

H₂-forming N⁵,N¹⁰-methylenetetrahydromethanopterin dehydrogenase: mechanism of H₂ formation analyzed using hydrogen isotopes

Andreas R. Klein^a, Victor M. Fernández^b, Rudolf K. Thauer^{a,*}

^aLaboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität Marburg and Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany

^bUnidad de Biocatalisis, Instituto de Catalisis C.S.I.C., Universidad Autónoma, Cantoblanco, E-28049 Madrid, Spain

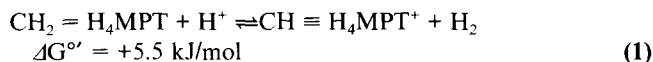
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Abstract H₂-forming N⁵,N¹⁰-methylenetetrahydromethanopterin dehydrogenase catalyzes the reversible dehydrogenation of N⁵,N¹⁰-methylenetetrahydromethanopterin (CH₂ = H₄MPT) to N⁵,N¹⁰-methenyltetrahydromethanopterin (CH = H₄MPT⁺) and H₂. In D₂O both HD and D₂ are formed from CH₂ = H₄MPT and in H₂O both HD and H₂ from CD₂ = H₄MPT. Evidence is presented that HD is not an intermediate in the formation of D₂ and H₂, respectively.

Key words: Hydrogenase; Tetrahydromethanopterin; Enzyme mechanism; Methanogenic Archaea; *Methanobacterium thermoautotrophicum*

1. Introduction

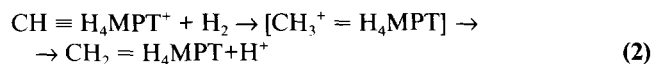
H₂-forming N⁵,N¹⁰-methylenetetrahydromethanopterin dehydrogenase (H₂-forming methylene-H₄MPT dehydrogenase) is a novel type of hydrogenase found in many methanogenic Archaea growing on H₂ and CO₂ as energy sources [1,2]. It catalyzes the reversible dehydrogenation of N⁵,N¹⁰-methylenetetrahydromethanopterin (CH₂ = H₄MPT) to N⁵,N¹⁰-methenyltetrahydromethanopterin (CH = H₄MPT⁺) and H₂ (reaction 1, Fig. 1).



The enzyme, which is composed of only one type of subunits with an apparent molecular mass of 43 kDa, differs from all other known hydrogenases in the following properties: it does not contain nickel and/or iron-sulfur clusters; it is not inhibited by CO, acetylene, NO, azide or cyanide; it does not catalyze the reduction of dyes with H₂; and in the absence of an electron acceptor, in this case CH = H₄MPT⁺, it does not catalyze an H₂/H⁺ exchange [3].

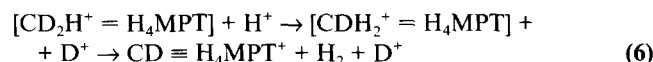
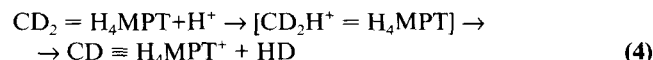
The mechanism of CH = H₄MPT⁺ reduction has been studied by two-dimensional NMR spectroscopic analysis of the product formed from CH = H₄MPT⁺ and H₂ or D₂ in H₂O or D₂O. The results indicate that the H₂-forming methylene-H₄MPT dehydrogenase catalyzes a stereospecific hydride transfer from H₂ into the *pro-R* site of the C(14a) methenyl group of CH = H₄MPT⁺ (Fig. 1) and a stereospecific direct exchange of the *pro-R* hydrogen of the C(14a) methylene group

of CH₂ = H₄MPT with protons of water [4–6]. The findings were interpreted to indicate that CH = H₄MPT⁺ reduction with H₂ proceeds via a pentacoordinated carbonium cation CH₃⁺ = H₄MPT as intermediate, in which the *pro-R* hydrogen bond in the C(14a) methylene group is protonated and thus activated and expected to exchange with protons of water [6,7] (reactions 2 and 3).



The mechanism of CH₂ = H₄MPT dehydrogenation has been studied by mass spectroscopic analysis of the dihydrogen generated. It was found that both HD and D₂ are formed from CH₂ = H₄MPT in D₂O and both HD and H₂ are formed from CD₂ = H₄MPT in H₂O and that the enzyme catalyzes a CH = H₄MPT⁺-dependent exchange between H₂ and D₂O yielding HD and D₂ and between D₂ and H₂O yielding HD and H₂ [8].

In this communication we have addressed the question how H₂ is formed from CD₂ = H₄MPT in H₂O and how D₂ is formed from CH₂ = H₄MPT in D₂O. Two mechanisms, formulated for H₂ formation, were considered: H₂ formation proceeds via reactions 4 and 5 (mechanism *a*) or via reaction 4 and 6 (mechanism *b*).



