JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5351–5356 Vol. 180, No. 20
0021-9193/98/$04.00+0


# The NADP-Dependent Methylene Tetrahydromethanopterin Dehydrogenase in *Methylobacterium extorquens* AM1

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Received 9 June 1998/Accepted 12 August 1998

**An NADP-dependent methylene tetrahydromethanopterin ($H_4$MPT) dehydrogenase has recently been proposed to be involved in formaldehyde oxidation to $CO_2$ in *Methylobacterium extorquens* AM1. We report here on the purification of this novel enzyme to apparent homogeneity. Via the N-terminal amino acid sequence, it was identified to be the *mtdA* gene product. The purified enzyme catalyzed the dehydrogenation of methylene $H_4$MPT with $NADP^+$ rather than with $NAD^+$, with a specific activity of approximately 400 U/mg of protein. It also catalyzed the dehydrogenation of methylene tetrahydrofolate (methylene $H_4$F) with $NADP^+$. With methylene $H_4$F as the substrate, however, the specific activity (26 U/mg) and the catalytic efficiency ($V_{max}/K_m$) were approximately 20-fold lower than with methylene $H_4$MPT. Whereas the dehydrogenation of methylene $H_4$MPT ($E_0 = -390$ mV) with $NADP^+$ ($E_0 = -320$ mV) proceeded essentially irreversibly, the dehydrogenation of methylene $H_4$F ($E_0 = -300$ mV) was fully reversible. Comparison of the primary structure of the NADP-dependent dehydrogenase from *M. extorquens* AM1 with those of methylene $H_4$F dehydrogenases from other bacteria and eucarya and with those of methylene $H_4$MPT dehydrogenases from methanogenic archaea revealed only marginally significant similarity (<15%).**

*Methylobacterium extorquens* AM1 is an aerobic methylotrophic bacterium which belongs to the α-subgroup of the proteobacteria (22, 30). In this organism methanol has been proposed to be metabolized to $CO_2$ via formaldehyde, $N^5$,$N^{10}$-methylene tetrahydrofolate (methylene $H_4$F), $N^5$,$N^{10}$-methenyl tetrahydrofolate (methenyl $H_4$F), $N^{10}$-formyl tetrahydrofolate (formyl $H_4$F), and formate as intermediates in reactions involving pyrroloquinolinequinone-dependent methanol dehydrogenase, NADP-dependent methylene $H_4$F dehydrogenase, methenyl $H_4$F cyclohydrolase, formyl $H_4$F synthetase, and NAD-dependent formate dehydrogenase (7, 19, 21).

Recently, *M. extorquens* AM1 was shown to contain genes thought to be unique for methanogenic archaea, namely, genes predicted to encode methenyl tetrahydromethanopterin ($H_4$MPT) cyclohydrolase, formylmethanofuran:$H_4$MPT formyltransferase, and formylmethanofuran dehydrogenase (6). These enzymes catalyze the first three steps of methanogenesis during the growth of methanogens on $CO_2$ and $H_2$ and the last three steps of $CO_2$ formation during the growth of methanogens on methanol (15, 28). The genes in *M. extorquens* AM1 encoding the three methanogenic enzymes were shown by mutagenesis to be required for growth on $C_1$ compounds, suggesting an involvement of the corresponding enzymes in formaldehyde oxidation to $CO_2$ via $N^5$,$N^{10}$-methylene $H_4$MPT (methylene $H_4$MPT), $N^5$,$N^{10}$-methenyl $H_4$MPT (methenyl $H_4$MPT), $N^5$-formyl $H_4$MPT (formyl $H_4$MPT), and formylmethanofuran as intermediates (6).

The genetic evidence for this novel metabolic pathway was substantiated by the finding that cell extracts of methanol-grown *M. extorquens* AM1 exhibit methenyl $H_4$MPT cyclohydrolase activity and formylmethanofuran:$H_4$MPT formyltransferase activity and contain dephospho-$H_4$MPT (6). $H_4$MPT is an $H_4$F analogue previously found only in methanogenic and sulfate-reducing archaea. Its properties are significantly different from those of $H_4$F (Fig. 1). Thus, the redox potential of the $N^5$,$N^{10}$-methenyl $H_4$MPT–$N^5$,$N^{10}$-methylene $H_4$MPT couple (−390 mV) is 90 mV more negative than that of the $N^5$,$N^{10}$-methenyl $H_4$F–$N^5$,$N^{10}$-methylene $H_4$F couple (−300 mV) (13, 29, 37).

