Tetrahydromethanopterin, a Carbon Carrier in Methanogenesis*

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Evidence obtained by 13C NMR spectroscopy indicates that tetrahydromethanopterin (H4MPT) serves as a carbon carrier for C1 units at the methine, methylene, and methyl levels of oxidation. All three derivatives of H4MPT served as substrates for methanogenesis by cell extracts under a hydrogen atmosphere; in each instance, methane evolved at a rate comparable to that obtained when 2-(methylthio)ethanesulfonic acid was used as the substrate. Each C1 derivative of H4MPT stimulated the reduction of CO2 as efficiently as 2-(methylthio)ethanesulfonic acid. High resolution fast atom bombardment mass spectrometry indicated that the product of the spontaneous reaction of formaldehyde with H4MPT was methylene-H4MPT, with the molecular formula C31H45N6O16P. 13C NMR spectroscopy of hexamethylenetetramine, a model compound, suggested that the methylene group in methylene-H4MPT was bound to two nitrogen atoms. Molecular formulas of C31H45N6O16P and C32H47N6O16P were assigned to methenyl-H4MPT and methyl-H4MPT, by high resolution fast atom bombardment mass spectrometry. 1H NMR spectroscopy of methyl-H4MPT indicated that the methyl group was bound to a nitrogen atom. Sensitivity of each derivative to oxygen was manipulated under a nitrogen atmosphere and has been isolated in a highly purified form from deproteinized extracts of Methanobacterium thermoautotrophicum strain ΔH (15). The chemical structure of H4MPT has been recently elucidated (26). In this communication, we present results obtained from UV spectroscopy, 13C and 1H NMR spectroscopy, and HRFABMS, which indicate that H4MPT is a carbon carrier in methanogenesis at the methine, methylene, and methyl levels of oxidation.

**EXPERIMENTAL PROCEDURES**

**Assay for H4MPT**—Anoxic conditions were maintained in all manipulations and assays. We use the term anoxic to mean that solutions were sparged with N2 to remove oxygen and that cells or extracts were manipulated under a blanket of N2. An anaerobic chamber (Coy Scientific, Ann Arbor, MI) that contained an atmosphere of 97% N2 and 3% H2 was used where indicated. Gases were scrubbed free of oxygen by passage over heated, reduced copper at 350 °C. The assay for H4MPT employed its chemical reaction with HCHO to form methane-H4MPT, coupled to the enzymatically catalyzed oxidation of methylene-H4MPT to methenyl-H4MPT monitored by the increase in absorbance at 340 nm. Reaction cells (Bausch and Lomb, i.d. = 1.0 cm) containing HCHO (0.8 μmol) were transferred into an anaerobic chamber. Potassium phosphate buffer at pH 7.0 (20 mM) was sparged with N2 for 40 min (500 ml/min/liter of solution), after which 2-mercaptoethanol was added to a final concentration of 10 mM. The assay mixture was prepared inside an anaerobic chamber to protect H4MPT from exposure to O2. H4MPT or boiled cell extract was added after the addition of the buffer. The final volume was 3.0 ml. Each reaction cell was stoppered with a rubber serum stopper (Fisher Scientific Co., Springfield, NJ) and transferred out of the chamber. The atmosphere in the reaction cell was replaced with N2 by periodic mixing of the reaction mixture throughout a 5-min gassing period (50 ml of N2/reaction cell/min). Following preincubation for 10 min at 60 °C in a water bath, Sephadex G-25-treated extract (1-10 μg of protein) was added to start the reaction. The absorbance at 340 nm was monitored as described (2). The assay conditions were chosen so that the initial velocities were linear over a 0.2 A260 unit range. M1 = 766 (15) and an apparent molar extinction coefficient of 20,800 at 340 nm were used in calculating the rate of the reaction and the concentration of H4MPT.

**Assay for Hydrogen**—Production of H2 was followed by gas chromatography. The reaction mixture contained HCHO, 4.2 μmol, and H4MPT, 0.35 μmol. The atmosphere of the headspace of the reaction cell was N2. After the reaction mixture was preincubated at 60 °C for 10 min, the reaction was started by the addition of Sephadex G-25-treated extract (0.5 mg of protein). After 45 min of incubation at 60 °C, a 200-μl sample was withdrawn from the headspace of the reaction cell into a gas-tight Pressure-Lok syringe (Precision Sampling Co., Baton Rouge, LA) and injected into a Packard Model 428 gas chromatograph equipped with a thermal conductivity detector and a Porapak Q column to detect H2. To follow the reaction by dye reduction, 4.5 μmol of methyl viologen was added to the reaction mixture that contained 1.4 μmol of HCHO and 10 μg of protein (Sephadex G-25-treated extract); a molar extinction coefficient of 11,300 at 603 nm was used for reduced methyl viologen (16).

