Two $N^5, N^{10}$-methyleneetetrahidromethanopterin dehydrogenases in the extreme thermophile *Methanopyrus kandleri*: characterization of the coenzyme F$_{420}$-dependent enzyme

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**Abstract.** It was recently reported that the extreme thermophile *Methanopyrus kandleri* contains only a H$_2$-forming $N^5, N^{10}$-methyleneetetrahidromethanopterin dehydrogenase which uses protons as electron acceptor. We describe here the presence in this Archaeon of a second $N^5, N^{10}$-methyleneetetrahidromethanopterin dehydrogenase which is coenzyme F$_{420}$-dependent. This enzyme was purified and characterized. The enzyme was colourless, had an apparent molecular mass of 300 kDa, an isoelectric point of $3.7 \pm 0.2$ and was composed of only one type of subunit of apparent molecular mass of 36 kDa. The enzyme activity increased to an optimum with increasing salt concentrations. Optimal salt concentrations were e.g. 2 M (NH$_4$)$_2$SO$_4$, 2 M Na$_2$HPO$_4$, 1.5 M K$_2$HPO$_4$, and 2 M NaCl. In the absence of salts the enzyme exhibited almost no activity. The salts affected mainly the $V_{max}$ rather than the $K_m$ of the enzyme. The catalytic mechanism of the dehydrogenase was determined to be of the ternary complex type, in agreement with the finding that the enzyme lacked a chromophoric prosthetic group. In the presence of 1 M (NH$_4$)$_2$SO$_4$ the $V_{max}$ was 4000 U/mg ($k_{cat} = 2400$ s$^{-1}$) and the $K_m$ for $N^5, N^{10}$-methyleneetetrahidromethanopterin and for coenzyme F$_{420}$ were 80 $\mu$M and 20 $\mu$M, respectively. The enzyme was relatively heat-stable and lost no activity when incubated anaerobically in 50 mM K$_2$HPO$_4$ at 90 °C for one hour. The $N$-terminal amino acid sequence was found to be similar to that of the F$_{420}$-dependent $N^5, N^{10}$-methyleneetetrahidromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum*, *Methanosarcina barkeri*, and *Archaeoglobus fulgidus*.

**Key words:** Coenzyme F$_{420}$ — Tetrahydromethanopterin — Hydrogenase — H$_2$-forming methyleneetetrahidromethanopterin dehydrogenase — *Methanobacterium thermoautotrophicum* — *Methanosarcina barkeri* — *Archaeoglobus fulgidus*

*Methanopyrus kandleri* is an abyssal hyperthermophilic Archaeon, that grows optimally at 98 °C. *M. kandleri* obtains energy for growth by reducing CO$_2$ with H$_2$ to CH$_4$ (Huber et al. 1989; Kurr et al. 1991). The organism is the most thermophilic methanogen known and is phylogenetically distant from other methanogenic Archaea (Burggraf et al. 1991). The pathway of CO$_2$ reduction to CH$_4$ in *M. kandleri* has, however, been shown to be identical to that used in the orders of Methanobacteriales, Methanomicrobiales, and Methanococcales (Rosper et al. 1991; Breitung et al. 1991, 1992; Ma et al. 1991a, b).

The reduction of $N^5, N^{10}$-methylenetetrahydromethanopterin (CH$_2=H_4$MPT$^+$) to $N^5, N^{10}$-methyleneetetrahydromethanopterin (CH$_2=H_2$MPT) is a central step in the pathway of CO$_2$ reduction to CH$_4$ (Wolfle 1991). Two enzymes are known to catalyze this reversible reaction. One uses reduced coenzyme F$_{420}$ (F$_{420}$H$_2$) and the other H$_2$ as direct electron donor (Mukhopadhyay and Daniels 1989; Zirngibl et al. 1990).

\[
\text{CH}_2\text{H}_4\text{MPT}^+ + \text{F}_{420}\text{H}_2 \rightleftharpoons \text{CH}_2=\text{H}_2\text{MPT}^+ + \text{F}_{420} + \text{H}_2 + \text{H}_2 \\
\Delta G^\circ = -6.5 \text{ KJ/mol}
\]

\[
\text{CH}_2\text{H}_4\text{MPT}^+ + \text{H}_2 \rightleftharpoons \text{CH}_2=\text{H}_4\text{MPT}^+ + \text{H}_2^+ \\
\Delta G^\circ = +5.5 \text{ KJ/mol}
\]

The Methanobacteriales and the Methanococcales all appear to contain both $N^5, N^{10}$-methyleneetetrahydromethanopterin dehydrogenases (from here on abbreviated methylene-H$_4$MPT dehydrogenase). In members of the order Methanomicrobiales only the F$_{420}$-dependent enzyme and in *Methanopyrus kandleri* only the H$_2$-forming

Abbreviations: H$_4$MPT, tetrahydromethanopterin; F$_{420}$, coenzyme F$_{420}$; CH$_2=H_4$MPT, $N^5, N^{10}$-methyleneetetrahydromethanopterin; CH$_2=H_2$MPT, $N^5, N^{10}$-methyleneetetrahydromethanopterin; Mops, N-morpholino propane sulfonic acid; Tricine, N-[Tris(hydroxymethyl)-methyl]glycine; 1 U = 1 nmol/mm

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enzyme has been found (Schwörer and Thauer 1991; Rospert et al. 1991). The later observations suggested that the two enzymes can completely substitute for each other. The H₂-forming methylene-H₄MPT dehydrogenase from M. kandleri has been purified and characterized and its gene cloned and sequenced (Ma et al. 1991b; Zirzagil et al. 1992).

Evidence has recently been presented that the F₄₂₀-dependent methylene-H₄MPT dehydrogenase can be overlooked when the H₂-forming enzyme is present in high activity (von Bünau et al. 1991). We therefore reinvestigated whether M. kandleri contains the F₄₂₀-dependent enzyme.

Materials and methods

FPLC columns. Blue-Sepharose CL-6B and molecular mass standards were from Pharmacia (Freiburg, Germany). Methanopyrus kandleri (strain AV-1; DSM 6224) and Methanothermobacter thermautotrophicus (strain Marburg; DSM 2133) are the strains deposited at the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). Tetrahydromethanopterin (H₄MPT) and coenzyme F₄₂₀ were purified from Methanobacterium thermautotrophicus (strain Marburg) (Breitling et al. 1992).

Purification of F₄₂₀-dependent methylene-H₄MPT dehydrogenase

Where possible, purification steps were performed in an anaerobic chamber under an atmosphere of 95% N₂ and 5% H₂ since the dehydrogenase was slowly inactivated in the presence of O₂.

Frozen cells (22 g wet mass) of M. kandleri were suspended in 50 mM Mops/KOH pH 7.0 (22 ml) containing 0.5 mg DNAse I. The cell suspension was subsequently passed three times through a French pressure cell (American Instruments Company, Silver Spring, Md., USA) at 110 MPa. Cell debris were removed by centrifugation at 27,000 × g for 30 min at 4°C. The 35 ml supernatant with a protein concentration of 55 mg/ml is designated cell extract. Portions of 10 ml were stored at -20°C until purification of the methylene-H₄MPT dehydrogenase.

Ten ml cell extract cooled to 0°C in ice water were supplemented with 15 ml saturated ammonium sulfate solution in 100 mM Tris/HCl pH 7.0 to give a final concentration of 60% saturation. After 30 min of stirring, the precipitated protein was removed by 30 min centrifugation at 27,000 × g. The supernatant was applied to a HiLoad 26/10 Phenyl-Sepharose high performance column equilibrated with 2.0 mM ammonium sulfate in 50 mM Tris/HCl pH 7.8.

The column was washed with 3 ml (NH₄)₂SO₄ and the protein was eluted with a decreasing gradient (in 50 mM Tris/LiCl pH 7.8) of (NH₄)₂SO₄: 2 M to 1 M in 100 ml; 1 M to 0.6 M in 50 ml; 0.6 M to 0 M in 100 ml; 0.5 M CH₃SO₄; and 23 μM F₄₂₀; and, unless otherwise indicated, 1 M (NH₄)₂SO₄. The reaction was started by the addition of 5 to 15 μl enzyme solution. The formation of CH₃H₄MPT⁻ was monitored by following the increase in absorbance at 335 nm ($\tau = 21.6$ μM⁻¹ cm⁻¹) (DiMarco et al. 1990). The H₂-forming methylene-H₄MPT dehydrogenase was measured using the same assay with the exception that F₄₂₀ was omitted.

Where indicated we also determined the F₄₂₀-dependent N₇,N₉-methylene-H₄MPT dehydrogenase by following the F₄₂₀/H₂-dependent reduction of CH₃H₄MPT⁻ at pH 8.0 and 65°C. The 0.7-ml assay contained: 120 mM potassium phosphate pH 8.0; 35 μM CH₃H₄MPT⁻; and 23 μM F₄₂₀ for the reduction of F₄₂₀ to F₄₂₀H₂. After 10 min at 65°C 15 mM formaldehyde was added which quenched the excess of dithionite. The reaction was started by the addition of 5 to 15 μl enzyme solution. The conversion of CH₃H₄MPT⁻ to CH₃H₂MPT was monitored by the decrease in absorbance at 335 nm. The assay could not be employed to test the activity in crude extracts and in the 60% ammonium sulfate supernatant due to the presence of high activities of N₇,N₉-methylene tetrahydromethanopterin cyclohydrolase and F₄₂₀-reducing hydrogenase.

Determination of the isoelectric point

The isoelectric point of the N₇,N₉-methylene-H₄MPT dehydrogenase was determined by analytic isoelectric focusing (Görg et al. 1988) in the presence of 8 M urea using trypsinogen (pI = 9.3), lentil lectin-basic band (pI = 6.65), lentil lectin-middle band (pI = 8.45), lentil lectin-acidic band (pI = 8.15), myoglobin-basic band (pI = 7.35), myoglobin-acidic band (pI = 6.85), human carbonic anhydrase (pI = 6.55), bovine carbonic anhydrase (pI = 5.85), β-lactoglobulin A (pI = 5.2), soybean trypsin inhibitor (pI = 4.55), and amyloglucosidase (pI = 3.5) for the calibration of the gradient profile (marker proteins were from Serva, Heidelberg, Germany).