Further support for the novel metabolic pathway came from the finding that cell extracts of *M. extorquens* AM1 contain an NADP-dependent methylene $H_4$MPT dehydrogenase activity catalyzing the formation of methenyl $H_4$MPT from methylene $H_4$MPT (6). The presence of an NADP-dependent methylene $H_4$MPT dehydrogenase in *M. extorquens* AM1 is of special interest because the methylene $H_4$MPT dehydrogenases in methanogenic and sulfate-reducing archaea are specific for coenzyme $F_{420}$, which is a 5′ deazaflavin derivative (8). The redox potential of the $F_{420}$–$F_{420}H_2$ couple (−360 mV) is 40 mV more negative than that of the $NAD(P)^+$–NAD(P)H couple (−320 mV) (12, 34). Apparently, *M. extorquens* AM1 contains a novel enzyme combining features of both the $F_{420}$-dependent methylene $H_4$MPT dehydrogenase from methanogens and the NAD(P)-dependent methylene $H_4$F dehydrogenase found in bacteria and eucarya. We report here on the purification and the characterization of the novel enzyme.

## MATERIALS AND METHODS

**Coenzymes.** $H_4$MPT, methenyl $H_4$MPT, and coenzyme $F_{420}$ were purified from *Methanobacterium thermoautotrophicum* Marburg (DSM 2133) (5). $H_4$F was purchased from Sigma. Anoxic stock solutions of $H_4$MPT and $H_4$F were prepared in 50 mM Tricine-KOH (pH 7.0) containing 2 mM dithiothreitol. In giving concentrations of $H_4$F, it was considered that less than 50% of the commercially available $H_4$F was biologically active as determined enzymatically, due to the presence of the biologically inactive *R* isomer and dihydrofolate (36). Methenyl $H_4$F was generated from methylene $H_4$F by dehydrogenation via

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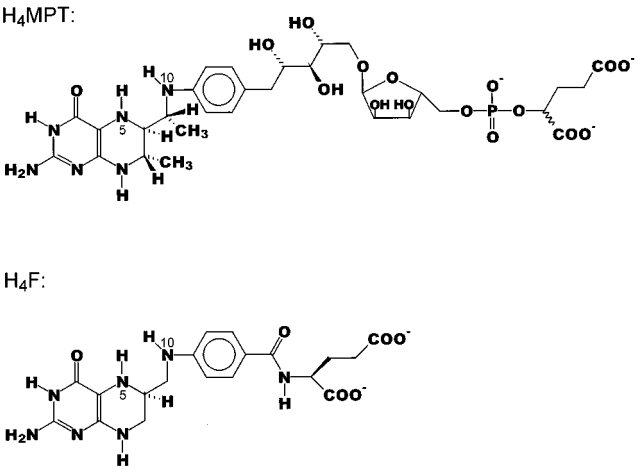


FIG. 1. Structures of $H_4$MPT (25, 32, 33) and $H_4$F. Functionally, the most important difference between $H_4$MPT and $H_4$F is that $H_4$MPT has an electron-donating methylene group in conjugation to $N^{10}$ via the aromatic ring, whereas $H_4$F has an electron-withdrawing carbonyl group in this position. One consequence is that the redox potential of the $N^5$,$N^{10}$-methenyl $H_4$MPT–$N^5$,$N^{10}$-methylene $H_4$MPT couple (−390 mV) is almost 100 mV more negative than that of the $N^5$,$N^{10}$-methenyl $H_4$F–$N^5$,$N^{10}$-methylene $H_4$F couple (−300 mV) (29). The $H_4$MPT derivative found in *M. extorquens* AM1 lacks the α-hydroxyglutaryl phosphate unit (6). The dephospho-$H_4$MPT is also present in methanogenic archaea (35).

NADP-dependent methylene $H_4$MPT dehydrogenase from *M. extorquens* AM1 and was purified by high-performance liquid chromatography (HPLC). Methylene $H_4$F was generated from $H_4$F and formaldehyde by spontaneous reaction. Methylene $H_4$F was not purified prior to the enzymatic conversion to methenyl $H_4$F.

**Growth of bacteria.** *M. extorquens* AM1 was grown on methanol (100 mM) at 30°C in minimal medium as described previously (11). The cultures were harvested in the late-exponential phase at a cell concentration of 3 g (wet mass)/liter. Cells were pelleted by centrifugation at 5,000 × *g* and stored at −20°C.

**Preparation of cell extracts.** Frozen cells (9 g) were suspended in 18 ml of 50 mM morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.0) at 4°C and passed three times through a French pressure cell at $1.2 \times 10^8$ Pa. Cell debris and unbroken cells were removed by centrifugation at 27,000 × *g* for 30 min. The resulting supernatant is referred to as cell extract.

Protein concentration was determined by the Bradford assay (4) by using the Bio-Rad reagent with bovine serum albumin as the standard.

**Determination of methylene $H_4$MPT dehydrogenase activity.** The assays were performed routinely at 30°C in 1-ml cuvettes (depth, 1 cm) in a total volume of 0.7 ml. The reactions were monitored photometrically by measuring the increase or decrease in absorbance at 340 nm. For the calculations, $\varepsilon_{340}$ values of 6.2 $mM^{-1}$ $cm^{-1}$ for NADPH, 20.8 $mM^{-1}$ $cm^{-1}$ for methenyl $H_4$MPT (10), and 21.7 $mM^{-1}$ $cm^{-1}$ for methenyl $H_4$F (18) were used. Units of enzyme activities are defined as 1 μmol/min at 30°C.

Methylene $H_4$MPT dehydrogenation with $NADP^+$ was routinely measured in 120 mM potassium phosphate (pH 6.0) at a methylene $H_4$MPT concentration of 35 μM and an $NADP^+$ concentration of 0.2 mM. The methylene $H_4$MPT was generated in the cuvette by a spontaneous reaction of $H_4$MPT (35 μM) with formaldehyde (3 mM). The excess of formaldehyde did not adversely affect the enzyme activity.

Methylene $H_4$F dehydrogenation with $NADP^+$ was measured in 120 mM potassium phosphate (pH 6.0) at a methylene $H_4$F concentration of 70 μM and an $NADP^+$ concentration of 0.2 mM. The methylene $H_4$F was generated in the cuvette by a spontaneous reaction of $H_4$F (70 μM) with formaldehyde (3 mM). The excess of formaldehyde did not adversely affect the enzyme activity.

Methenyl $H_4$MPT reduction with NADPH was measured in 120 mM potassium phosphate (pH 7.0) at a methenyl $H_4$MPT concentration of 30 μM and an NADPH concentration of 50 μM. The NADPH was regenerated with glucose-6-phosphate (2 mM) and glucose-6-phosphate dehydrogenase (10 U).

Methenyl $H_4$F reduction with NADPH was measured in 120 mM potassium phosphate (pH 7.0) at a methenyl $H_4$F concentration of 30 μM and an NADPH concentration of 50 μM.

**Purification of methylene $H_4$MPT dehydrogenase.** All purification steps were performed at 4°C under aerobic conditions. To 23 ml of cell extract stirred on ice, 34.5 ml of saturated ammonium sulfate in 50 mM Tris-HCl (pH 7.0) was added to a final concentration of 60% saturation. After 20 min of stirring, the precipitated protein was removed by 30 min of centrifugation at 20,000 × *g*. The supernatant was applied to a phenyl Sepharose (High Performance 26/10; Pharmacia Biotech) column equilibrated with 2 M ammonium sulfate in 50 mM Tris-HCl (pH 7.0). With a linear gradient decreasing from 2 to 0 M $(NH_4)_2SO_4$ (600 ml), the dehydrogenase activity eluted at about 0.25 M $(NH_4)_2SO_4$. Combined active fractions were concentrated with Centricon 30 microconcentrators (Millipore), washed with 50 mM MOPS-KOH (pH 7.0) (1:5), and subjected to anion-exchange chromatography on a Q Sepharose column (High Performance 16/10; Pharmacia Biotech). The enzyme activity was recovered in the flowthrough of the column (50 mM MOPS-KOH [pH 7.0]). Active fractions were pooled, washed, and concentrated by using Centricon 30 microconcentrators and 50 mM morpholineethanesulfonic acid (MES)-NaOH (pH 5.5). The enzyme was further purified by cation-exchange chromatography on a Mono S column (5/5; Pharmacia Biotech) via a linear gradient from 0 to 0.2 M NaCl in 50 mM MES-NaOH (pH 5.5) (57 ml). Methylene $H_4$MPT dehydrogenase was recovered at 0.16 M NaCl, diluted (1:3) with 50 mM MES-NaOH (pH 5.5), and rechromatographed on a Mono S column by using the same gradient.

**Determination of the N-terminal amino acid sequence.** Purified enzyme was electrophoresed in the presence of sodium dodecyl sulfate (SDS), and the 32-kDa band was electroblotted onto a poly(vinyl trifluoride) membrane (Applied Biosystems). Sequence determination was performed on a 477 protein/peptide sequencer from Applied Biosystems by D. Linder, Giessen, Germany.