To follow the effect of H2 on the reaction, a known amount of it was injected into each reaction vial after the atmosphere in the headspace had been exchanged with N2. Before adding H2, an equal volume of gas was removed from the headspace of the cell. A vial to...
which no H₂ was injected was used as a control. The partial pressure of H₂ in the reaction vial was estimated by assuming ideal behavior of H₂ (volume of the headspace of the reaction cell was 6.5 ml).

Preparation of Ammonium Sulfate-treated Extracts—For certain experiments, a 70% ammonium sulfate-treated extract was prepared by adding 43.6 g of finely ground ammonium sulfate (enzyme grade; Schwarz/Mann, Inc., Spring Valley, NY) to 100 ml of cell extract that contained 10 mM 2-mercaptoethanol. The addition of the salt was performed at 4 °C over a period of 1 h. The solution was allowed to stand for 15 min before centrifugation at 27,500 g for 20 min at 4 °C. The supernatant fluid was extensively dialyzed in anoxic 20 mM potassium phosphate buffer at pH 7 that contained 10 mM 2-mercaptoethanol before use in the assay.

Assay for Reversibility of the Reaction—To demonstrate reversibility of the reaction, a Nicolet Instrument Corporation 360-MHz NMR spectrometer, employing 5-mm tubes. The internal standards were dioxane (66.5 ppm) for 13C spectra and sodium 3-(trimethylsilyl)propionate (Aldrich Chemical Co.) for the 1H spectra. Three repeated 1H-labeled aliquots of the sample in D₂O ensured the exchange of protons for deuterons in (N₂) and reductive (H₂) atmospheres. To ensure a uniform gaseous atmosphere, the samples were transferred into the anaerobic chamber, and transferred to a quartz cuvette, the contents were mixed, and the UV spectrum was determined again. After completion of the reaction (20 min) the reaction mixture was transferred back to a quartz cuvette and the UV spectrum was recorded again under anoxic conditions.

13C and 1H NMR Spectroscopy—Spectra were obtained in D₂O (100.0 atom % 2H; Aldrich Chemical Company, Milwaukee, WI) with a Nicolet Instrument Corporation 360-MHz NMR spectrometer, employing 5-mm tubes. The internal standards were dioxane (66.5 ppm) for 13C spectra and sodium 3-(trimethylsilyl)propionate (Aldrich Chemical Co.) for the 1H spectra. Three repeated 1H-labeled aliquots of the sample in D₂O ensured the exchange of protons for deuterons in (N₂) and reductive (H₂) atmospheres. To ensure a uniform gaseous atmosphere, the samples were transferred into the anaerobic chamber, and transferred to a quartz cuvette, the contents were mixed, and the UV spectrum was determined again. After completion of the reaction (20 min) the reaction mixture was transferred back to a quartz cuvette and the UV spectrum was recorded again under anoxic conditions.

RESULTS

When the oxidation of methylene-H₄MPT to methenyl-H₄MPT by Sephadex G-25-treated extract was followed spectrophotometrically, an increase in absorbance at 340 nm was observed; the total change in absorbance was proportional...
to the amount of HCHO added to the reaction mixture (Fig. 1). In the absence of HCHO, Sephadex G-25-treated extract, or H4MPT, no change in absorbance at 340 nm was observed. The specific activity remained unchanged (0.4 µmol of product min⁻¹ mg⁻¹ of protein) when the amount of protein used was doubled or tripled indicating that the rate of the reaction was proportional to the amount of protein added. The reaction required neither ATP nor Mg²⁺. More reduced or oxidized C₁ compounds, CH₂OH or HCOO⁻, failed to substitute for HCHO. The apparent molar extinction coefficient of the product of the reaction at 340 nm was found to be 20,800 (Table I). Acetaldehyde, glyceraldehyde, butyraldehyde, and glutaraldehyde did not substitute for HCHO. A number of known coenzymes were tested at a concentration of 50 µM in the spectrophotometric assay as substitutes for H₄MPT. Pyridoxal phosphate, thiamin pyrophosphate, HS-CoA, lipoic acid, biotin, ascorbic acid, tetrahydrofolate, NAD⁺, FAD, FMN, and glutathione did not substitute for H₄MPT.