In mechanism *a* (reactions 4 and 5) HD is an intermediate whereas in mechanism *b* (reactions 4 and 6) it is not. The two mechanisms can therefore be discriminated kinetically.

2. Materials and methods

D₂O (99.9%) was from Sigma, HD (>96% HD, 2% H₂, 0.2% D₂ and 0.7% N₂) and CD₂O (98%) were purchased from Cambridge Isotope Laboratories. CH₂O was prepared from paraformaldehyde by hydrolysis for 12 h at 70°C. Tetrahydromethanopterin (H₄MPT) and CH = H₄MPT⁺ were isolated from *Methanobacterium thermoautotrophicum* (strain Marburg) [8,9]. CH₂ = H₄MPT was formed from H₄MPT and CH₂O by spontaneous reaction [10].

*Corresponding author. Fax: (49) (6421) 28-5833.
E-mail: thauer@mail.uni-marburg.de

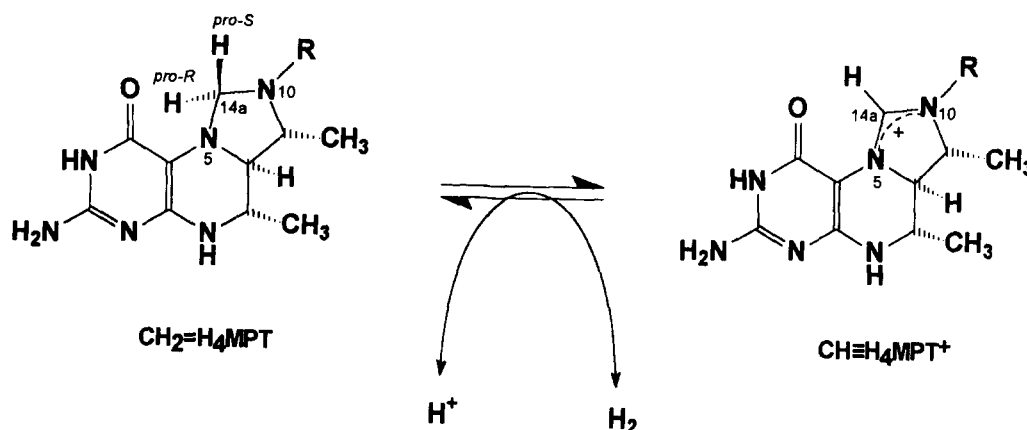


Fig. 1. Reaction catalyzed by the H_2 -forming N^5,N^{10} -methylene- H_4 MPT dehydrogenase. For the complete structures see [4,5].

2.1. Enzyme purification

The H_2 -forming N^5,N^{10} -methylenetetrahydromethanopterin dehydrogenase (EC 1.12.99.-) was purified from *M. thermoautotrophicum* strain Marburg [3]. One unit of enzyme activity refers to the amount of enzyme which catalyzes the dehydrogenation of 1 μmol $\text{CH}_2=\text{H}_4\text{MPT}$ per min at 40°C in 120 mM potassium phosphate pH 6.75 and a $\text{CH}_2=\text{H}_4\text{MPT}$ concentration of 17 μM .

2.2. Assay conditions

The assays were performed at 40°C in a 10 ml reaction vessel (15 mm in diameter). The bottom of the vessel consisted of a stainless steel grid covered with a 12.5 μm thick teflon membrane. For details of the construction, the reader is referred to [11]. The vessel was filled with 7 ml anaerobic 120 mM potassium phosphate pH 6.7 in H_2O or pD 6.7 in D_2O . The buffer was equilibrated with N_2 (dehydrogenation assay) or HD (97%) (exchange assay). Then, either 120 nmol H_4MPT plus 2 μmol CH_2O or CD_2O (dehydrogenation assay) or 120 nmol $\text{CH}\equiv\text{H}_4\text{MPT}^+$ (exchange assay) were added. The assay mixture was continuously stirred by a magnetic bar. The reaction was routinely started by the addition of purified enzyme (50 mU).

2.3. Determination of H_2 , HD and D_2

The formation of H_2 , HD or D_2 was followed on-line by mass spectrometry of the dissolved gases which diffuses through the teflon membrane to a vacuum line directly connected to the ion source of a mass spectrometer (Mastorr 200 DX quadrupole, VG Quadrupoles Ltd.). After passage through a cold trap (liquid nitrogen), where water vapour condensed, the gases were admitted into the ion source, where they were ionized and analyzed. In the dehydrogenation assay, all 3 masses were analyzed in one single experiment by scanning a mass range from 2 to 7 within 10 s. In the exchange experiment, the kinetics of H_2 , HD or D_2 formation were determined in separate experiments.