**Determination of dephospho-$H_4$MPT and $H_4$F concentrations.** A cell extract (3 ml) of *M. extorquens* AM1 was ultrafiltrated with Centricon 3 microconcentrators. The filtrate, containing the low-molecular-mass compounds, was then supplemented with 15 mM formaldehyde, 2 mM $NADP^+$, and 2 U of purified methylene $H_4$MPT dehydrogenase in order to convert dephospho-$H_4$MPT and $H_4$F into dephospho-methenyl $H_4$MPT and methenyl $H_4$F, respectively. Up to this point, all steps were performed under strictly anaerobic conditions. Subsequently, the filtrate was subjected to HPLC with a LiChrospher RP-18 column (14 mm by 125 mm) (Merck). Absorbed compounds were eluted (1 ml/min) with a linear gradient (30 ml) of 0 to 50% methanol in 25 mM sodium formate (pH 3.0). The eluate was continuously monitored for absorbance at 335, 356, and 274 nm, and UV-visible spectra were recorded by using a Hewlett-Packard 1050 diode array detector. The retention times were 20.0 min for dephospho-methenyl $H_4$MPT, 18.8 min for methenyl $H_4$MPT, and 17.9 min for methenyl $H_4$F. The methenyl $H_4$MPT had been added to the filtrate for calibration. The methenyl derivatives were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI/TOF) mass spectrometry by J. Kahnt, Marburg, Germany, using Voyager-DE RP (PerSeptive Biosystems).

## RESULTS

Cell extracts of *M. extorquens* AM1 grown on methanol catalyzed the reduction of $NADP^+$ with methylene $H_4$MPT (2.6 U/mg), the reduction of $NADP^+$ with methylene $H_4$F (0.2 U/mg), and the reduction of $NAD^+$ with methylene $H_4$MPT (0.6 U/mg) rather than with methylene $H_4$F (<0.01 U/mg). The activities were associated with the soluble cell fraction. The two NADP-dependent activities were found to copurify and to be separated from the NAD-dependent activity upon ammonium sulfate precipitation. Purification was possible under aerobic conditions.

Cell extracts of *M. extorquens* AM1 did not catalyze the reduction of coenzyme $F_{420}$ with methylene $H_4$MPT or methylene $H_4$F. They also appeared not to contain coenzyme $F_{420}$, as determined spectrofluorometrically.

**Purification and molecular properties.** The two NADP-dependent activities were purified by hydrophobic chromatography on a phenyl Sepharose column, by anion-exchange chromatography on a Q Sepharose column, and by cation-exchange chromatography on a Mono S column. In each step a complete copurification of NADP-dependent methylene $H_4$MPT dehydrogenase and NADP-dependent methylene $H_4$F dehydrogenase was observed. Purification of both activities was approximately 130-fold with 24% yields (Table 1).

After the second chromatography on Mono S, the preparation contained only one polypeptide, with an apparent molecular mass of 32 kDa, as revealed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). The N-terminal amino acid sequence (SKKLLFQFDTDATPSVFDVV) determined by Edman degradation and the apparent molecular mass conformed with those predicted for the *mtdA* product (7).

The purified enzyme eluted from Superdex 200, a gel filtration column, with an apparent molecular mass of 93 kDa (data

TABLE 1. Copurification of NADP-dependent methylene $H_4F$ dehydrogenase and NADP-dependent methylene $H_4MPT$ dehydrogenase activities from *M. extorquens* AM1 grown on methanol

| Purification step | Protein (mg) | Activity (U)[a] with: | | Fold purification[b] | Yield (%)[b] |
|---|---|---|---|---|---|
| | | $H_4F$ | $H_4MPT$ | | |
| Cell extract | 642 | 127 | 1,652 | 1 | 100 |
| $(NH_4)_2SO_4$ supernatant | 129 | 107 | 1,408 | 4 | 85 |
| Phenyl Sepharose | 28 | 88 | 1,278 | 18 | 77 |
| Q Sepharose | 3.4 | 61 | 1,020 | 115 | 62 |
| Mono S I[c] | 2.5 | 55 | 812 | 125 | 49 |
| Mono S II | 1.2 | 31 | 495 | 128 | 24 |

[a] Determined at 30°C under standard assay conditions.
[b] Calculated for activities determined with methylene $H_4MPT$.
[c] Mono S I and Mono S II, first and second chromatographies, respectively, on a Mono S column.

not shown), suggesting that the dehydrogenase has a homotrimeric structure.

The UV-visible spectrum of the enzyme was that of a protein lacking a chromophoric prosthetic group (data not shown). The $\varepsilon_{280}$ determined and the $\varepsilon_{280}$ calculated from the tyrosine, phenylalanine, and tryptophan contents of the enzyme coincided within a 5% range.

The calculated pI for MtdA is 7.2, conforming with the property of the enzyme to bind to cation-exchange resins at pHs below 7.

The enzyme could be stored at −20°C under aerobic conditions for several weeks without loss of activity.

**Catalytic properties.** The purified enzyme was found to be similarly active with the $H_4MPT$ and the dephospho-$H_4MPT$ derivatives, allowing for determination of the catalytic properties by using the more readily available $H_4MPT$. Apparently, the modification in the side chain (Fig. 1) has little effect on the enzyme activity, a phenomenon also observed for the various $H_4MPT$-dependent enzymes in methanogenic archaea (14, 23, 26, 27).

Under the standard assay conditions described in Materials and Methods, the purified enzyme catalyzed the dehydrogenation of methylene $H_4MPT$ with $NADP^+$ with a specific activity of 413 U/mg and the dehydrogenation of methylene $H_4F$ with $NADP^+$ with a specific activity of 26 U/mg. It also catalyzed the reverse reactions, the reduction of methenyl $H_4MPT$ to methylene $H_4MPT$ (9 U/mg) and of methenyl $H_4F$ to methylene $H_4F$ (15 U/mg) using NADPH. For thermodynamic reasons (see below), the reduction of methenyl $H_4MPT$ to methylene $H_4MPT$ could be monitored only when an NADPH-regenerating system was employed.

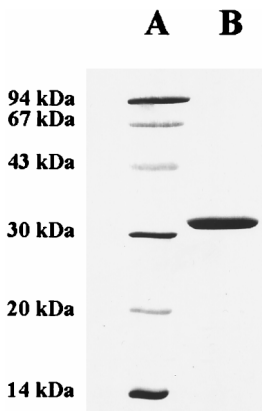


FIG. 2. SDS-PAGE analysis of purified methylene $H_4MPT$ dehydrogenase from *M. extorquens* AM1. Protein was separated on a 16% polyacrylamide gel and subsequently stained with Coomassie brilliant blue (17). Lane A, low-molecular-mass standards (Pharmacia Biotech); lane B, 5 μg of purified MtdA.

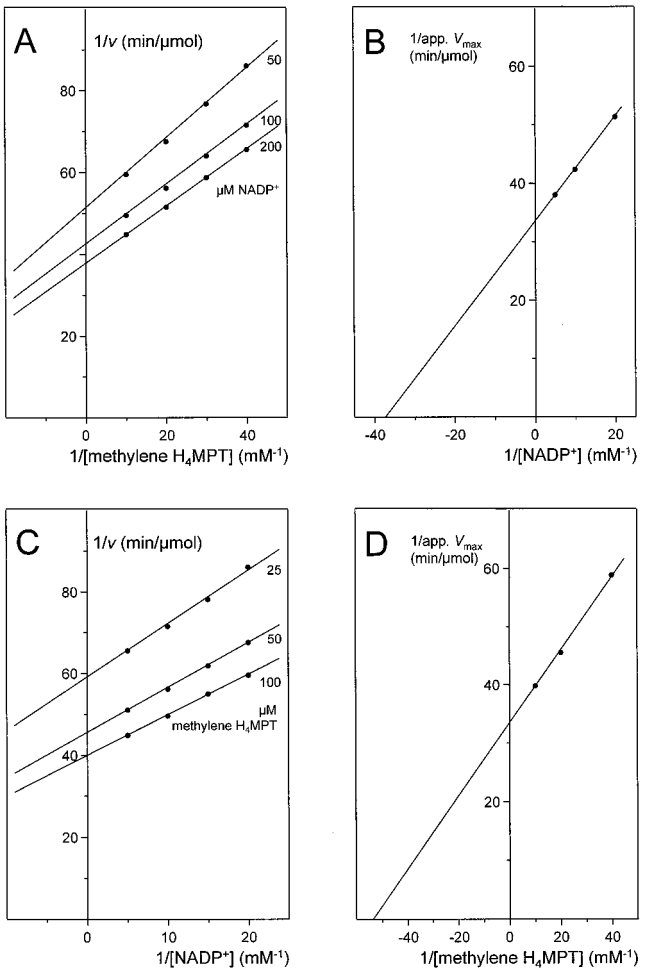


FIG. 3. Kinetics of methylene $H_4MPT$ dehydrogenation with $NADP^+$ as catalyzed by $NADP^+$-dependent methylene $H_4MPT$ dehydrogenase from *M. extorquens* AM1. (A and C) Plots of 1/*v* versus 1/[S], where velocity (*v*) is given as micromoles of substrate converted per minute. (B and D) Replots of 1/apparent (app.) $V_{max}$ versus 1/[S]. Assay conditions were as follows: temperature, 30°C; pH 6.0; $NADP^+$ and methylene $H_4MPT$ concentrations as indicated; 50 ng of purified methylene $H_4MPT$ dehydrogenase.

The dependence of the rate of methylene $H_4MPT$ dehydrogenation with $NADP^+$ on the concentrations of the substrates was determined. Reciprocal plots of the initial rates versus the concentration of one substrate at different fixed concentrations of the second substrate yielded straight lines (Fig. 3A and C). From reciprocal plots of 1/apparent $V_{max}$ versus 1/[S], where [S] is the concentration of the substrate, a $V_{max}$ of 600 U/mg, a $K_m$ for methylene $H_4MPT$ of approximately 20 μM, and a $K_m$ for $NADP^+$ of approximately 30 μM were obtained (Fig. 3B and D). With methylene $H_4F$ as the substrate, the $V_{max}$ was approximately 30 U/mg, the $K_m$ for methylene $H_4F$ was approximately 30 μM, and the $K_m$ for $NADP^+$ was approximately 10 μM (data not shown). Thus, the enzyme catalyzed the dehydrogenation of methylene $H_4MPT$ with an approximately 20-fold-higher cat-

alytic efficiency ($V_{max}/K_m$) than the dehydrogenation of methylene $H_4F$.

Due to the relatively low $K_m$ values for both substrates, it was not possible to accurately measure the rate dependence on the substrate concentration at concentrations much lower than the $K_m$. Such measurements would be required to ascertain whether the lines in the double reciprocal plots shown in Fig. 3A and C really converge or are parallel, which would indicate a ternary complex (sequential) or a ping-pong catalytic mechanism, respectively. The absence of a chromophoric prosthetic group argues for a ternary complex (sequential) catalytic mechanism.

The optimum pH for methylene $H_4MPT$ dehydrogenation was near 6.0, and the optimum temperature was near 45°C. The activity was slightly stimulated by salts, and maximal activity was reached at a potassium phosphate concentration of 120 mM (pH 6.0). NaCl concentrations higher than 100 mM were inhibitory.

The purified enzyme did not catalyze the reduction of $NAD^+$, flavin adenine dinucleotide, flavin mononucleotide, coenzyme $F_{420}$, or dyes such as methylene blue or benzyl viologen using either methylene $H_4MPT$ or methylene $H_4F$. It also did not exhibit methenyl $H_4MPT$ cyclohydrolase or methenyl $H_4F$ cyclohydrolase activity.

**Thermodynamics.** Methylene $H_4MPT$ dehydrogenation using $NADP^+$ was found to proceed almost to completion. The reverse reaction, the reduction of methenyl $H_4MPT$ using NADPH, ceased after less than 1% of the methylene $H_4MPT$ had been reduced, as predicted from the $E_0$ of −390 mV of the $N^5$,$N^{10}$-methenyl $H_4MPT$–$N^5$,$N^{10}$-methylene $H_4MPT$ couple and the $E_0$ of −320 mV of the $NADP^+$-NADPH couple. In the case of methylene $H_4F$ dehydrogenation, equilibrium was attained when about 30% of the methylene $H_4F$ had been converted to methenyl $H_4F$, in agreement with the $E_0$ of −300 mV of the methylene $H_4F$-methenyl $H_4F$ couple. From the concentrations at equilibrium, free energy changes ($\Delta G°$ values) of −13 and +3 kJ/mol were calculated for methylene $H_4MPT$ dehydrogenation and methylene $H_4F$ dehydrogenation, respectively.

**Intracellular concentrations of dephospho-$H_4MPT$ and $H_4F$.** Dephospho-$H_4MPT$ and $H_4F$ in cell extracts of *M. extorquens* AM1 were converted into more stable and readily detectable methenyl derivatives, which were subsequently subjected to reversed-phase HPLC and quantitated spectroscopically. Per milligram of cell extract protein, 1.4 nmol of dephospho-methenyl $H_4MPT$ and 0.5 nmol of methenyl $H_4F$ were detected, corresponding to intracellular concentrations of 0.4 and 0.15 mM, respectively, assuming that the intracellular volume is 3.3 μl/mg of protein, as in other procaryotes (3).

Dephospho-methenyl $H_4MPT$ and methenyl $H_4F$ were identified by their retention times on the HPLC column (internal standardization [data not shown]) and by the UV-visible spectra (Fig. 4), and their masses were determined by MALDI/TOF mass spectrometry (data not shown). The dephospho-methenyl $H_4MPT$ isolated from *M. extorquens* AM1 was used as the substrate by the NADP-dependent methylene $H_4MPT$ dehydrogenase with a specific activity similar to that for methenyl $H_4MPT$.

The HPLC analysis did not yield evidence for the presence of tetrahydropterin derivatives other than dephospho-$H_4MPT$ and $H_4F$.

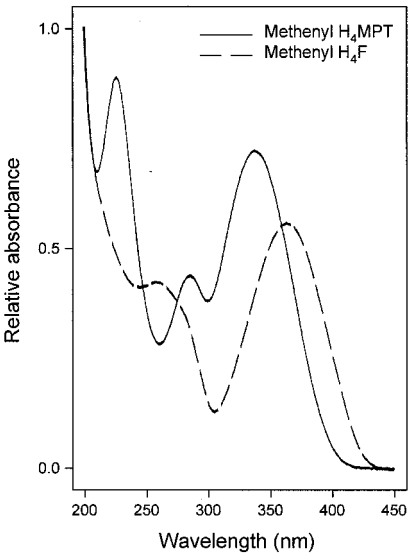


FIG. 4. UV-visible spectra of dephospho-methenyl $H_4MPT$ and methenyl $H_4F$ isolated from *M. extorquens* AM1 grown on methanol. The compounds were separated by HPLC, and their spectra were recorded online by diode array detection.

## DISCUSSION

The NADP-dependent methylene $H_4MPT$ dehydrogenase from *M. extorquens* AM1 has several properties in common with the two kinds of methylene $H_4MPT$ dehydrogenases found in methanogenic archaea, the $F_{420}$-dependent methylene $H_4MPT$ dehydrogenase (16) and the $H_2$-forming methylene $H_4MPT$ dehydrogenase (29). The three types of dehydrogenases are all composed of only one type of subunit with a molecular mass between 30 and 40 kDa, and they all lack a chromophoric prosthetic group and exhibit a ternary complex (sequential) catalytic mechanism. However, the three enzymes show only a very low degree of sequence similarity (<15%). Only minor sequence similarities are also found with NAD(P)-dependent methylene $H_4F$ dehydrogenases from bacteria and eucarya, which, with a few exceptions (2, 20, 24, 31, 38), are generally more complex, multifunctional enzymes containing additionally methenyl $H_4F$ cyclohydrolase in bacteria and both methenyl $H_4F$ cyclohydrolase and $N^5$-formyl $H_4F$ synthetase activities (1, 9) in eucarya. Therefore, based on differences in amino acid sequence and coenzyme specificity, four families of methylene tetrahydropterin dehydrogenases can be defined; they catalyze the following reactions:

$$\text{methylene } H_4MPT + NADP^+ \rightleftharpoons \text{methenyl } H_4MPT^+ + NADPH \quad (\Delta G° = -13 \text{ kJ/mol})$$

$$\text{methylene } H_4MPT + F_{420} + H^+ \rightleftharpoons \text{methenyl } H_4MPT^+ + F_{420}H_2 \quad (\Delta G° = -5.5 \text{ kJ/mol})$$

$$\text{methylene } H_4MPT + H^+ \rightleftharpoons \text{methenyl } H_4MPT^+ + H_2 \quad (\Delta G° = +5.5 \text{ kJ/mol})$$

$$\text{methylene } H_4F + NAD(P)^+ \rightleftharpoons \text{methenyl } H_4F^+ + NAD(P)H \quad (\Delta G° = +3.5 \text{ kJ/mol})$$

Within each of the four families, all enzymes show sequence similarity, even when they belong to organisms that are very distantly related phylogenetically.

In addition to the NADP-dependent methylene $H_4MPT$ dehydrogenase, *M. extorquens* AM1 cell extracts contain an NAD-dependent methylene $H_4MPT$ dehydrogenase activity. Genetic evidence indicates that this enzyme is encoded by *orfX*

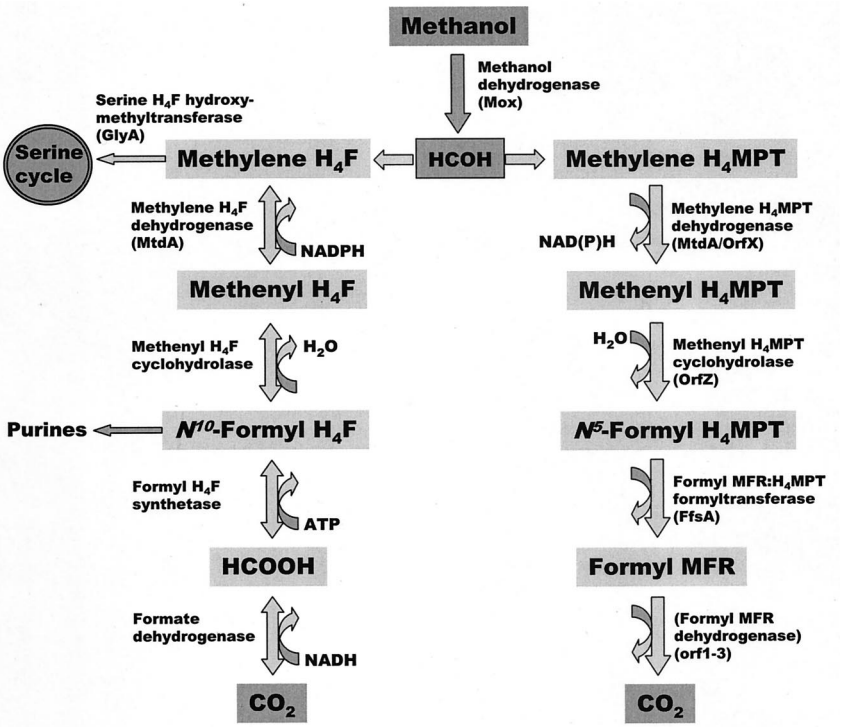


FIG. 5. Possible role of methylene $H_4$MPT dehydrogenase (MtdA) and other enzymes in growth of *M. extorquens* AM1 on methanol. Note that the presence of methanofuran in *M. extorquens* AM1 has not been proven. It is not known whether $N^5$-formyl $H_4$MPT can support purine biosynthesis.

(6). The amino acid sequence of OrfX is 30% identical to that of the NADP-dependent methylene $H_4$MPT dehydrogenase. While the NADP-dependent dehydrogenase (MtdA) exhibits some activity with methylene $H_4$F, the NAD-dependent methylene $H_4$MPT dehydrogenase is specific for $H_4$MPT (unpublished results).

For understanding possible functions of the pyridine nucleotide-dependent methylene $H_4$MPT dehydrogenases found so far only in *M. extorquens* AM1, it is important that they preferentially catalyze the $C_1$ oxidation reaction, whereas the three other methylene tetrahydropterin dehydrogenases can, in principle, operate in both directions. It is also of importance that the NADP-dependent methylene $H_4$MPT dehydrogenase exhibits some NADP-dependent methylene $H_4$F dehydrogenase activity and that *M. extorquens* AM1 contains dephospho-$H_4$MPT and $H_4$F at similar concentrations. Under certain conditions the NADP-dependent methylene $H_4$MPT dehydrogenase could therefore also function in the reversible dehydrogenation of methylene $H_4$F. There is evidence that methanol-grown *M. extorquens* AM1 contains an $H_4$F-specific serine hydroxymethyltransferase and an $H_4$F-specific methenyl $H_4$F cyclohydrolase (unpublished results). There is also evidence for the presence of an $H_4$MPT-specific methenyl $H_4$MPT cyclohydrolase and an $H_4$MPT-specific formylmethanofuran:$H_4$MPT formyltransferase. It therefore appears that in *M. extorquens* AM1 two independent $C_1$ transfer pathways are simultaneously operative, one that involves dephospho-$H_4$MPT as the $C_1$ carrier and is most likely to operate in the oxidative direction and one that involves $H_4$F and might operate in either direction, depending upon cellular pools of intermediates (Fig. 5). Purification and characterization of the enzymes involved in these pathways are an important step in the elucidation of the roles of the two $C_1$ transfer pathways in the anabolism and catabolism of *M. extorquens* AM1 growing on methanol.

## ACKNOWLEDGMENTS

This work was supported by the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft, and the Fonds der Chemischen Industrie (R.K.T.) and by an NIH grant to M.E.L. (GM36296).

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