Inclusion of methyl viologen in the reaction mixture in the absence of added H₄MPT resulted in the formation of 1.9 nmol of methenyl-H₄MPT⁺ and 21 nmol of reduced methyl viologen. When this reaction mixture was supplemented with 50 nmol of H₄MPT, 49.9 nmol of methenyl-H₄MPT⁺ and 116 nmol of reduced methyl viologen were formed. The ratio of reduced methyl viologen to methenyl-H₄MPT⁺ was 2.0. In the absence of methyl viologen, H₂ was produced from HCHO in the expected 1:1 stoichiometry; 350 nmol of HCHO was added, and 359 nmol mol of of H₂ was detected. No H₂ was evolved in the absence of HCHO, H₄MPT, or Sephadex G-25-treated extract.

H₂ inhibited the oxidation of methylene-H₄MPT by Sephadex G-25-treated extract. A logarithmically proportional relationship between the rate of the reaction and the partial pressure of H₂ was observed (Fig. 2). To follow the reverse reaction, the reduction of methenyl-H₄MPT⁺ under H₂ was followed spectrophotometrically (Fig. 3). This experiment was performed as described under “Experimental Procedures.” The dotted line represents the spectrum of H₄MPT. After the addition of HCHO to the reaction mixture, methylene-H₄MPT formed spontaneously as indicated by the shift of the absorption maximum from 302 to 287 nm (dashed curve). Incubation of the reaction mixture under N₂ in the presence of enzyme resulted in the UV spectrum of methenyl-H₄MPT⁺ (solid curve). When the atmosphere in the headspace of the reaction cell was exchanged for H₂ and the reaction mixture was incubated again (dash-dot curve), a marked decrease in the absorbance at 335 nm was observed together with the reappearance of the spectrum of methylene-H₄MPT. The rate of the reaction under N₂ was 4-fold faster than the reverse reaction that occurred under H₂, suggesting that the oxidation of methylene-H₄MPT to methenyl-H₄MPT⁺ was the most favorable reaction. Optimum assay conditions were determined using ammonium sulfate-treated extract. The temperature optimum for the assay was 60 °C, 14.7 µmol of methenyl-H₄MPT min⁻¹ mg⁻¹ of protein being formed whereas the values for other temperatures were: 40 °C, 5.6; 50 °C, 11.1; 70 °C, 5.2; 80 °C, 4.2. The pH optimum was 6.1 where the rate

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>λₘₜₜ</th>
<th>Extinction coefficients of H₄MPT and its derivatives</th>
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</thead>
<tbody>
<tr>
<td>H₄MPT⁺</td>
<td>302 (16.2)</td>
<td>302 (15.2) 247 (22.5) 220 (32.9)</td>
</tr>
<tr>
<td>Methylene-H₄MPT⁺</td>
<td>340 (20.8)</td>
<td>335 (21.6) 287 (13.3) 225 (30.7)</td>
</tr>
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<td>Methylene-H₄MPT⁺</td>
<td>287 (16.9)</td>
<td>250 (32.1) 220 (34.3)</td>
</tr>
<tr>
<td>Methyl-H₄MPT⁺</td>
<td>205 (8.6)</td>
<td>238 (15.4) 218 (22.9)</td>
</tr>
</tbody>
</table>

* The concentration of H₄MPT was determined based on enzymic conversion to methenyl-H₄MPT⁺.

* Extinction coefficients were determined spectrophotometrically based on the apparent molar extinction coefficient of 340 nm at 20,800.

* Extinction coefficients were estimated by measuring the absorbance of a known amount of H₄MPT in the presence of an excess of HCHO to ensure that all the H₄MPT in solution would be in the form of methenyl-H₄MPT.

* The concentration of methyl-H₄MPT was determined by dividing the absorbance of methyl-H₄MPT by the concentration of C₁ units converted to CH₄ sh, shoulder.
of the reaction was 14.7 μmol of methenyl-H4MPT· min⁻¹
mg⁻¹ of protein whereas the rates for other pH values were:
7.0, 6.6; 6.5, 11.9; 5.5, 2.5; 5.3, 1.6. A marked increase in the
rate of the reaction was observed as the concentration of NaCl
in the reaction mixture was increased. No further increase
was noted beyond 120 mM NaCl. Similar results were obtained
with other salts (data not shown), indicating that the effect
observed with NaCl was due to optimization of the ionic
strength for the reaction and not to a requirement for Na⁺ or
Cl⁻ ions.