The mass spectrometer was calibrated with H_2 , HD and D_2 dissolved in H_2O . At a partial pressure of 10^5 Pa and a temperature of 40° the H_2 concentration in H_2O is 0.68 mM and the D_2 concentration in H_2O is 0.73 mM. The concentration of HD in H_2O was assumed to be 0.70 mM [12].

2.4. Determination of pH and pD

The pH of assay mixtures in H_2O were determined with commercially available glass electrodes standardized with buffer solutions in H_2O .

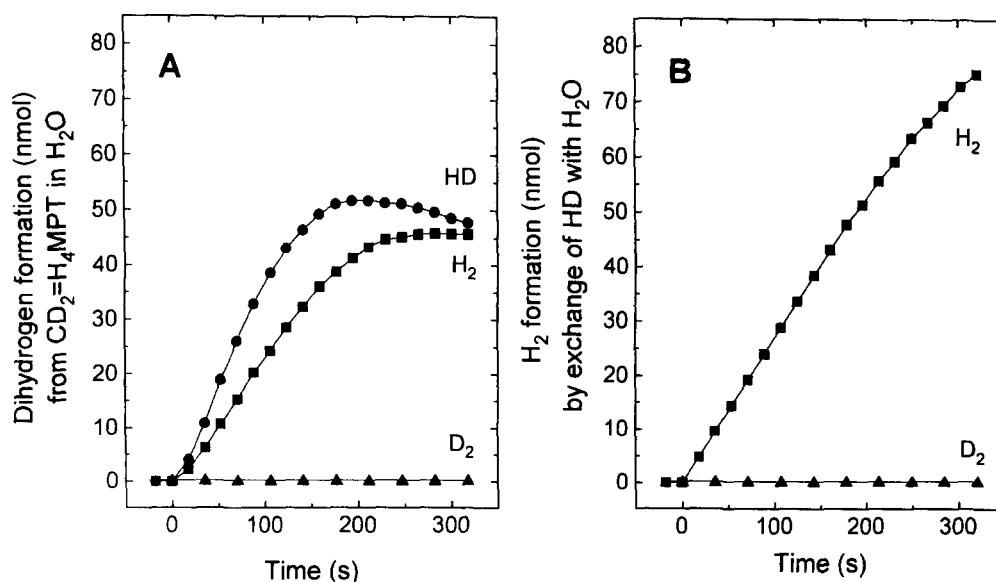


Fig. 2. (A) HD and H_2 formation from $\text{CD}_2=\text{H}_4\text{MPT}$ in H_2O at pH 6.7. (B) Isotopic exchange between HD and H_2O in the presence of $\text{CH}\equiv\text{H}_4\text{MPT}^+$ at pH 6.7. The 7 ml anaerobic assay mixture contained: (A) 840 μmol potassium phosphate pH 6.7; 120 nmol H_4MPT and 2 μmol CD_2O ; the solution was equilibrated with N_2 at 10^5 Pa. (B) 840 μmol potassium phosphate pH 6.7 and 120 nmol $\text{CH}\equiv\text{H}_4\text{MPT}^+$; the solution was equilibrated with 97% HD at 10^5 Pa. The reaction was started by the addition of 50 mU purified enzyme. Dihydrogen isotopes were determined on-line by mass spectrometry. (■) H_2 ; (●) HD; (▲) D_2 .

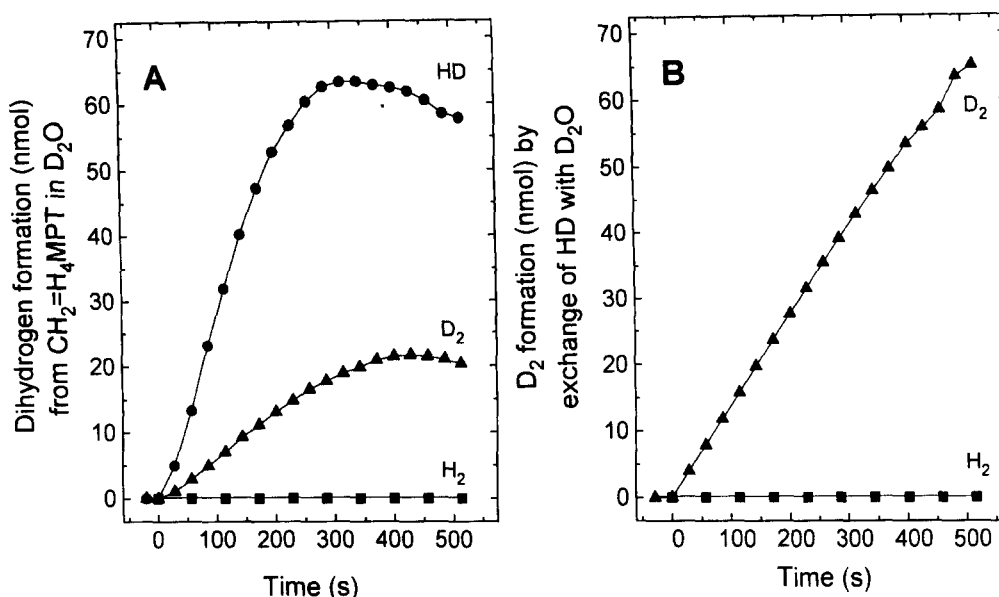


Fig. 3. (A) HD and D₂ formation from CH₂=H₄MPT in D₂O at pD 6.7. (B) Isotopic exchange between HD and D₂O in the presence of CH≡H₄MPT⁺ at pD 6.7. The 7 ml anaerobic assay mixture contained: (A) 840 μmol potassium phosphate pD 6.7; 120 nmol H₄MPT and 2 μmol CH₂O; the solution was equilibrated with N₂ at 10⁵ Pa. (B) 840 μmol potassium phosphate pD 6.7 and 120 nmol CH≡H₄MPT⁺; the solution was equilibrated with 97% HD at 10⁵ Pa. The reaction was started by the addition of 50 mU purified enzyme. Dihydrogen isotopes were determined on-line by mass spectrometry. (■) H₂; (●) HD; (▲) D₂.

The pD of assay mixtures in D₂O was measured by using the same calibrated electrode, taking into account that pD = pH + 0.41, where pH is the pH meter reading [13,14].

3. Results and discussion

The following experiments were performed with highly purified H₂-forming methylene-H₄MPT dehydrogenase from *Methanobacterium thermoautotrophicum* (strain Marburg). Almost identical results were, however, also obtained for the enzyme from *Methanopyrus kandleri*, *Methanothermobacter fervidus* and *Methanococcus igneus* (data not shown).

The kinetics of 4 reactions catalyzed by the H₂-forming methylene-H₄MPT dehydrogenase were analyzed: H₂ and HD formation from CD₂=H₄MPT in H₂O (Fig. 2A); H₂ formation from HD in H₂O containing CH≡H₄MPT⁺ (Fig. 2B); HD and D₂ formation from CH₂=H₄MPT in D₂O (Fig. 3A); and D₂ formation from HD in D₂O containing CH≡H₄MPT⁺ (Fig. 3B). The reactions were started by the addition of 50 mU enzyme activity. The following results were obtained:

– H₂ and HD formation from CD₂=H₄MPT in H₂O both began directly after the start of the reaction with the enzyme. Within the time resolution of the method employed H₂ and HD formation began simultaneously. The initial rate of H₂ formation (15 nmol/min) was only slightly lower than the rate of HD formation (23 nmol/min) and the rate of H₂ formation did not increase with increasing HD concentrations (Fig. 2A). Equivalent results were obtained for D₂ formation (18 nmol/min) and HD formation (4 nmol/min) from CH₂=H₄MPT in D₂O (Fig. 3A).

– The rate of H₂ formation from HD (700 μM) and H₂O (pH 6.7) in the presence of CH≡H₄MPT⁺ (17 μM) was 17 nmol/min (Fig. 2B) and the rate of D₂ formation from HD (700 μM) and D₂O (pD=6.7) was 8 nmol/min (Fig. 3B).

– The *K_m* from HD in the CH≡H₄MPT⁺-dependent exchange reaction was estimated to be 50 μM equivalent to 10% HD in the gas phase (data not shown).

The results indicate that at an HD concentration of 700 μM the rate of H₂ formation from HD in H₂O (17 nmol/min) is only a little bit higher than the rate of H₂ formation from CD₂=H₄MPT in H₂O (15 nmol/min) and that the rate of D₂ formation from HD in D₂O (8 nmol/min) is only approximately two-fold higher than the rate of D₂ formation from CH₂=H₄MPT in D₂O (4 nmol/min). At an HD concentration of only 1 μM, determined 20 s after start of the reaction (Figs. 2A and 3A), the rates of HD exchange, calculated with a *K_m* for HD of 50 μM (see above), are, however, only a few percent of the rate of H₂ and D₂ formation from CD₂=H₄MPT in D₂O and from CH₂=H₄MPT in H₂O, respectively. The results clearly indicate that free HD cannot be an intermediate in H₂ formation from CD₂=H₄MPT in H₂O and in D₂ formation from CH₂=H₄MPT in D₂O excluding mechanism *a* (reactions 4 and 5). The kinetics are, however, consistent with mechanism *b* (reactions 4 and 6), in which CH₃⁺=H₄MPT is assumed to be a transition state intermediate which stereospecific exchanges with protons of water. This mechanism predicts that the rate of H₂ formation from CD₂=H₄MPT in H₂O and the rate of D₂ formation from CH₂=H₄MPT in D₂O should be independent of the HD concentration, which is exactly what we found.

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