in 15 mL of 50 mM tris(hydoxymethyl)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 $(NH_4)_2SO_4$] 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-H_4MPT (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 H_2O 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 ultrafiltrated 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% N_2/5% H_2 and containing a palladium catalyst for the continuous removal of O_2.

Determination of [Fe] hydrogenase activities

The rate of methylene-H_4MPT dehydrogenation was determined at pH 6.0 and 25 °C by following the formation of methenyl-H_4MPT^+ from methylene-H_4MPT (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-H_4MPT^+ formed per minute.

The rate of T_2/H_2O 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-H_4MPT^+ and [Fe] hydrogenase in the amounts indicated in the legends to the figures. The 2.5 mL N_2 gas phase at 1.2×10^5 Pa contained either 14 or 24% tritium-labeled H_2 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 H_2 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 H_2 exchanged into water per minute.

It was previously shown that the methenyl-H_4MPT^+-dependent exchange between D_2 and H_2O and that between H_2 and D_2O catalyzed by [Fe] hydrogenase proceed with almost the same specific activity, indicating the absence of major isotope effects [25].

Results

The H_2/H^+ exchange activity of [Fe] hydrogenase was measured by following the incorporation of tritium from tritium-labeled H_2 (2.4 kBq μmol^{-1}) into 1 mL H_2O. This method is much more sensitive than following the formation of HD and D_2 from H_2 in D_2O or of HD and H_2 from D_2 in H_2O 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 H_2 exchanged into 0.1 mL H_2O) 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 from *M. 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*.

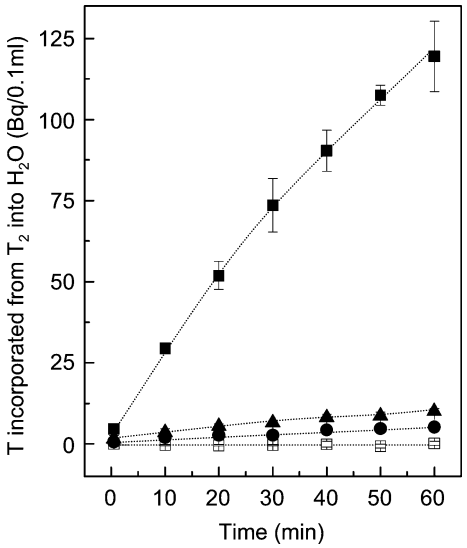

Fig. 2 Detection limit of the method employed for the determination of the T_2/H_2O exchange activity of [Fe] hydrogenase. The assays were performed in 3.5-mL vials closed with a rubber stopper. The vials contained 1 mL standard assay mixture: 120 mM potassium phosphate pH 6.0, 1 mM EDTA and either no 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 H_2 (2.4 kBq μmol^{-1}) and 76% N_2 at 1.2×10^5 Pa. At the times indicated, 0.1 mL liquid samples were withdrawn and analyzed for tritium radioactivity. From the specific radioactivity of tritium-labeled H_2 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 H_2 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% NH_3 in H_2O; pH $\sim$ 11) any contaminating methenyl-H_4MPT^+ would have been destroyed. Other contaminants and the destroyed methenyl-H_4MPT^+ 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 T_2/H_2O 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-H_4MPT (20 μM) to methenyl-H_4MPT^+ and H_2 with a specific activity of 150 U mg^{-1} at pH 6.0 and room

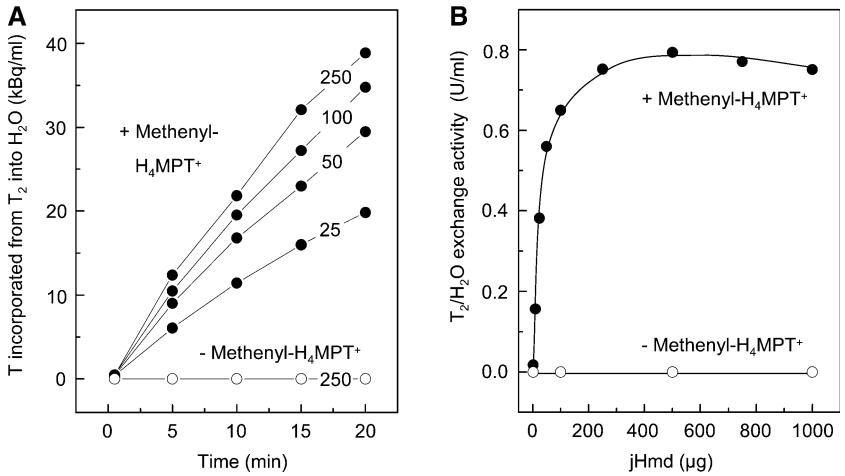

Fig. 3 T_2/H_2O exchange activity of [Fe] hydrogenase in the presence and absence of methenyl-H_4MPT^+. **a** Time dependence at different protein concentrations; **b** protein dependence. The assay conditions were essentially the same as described in the legend to Fig. 2. Instead of [Fe] hydrogenase from *M. marburgensis*, the assays contained the indicated microgram amounts of reconstituted [Fe] hydrogenase from *Methanocaldococcus jannaschii* (*jHmd*). Where indicated, the assays contained 5 µM methenyl-H_4MPT^+. One unit is equivalent to 1 µmol H_2 exchanged into water per minute

temperature. The apparent K_m for methylene-H_4MPT was 10 µM and the apparent V_{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_4MPT^+ with a specific activity of 10 U mg^{-1} under our standard assay conditions (5 µM methenyl-H_4MPT^+, 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 µg 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_4MPT^+ 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_4MPT^+ concentration is shown at two different H_2 concentrations. Reciprocal plots of the rates versus the methenyl-H_4MPT^+ concentration yielded two straight lines intersecting on the abscissa to the left of the ordinate at a K_A of 4 µM. At zero methenyl-H_4MPT^+ concentration the rate was essentially zero. At infinite methenyl-H_4MPT^+ concentration the apparent V_{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_4MPT^+ concentrations was 40 U mg^{-1} (V_{max}) (results not shown). V_{max} for the exchange reaction (40 U mg^{-1}) is thus 18% of V_{max} of

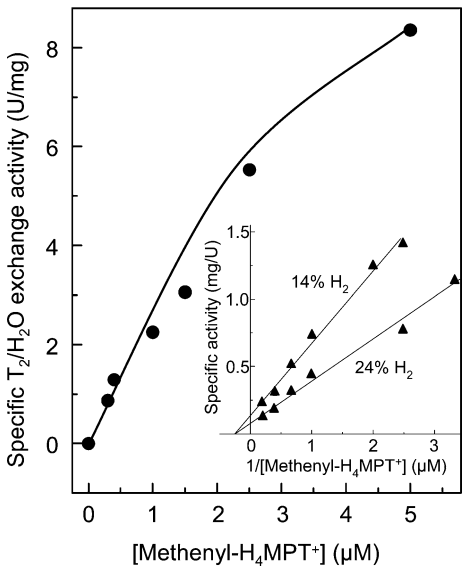

Fig. 4 Dependence of the T_2/H_2O exchange activity of [Fe] hydrogenase on the methenyl-H_4MPT^+ concentration at two different H_2 concentrations. The 1 mL assay contained 120 mM potassium phosphate pH 6.0, 1 mM EDTA, 2.0 µg reconstituted [Fe] hydrogenase from *M. jannaschii* and methenyl-H_4MPT^+ at the concentrations indicated. The gas phase was either 24% tritium-labeled H_2/76% N_2 or 14% tritium-labeled H_2/78% N_2. The specific radioactivity of the tritium-labeled H_2 was 3.3 kBq $µmol^{-1}$. One unit is equivalent to 1 µmol H_2 exchanged into water per minute

methylene-H_4MPT dehydrogenation to methenyl-H_4MPT$^+$ and H_2 (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 T_2/H_2O exchange activity of [Fe] hydrogenase is absolutely dependent on methenyl-H_4MPT$^+$, 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 T_2/H_2O exchange in the absence of added methenyl-H_4MPT$^+$: 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 T_2O in the T_2 gas, which was generated from T_2O and Li(0).

Discussion

The results show that [Fe] hydrogenase has a relatively high affinity for methenyl-H_4MPT$^+$ (K_A = 4 μM; K_{eq} = 1/K_A = 0.25 × 10^6 M^{-1}) (Fig. 4) and that the enzyme catalyzes T_2/H_2O exchange only in the presence of methenyl-H_4MPT$^+$ even when determined at very high [Fe] hydrogenase concentrations (Fig. 3b). The high affinity of the enzyme for methenyl-H_4MPT$^+$ indicates that some methenyl-H_4MPT$^+$, if present, will partially copurify with [Fe] hydrogenase. This can explain why [Fe] hydrogenase holoenzyme purified from methanogenic archaea, which contain high H_4MPT and methenyl-H_4MPT$^+$ concentrations (more than 1 mM) [43], exhibit some exchange activity in the absence of added methenyl-H_4MPT$^+$ (approximately 1 compared with 10 U mg^{-1} in the presence of methenyl-H_4MPT$^+$). We therefore heterologously produced the apoenzyme in *E. coli* which does not contain H_4MPT and reconstituted the holoenzyme from the apoenzyme and highly purified iron-containing cofactor. In the absence of added methenyl-H_4MPT$^+$ 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-H_4MPT$^+$, did not catalyze the exchange reaction, indicating that for T_2/H_2O exchange the apoprotein, the iron-containing cofactor and methenyl-H_4MPT$^+$ are required.

It has been shown that C(14a) of methenyl-H_4MPT$^+$, when bound to [Fe] hydrogenase, has carbocation character [44–46]. Carbocations can bind H_2 either side-on or end-on, which is followed by a heterolytic cleavage of H_2, the hydride reacting with the carbocation and the proton with a base [36, 47, 48, 49]. For methenyl-H_4MPT$^+$ the activation barrier for the reduction reaction has been calculated to be reasonable only when in the transition state H_2 binds end-on to the carbocation and a base is positioned relative to H_2 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 H_2/H^+ exchange catalyzed by [Fe] hydrogenase should be absolutely dependent on the presence of methenyl-H_4MPT$^+$, 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 H_2, which strongly suggests that H_2 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 (η^2-H_2)Fe complexes that are in proton exchange with bulk water [57–59]. Since in the absence of methenyl-H_4MPT$^+$ [Fe] hydrogenase does not catalyze such an exchange, it has to be assumed that in the absence of methenyl-H_4MPT$^+$ H_2 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-H_4MPT$^+$ for H_2 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 H_2 and methenyl-H_4MPT$^+$ are present.

If the iron in [Fe] hydrogenase functions as Lewis acid, as suggested in the paragraph above, then how can the methenyl-H_4MPT$^+$ dependency of the H_2/H_2O exchange be explained? There are three possibilities which come to mind:

1. Methenyl-H_4MPT$^+$ induces a conformational change within the active site of the enzyme leading to an activation of the iron which catalyzes the H_2/H_2O exchange without the carbocation center of methenyl-H_4MPT$^+$ being involved.

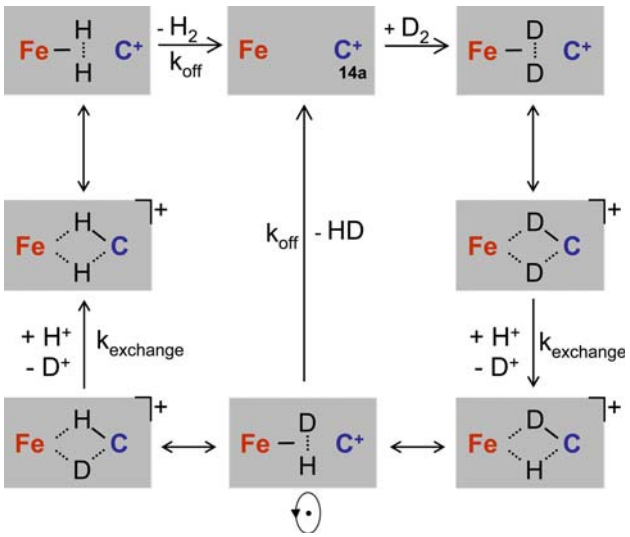

Fig. 5 Mechanism that can explain the parallel formation of HD and H_2 from D_2 and H_2O as catalyzed by [Fe] hydrogenase in the presence of methenyl-H_4MPT^+ (Fig. 6a). The carbocationic C(14a) of methenyl-H_4MPT^+ bound to the enzyme is shown as C^+. The carbocation is assumed to resemble a second transition metal center as do carbenes [49]. The catalytic cycle starts with the formation of the (η^2-D_2)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 (η^2-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 H_2 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

2. Methenyl-H_4MPT^+ binds to the enzyme such that its carbocationic C(14a) is juxtapositioned to the iron such as to allow the interaction of H_2 with both the iron and the carbocation. This interaction would lead to a polarization of H_2 to an extent in which a bridging hydride and a bridging proton are formed between the two nucleophiles (shown for D_2 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-H_4MPT^+-induced conformational change and the juxta position of the carbocationic C(14a) of methenyl-H_4MPT^+ are required for H_2 activation, which is a combination of possibilities 1 and 2.

A larger conformational change of [Fe] hydrogenase upon methenyl-H_4MPT^+ binding is indicated by the finding that [Fe] hydrogenase crystals crack upon soaking with methenyl-H_4MPT^+. Also, it was found that in the presence of methenyl-H_4MPT^+, [Fe] hydrogenase does not crystallize under the conditions under which the enzyme crystallizes in the absence of methenyl-H_4MPT^+ (unpublished results).

In Fig. 6a, the kinetics of exchange between D_2 and H_2O as catalyzed by [Fe] hydrogenase in the presence of methenyl-H_4MPT^+ are shown. HD (single exchange) and H_2 (double exchange) are formed at almost equal initial rates. The apparent V_{max} and K_m of the enzyme for D_2, HD and H_2 were shown to be very similar [25, 28]. Therefore, the almost identical initial single-exchange and double-exchange rates indicate that free HD is not an intermediate in H_2 formation from D_2 and H_2O.

The parallel formation of H_2 and HD from D_2 and H_2O (Fig. 6a) is explainable via the exchange mechanism

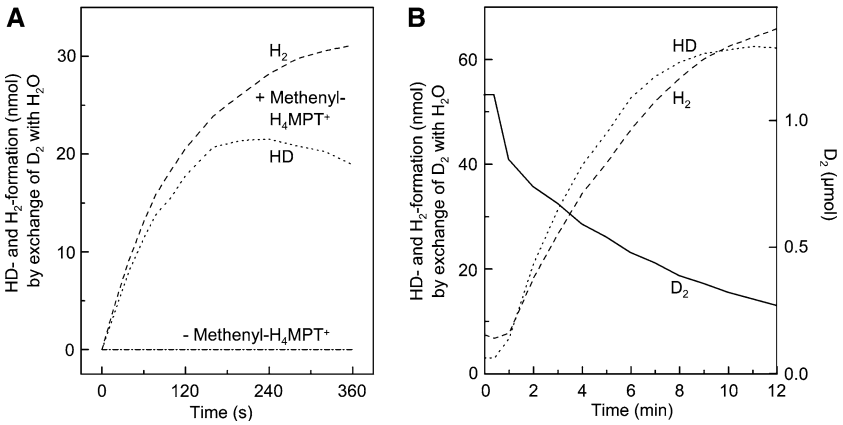

Fig. 6 Kinetics of H_2 and HD formation from D_2 and H_2O catalyzed **a** by [Fe] hydrogenase from *M. marburgensis* and **b** by the H_2-signaling [NiFe] hydrogenase from *Rhodobacter capsulatus*. **a** The 7 mL assay mixtures at 40 °C contained 120 mM potassium phosphate pH 6.0, 7 µM methenyl-H_4MPT^+ and 150 µM D_2 (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 H_2-signaling hydrogenase. The suspension was gassed with 100% D_2 before the vessel was closed and the formation of HD and that of H_2 were monitored by mass spectrometry. (**a** The data were taken from [27] with permission; **b** the data were taken from [67] with permission)

Table 1 H_2 and HD formation from D_2 and H_2O catalyzed by purified [Fe], [FeFe], [NiFe] and [NiFeSe] hydrogenases

Type	Organism	Topology	Initial rates of HD and H_2 formation	References
[Fe]	*Methanothermobacter marburgensis*	Cytoplasmic	HD ≅ H_2	[23]
[FeFe]	*Desulfovibrio vulgaris*	Periplasmic	HD > H_2	[61]
[FeFe]	*Desulfovibrio desulfricans*	Periplasmic	HD > H_2	[62]
[NiFe]	*Desulfovibrio gigas*	Periplasmic	HD > H_2	[61]
[NiFe]	*Desulfovibrio fructosovorans*	Periplasmic	HD > H_2	[63, 64]
[NiFeSe]	*Desulfovibrio baculatus*	Periplasmic	HD < H_2	[61, 64, 65]
[NiFeSe]	*Desulfovibrio baculatus*	Cytoplasmic	HD < H_2	[65]
[NiFeSe]	*Desulfovibrio baculatus*	Membrane-bound	HD < H_2	[65]
[NiFeSe]	*Desulfovibrio salexigens*	Periplasmic	HD < H_2	[61]
[NiFe]	*Thiocapsa roseopersicina*	Periplasmic	HD > H_2	[66]
[NiFe][a]	*Rhodobacter capsulatus*	Membrane-bound	HD < H_2	[67]
[NiFe][a]	*Rhodobacter capsulatus*	Cytoplasmic	HD ≅ H_2	[64, 67]
[NiFe]	*Methanosarcina barkeri*	Cytoplasmic	HD > H_2	[61]
[NiFe]	*Ralstonia eutropha*	Cytoplasmic	H_2 ≫ HD	[68]
[NiFe]	*Azotobacter vinelandii*	Membrane-bound	H_2 ≫ HD	[69]

[a] Cell suspensions

outlined in Fig. 5 in which it is assumed that the iron and the C(14a) of methenyl-H_4MPT$^+$ have to be juxtapositioned such that the H_2 can interact with both the iron and the C(14a) (possibilities 2 or 3 discussed above). The mechanism implies that upon binding of D_2 side-on to [Fe] hydrogenase in the presence of methenyl-H_4MPT$^+$ an (η^2-D_2)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 (η^2-HD)Fe complex. From the (η^2-HD)Fe complex the HD can dissociate off. Alternatively, after rotation of HD in the (η^2-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-H_4MPT$^+$ 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-H_4MPT. The second exchange yields a (μ-H)$_2$ complex in equilibrium with a (η^2-H_2)Fe complex, from which H_2 can dissociate off (Fig. 5). With respect to the kinetics it is assumed that the two proton exchange rates ($k_{exchange}$) are equal and that also the two off-dissociation rates (k_{off}) (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 H_2 are parallely formed from D_2 and H_2O at equal rates when $k_{exchange} \cong k_{off}$. If $k_{exchange} < k_{off}$ then more HD than H_2 is formed and if $k_{exchange} > k_{off}$ then more H_2 than HD is generated.

The cationic (μ-H)$_2$ complex proposed to be formed upon binding of methenyl-H_4MPT$^+$ and H_2 to [Fe] hydrogenase (shown for D_2 binding in Fig. 5) is most likely also formed upon binding of methylene-H_4MPT to the enzyme, which can explain the direct exchange of the *pro-R* hydrogen of methylene-H_4MPT with protons of bulk water (Eq. 7).

In Fig. 6b the kinetics of single and double exchange of D_2 with H_2O catalyzed by H_2-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 H_2 such that a hydride can be transferred to an acceptor, which is the C(14a) of methenyl-H_4MPT$^+$ 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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