Synthesis and Characterization of Methylene-H4MPT—The
UV spectrum of H₄MPT under anoxic conditions showed
absorption maxima at 302, 247, and 220 nm (Fig. 4) with
the presence of H⁺CHO was due to the spontaneous
absorption coefficients of
302, 250, and 3200, respectively (Table I). A 20-fold
250 nm (Fig. 4) with
300, and 220 nm, respectively (Table
reduction of H₄MPT induced by
H₄MPT (18 μmol) was dissolved in D₂O, and transferred to
an NMR tube. Dioxane (227 μmol) was also added as an
internal standard (66.5 ppm). The number of acquisitions
(500) was selected to allow detection of H⁺CHO in the spectrum.
The spectrum of the sample did not change after lyophili-
Anoxic ¹³C NMR spectroscopy was used to demon-
sitrate that the change in the UV spectrum of H₄MPT induced
by the presence of H⁺CHO was due to the spontaneous
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internal standard (66.5 ppm). The number of acquisitions
(500) was selected to allow detection of H⁺CHO in the spectrum.

The number of HCHO-binding sites per H₄MPT was de-
termined by ¹³C NMR spectroscopy as well; increasing
amounts of H⁺CHO were anoxically added to an NMR tube
that contained 18 μmol of H₄MPT, and the sample was
scanned after each addition. The intensity of the signals was
compared to that of the internal standard dioxane. The inten-
sity of the signals was plotted as relative peak intensity versus
micromoles of H⁺CHO added to the sample (Fig. 6). The relative intensity of the peak corresponding to methylene-
H₄MPT at 69.87 ppm remained unchanged after the addition

Fig. 4. UV spectra of H₄MPT and methylene-H₄MPT. --- UV spectrum of H₄MPT in 20 mM phosphate buffer at pH 7.0; --- UV spectrum of methylene-H₄MPT formed after the addition of HCHO to H₄MPT. The spectra were obtained under anoxic conditions.
of 17.6 μmol of H^13CHO, in agreement with a ratio of HCHO to H_4MPT of 1:1. The \(^1\)C NMR spectrum of [methylene-\(^1\)C]methylene-H_4MPT was compared to that of hexamethylenetetramine, (CH_2)_6N_4 (hexamine), which was used as a model compound for methylene groups bound to two nitrogen atoms. The chemical shift of the methylenes in hexamine was 71.82 ppm, very close to that of methylene-H_4MPT.

The chemical interaction of HCHO with H_4MPT also was documented by \(^1\)H NMR spectroscopy. H,MPT was reacted with a 10-fold excess of HCHO, and the sample was lyophilized to remove unreacted HCHO. The \(^1\)H NMR spectrum of methylene-H_4MPT in H_2O is shown in Fig. 7A. The product methylene-H_4MPT showed a doublet at 3.67 ppm and a multiplet of 2.58 ppm not found in H_4MPT. The integral for the doublet at 3.67 ppm accounts for one proton, whereas two protons are accounted for by the multiplet at 2.58 ppm. Identical changes in the \(^1\)H NMR spectrum occurred when the adduct was formed between \([\text{H}_2\text{H}]\text{HCHO}\) and H_4MPT, indicating that neither corresponded to the methylene group bound to H_4MPT (spectrum not shown). The location of the methylene signal in the spectrum of methylene-H_4MPT has not been identified and is probably buried under other signals in the complex region between 3.6 and 4.4 ppm. Free HCHO was located at 4.81 ppm (spectrum not shown). It should be noted that the singlet observed in H_4MPT at 1.24 ppm, which integrates to six protons, is split into two doublets at 1.24 and 1.38 ppm in methylene-H_4MPT, each one accounting for three protons.

The FAB mass spectrum of the adduct formed between HCHO and H_4MPT is shown in Fig. 8A. The signals at m/z 811, 833, and 855 represent M + Na, M - H + 2Na, M - 2H + 3Na, respectively. A high resolution measurement performed on the signal at m/z 855 gave the value of 855.2114 in agreement with the formula C_{31}H_{43}N_{6}O_{16}PNa_{3} corresponding to a nonsodiated molecular formula of C_{31}H_{42}N_{6}O_{16}P. The difference between H,MPT, C_{30}H_{42}N_{6}O_{16}P (M = 776) (15), and this \(C_1\)-derivative of H,MPT is 12 atomic mass units, i.e. one carbon, a methylene group bound to H,MPT. We refer to this derivative of H,MPT as methylene-H,MPT.

As shown in Fig. 9, the UV spectrum of methylene-H,MPT changes with time upon exposure to air. Under anoxic conditions, absorption maxima at 287, 250, and 220 nm were observed. