H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase: mechanism of H_2 formation analyzed using hydrogen isotopes

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Received 18 May 1995; revised version received 31 May 1995

Abstract **H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase catalyzes the reversible dehydrogenation of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2 = H_4MPT$) to N^5,N^{10}-methenyltetrahydromethanopterin ($CH \equiv H_4MPT^+$) and H_2. In D_2O both HD and D_2 are formed from $CH_2 = H_4MPT$ and in H_2O both HD and H_2 from $CD_2 = H_4MPT$. Evidence is presented that HD is not an intermediate in the formation of D_2 and H_2, respectively.**

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

1. Introduction

H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (H_2-forming methylene-H_4MPT dehydrogenase) is a novel type of hydrogenase found in many methanogenic Archaea growing on H_2 and CO_2 as energy sources [1,2]. It catalyzes the reversible dehydrogenation of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2 = H_4MPT$) to N^5,N^{10}-methenyltetrahydromethanopterin ($CH \equiv H_4MPT^+$) and H_2 (reaction 1, Fig. 1).

$$CH_2 = H_4MPT + H^+ \rightleftharpoons CH \equiv H_4MPT^+ + H_2 \quad \Delta G^{\circ\prime} = +5.5 \text{ kJ/mol} \qquad (1)$$

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 H_2; and in the absence of an electron acceptor, in this case $CH \equiv H_4MPT^+$, it does not catalyze an H_2/H^+ exchange [3].

The mechanism of $CH \equiv H_4MPT^+$ reduction has been studied by two-dimensional NMR spectroscopic analysis of the product formed from $CH \equiv H_4MPT^+$ and H_2 or D_2 in H_2O or D_2O. The results indicate that the H_2-forming methylene-H_4MPT dehydrogenase catalyzes a stereospecific hydride transfer from H_2 into the *pro-R* site of the C(14a) methenyl group of $CH \equiv H_4MPT^+$ (Fig. 1) and a stereospecific direct exchange of the *pro-R* hydrogen of the C(14a) methylene group of $CH_2 = H_4MPT$ with protons of water [4–6]. The findings were interpreted to indicate that $CH \equiv H_4MPT^+$ reduction with H_2 proceeds via a pentacoordinated carbonium cation $CH_3^+ = H_4MPT$ 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).

$$CH \equiv H_4MPT^+ + H_2 \rightarrow [CH_3^+ = H_4MPT] \rightarrow \rightarrow CH_2 = H_4MPT + H^+ \qquad (2)$$

$$[CH_3^+ = H_4MPT] + D^+ \rightarrow [CH_2D^+ = H_4MPT] + H^+ \qquad (3)$$

The mechanism of $CH_2 = H_4MPT$ dehydrogenation has been studied by mass spectroscopic analysis of the dihydrogen generated. It was found that both HD and D_2 are formed from $CH_2 = H_4MPT$ in D_2O and both HD and H_2 are formed from $CD_2 = H_4MPT$ in H_2O and that the enzyme catalyzes a $CH \equiv H_4MPT^+$-dependent exchange between H_2 and D_2O yielding HD and D_2 and between D_2 and H_2O yielding HD and H_2 [8].

In this communication we have addressed the question how H_2 is formed from $CD_2 = H_4MPT$ in H_2O and how D_2 is formed from $CH_2 = H_4MPT$ in D_2O. Two mechanisms, formulated for H_2 formation, were considered: H_2 formation proceeds via reactions 4 and 5 (mechanism *a*) or via reaction 4 and 6 (mechanism *b*).

$$CD_2 = H_4MPT + H^+ \rightarrow [CD_2H^+ = H_4MPT] \rightarrow \rightarrow CD \equiv H_4MPT^+ + HD \qquad (4)$$

$$HD + H^+ \rightarrow H_2 + D^+ \qquad (5)$$

$$[CD_2H^+ = H_4MPT] + H^+ \rightarrow [CDH_2^+ = H_4MPT] + + D^+ \rightarrow CD \equiv H_4MPT^+ + H_2 + D^+ \qquad (6)$$

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

D_2O (99.9%) was from Sigma, HD (>96% HD, 2% H_2, 0.2% D_2 and 0.7% N_2) and CD_2O (98%) were purchased from Cambridge Isotope Laboratories. CH_2O was prepared from paraformaldehyde by hydrolysis for 12 h at 70°C. Tetrahydromethanopterin (H_4MPT) and $CH \equiv H_4MPT^+$ were isolated from *Methanobacterium thermoautotrophicum* (strain Marburg) [8,9]. $CH_2 = H_4MPT$ was formed from H_4MPT and CH_2O by spontaneous reaction [10].

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SSDI 0014-5793(95)00642-7

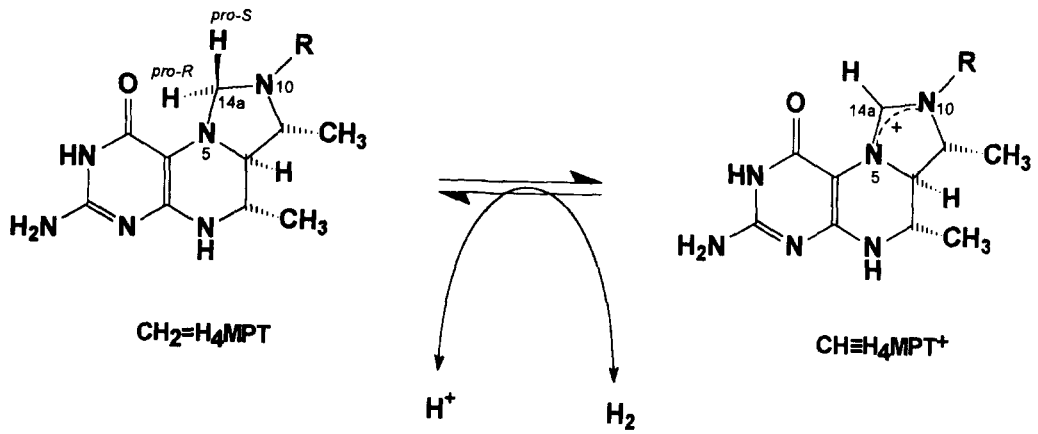

Fig. 1. Reaction catalyzed by the H_2-forming N^5,N^{10}-methylene-H_4 MPT dehydrogenase. For the complete structures see [4,5].

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 $CH_2 = H_4MPT$ per min at 40°C in 120 mM potassium phosphate pH 6.75 and a $CH_2 = H_4MPT$ 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 $CH \equiv H_4MPT^+$ (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 gasses 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.

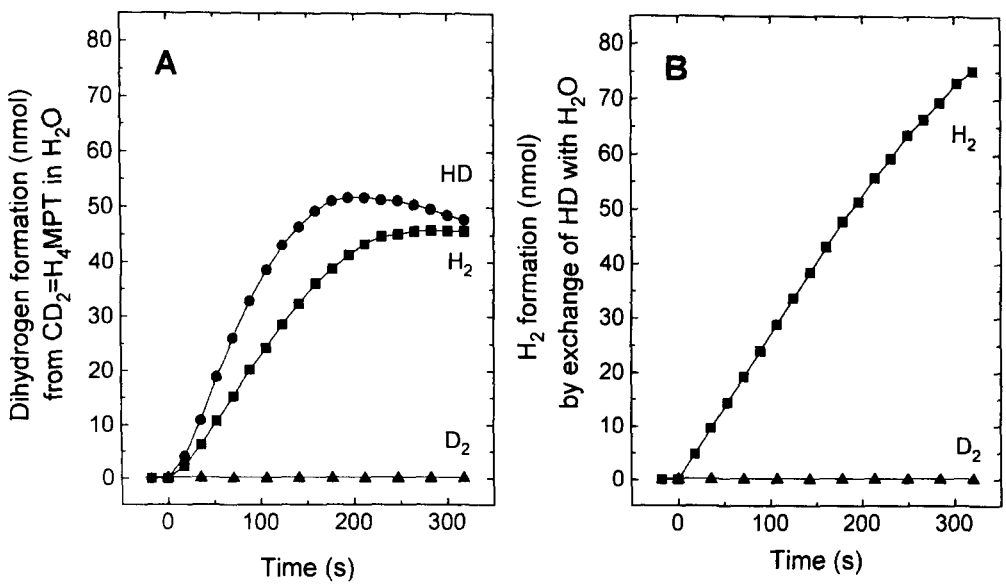

Fig. 2. (A) HD and H_2 formation from $CD_2 = H_4MPT$ in H_2O at pH 6.7. (B) Isotopic exchange between HD and H_2O in the presence of $CH \equiv H_4MPT^+$ at pH 6.7. The 7 ml anaerobic assay mixture contained: (A) 840 μmol potassium phosphate pH 6.7; 120 nmol H_4MPT and 2 μmol CD_2O; the solution was equilibrated with N_2 at 10^5 Pa. (B) 840 μmol potassium phosphate pH 6.7 and 120 nmol $CH \equiv H_4MPT^+$; 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.

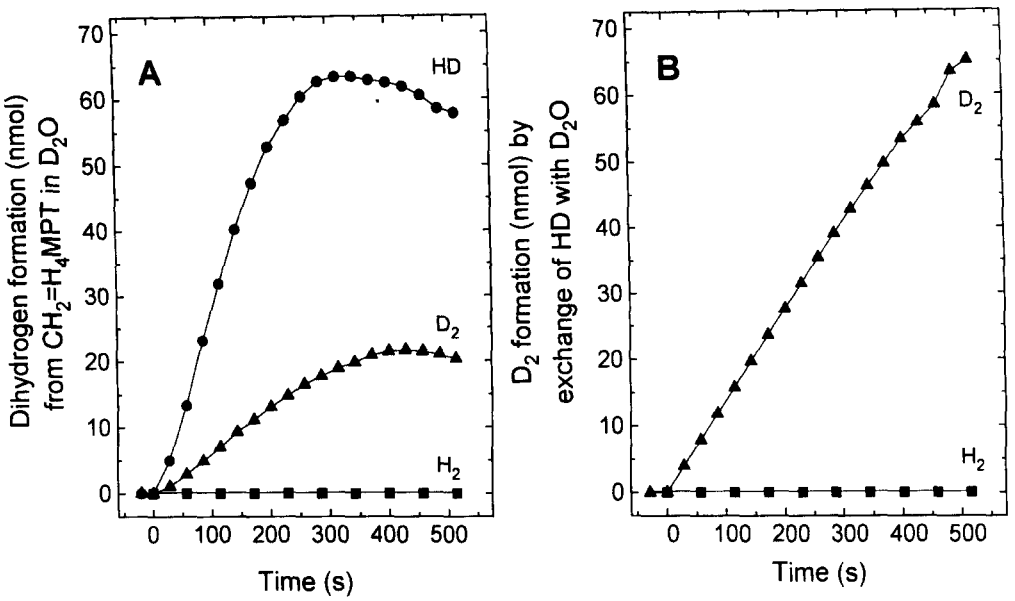

Fig. 3. (A) HD and D_2 formation from $CH_2 = H_4MPT$ in D_2O at pD 6.7. (B) Isotopic exchange between HD and D_2O in the presence of $CH \equiv H_4MPT^+$ at pD 6.7. The 7 ml anaerobic assay mixture contained: (A) 840 μmol potassium phosphate pD 6.7; 120 nmol H_4MPT and 2 μmol CH_2O; the solution was equilibrated with N_2 at 10^5 Pa. (B) 840 μmol potassium phosphate pD 6.7 and 120 nmol $CH \equiv H_4MPT^+$; 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.

The pD of assay mixtures in D_2O 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 H_2-forming methylene-H_4MPT dehydrogenase from *Methanobacterium thermoautotrophicum* (strain Marburg). Almost identical results were, however, also obtained for the enzyme from *Methanopyrus kandleri*, *Methanothermus fervidus* and *Methanococcus igneus* (data not shown).

The kinetics of 4 reactions catalyzed by the H_2-forming methylene-H_4MPT dehydrogenase were analyzed: H_2 and HD formation from $CD_2 = H_4MPT$ in H_2O (Fig. 2A); H_2 formation from HD in H_2O containing $CH \equiv H_4MPT^+$ (Fig. 2B); HD and D_2 formation from $CH_2 = H_4MPT$ in D_2O (Fig. 3A); and D_2 formation from HD in D_2O containing $CH \equiv H_4MPT^+$ (Fig. 3B). The reactions were started by the addition of 50 mU enzyme activity. The following results were obtained:

– H_2 and HD formation from $CD_2 = H_4MPT$ in H_2O both began directly after the start of the reaction with the enzyme. Within the time resolution of the method employed H_2 and HD formation began simultaneously. The initial rate of H_2 formation (15 nmol/min) was only slightly lower than the rate of HD formation (23 nmol/min) and the rate of H_2 formation did not increase with increasing HD concentrations (Fig. 2A). Equivalent results were obtained for D_2 formation (18 nmol/min) and HD formation (4 nmol/min) from $CH_2 = H_4MPT$ in D_2O (Fig. 3A).

– The rate of H_2 formation from HD (700 μM) and H_2O (pH 6.7) in the presence of $CH \equiv H_4MPT^+$ (17 μM) was 17 nmol/min (Fig. 2B) and the rate of D_2 formation from HD (700 μM) and D_2O (pD=6.7) was 8 nmol/min (Fig. 3B).

– The K_m from HD in the $CH \equiv H_4MPT^+$-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 H_2 formation from HD in H_2O (17 nmol/min) is only a little bit higher than the rate of H_2 formation from $CD_2 = H_4MPT$ in H_2O (15 nmol/min) and that the rate of D_2 formation from HD in D_2O (8 nmol/min) is only approximately two-fold higher than the rate of D_2 formation from $CH_2 = H_4MPT$ in D_2O (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 H_2 and D_2 formation from $CD_2 = H_4MPT$ in D_2O and from $CH_2 = H_4MPT$ in H_2O, respectively. The results clearly indicate that free HD cannot be an intermediate in H_2 formation from $CD_2 = H_4MPT$ in H_2O and in D_2 formation from $CH_2 = H_4MPT$ in D_2O excluding mechanism *a* (reactions 4 and 5). The kinetics are, however, consistent with mechanism *b* (reactions 4 and 6), in which $CH_3^+ = H_4MPT$ is assumed to be a transition state intermediate which stereospecific exchanges with protons of water. This mechanism predicts that the rate of H_2 formation from $CD_2 = H_4MPT$ in H_2O and the rate of D_2 formation from $CH_2 = H_4MPT$ in D_2O should be independent of the HD concentration, which is exactly what we found.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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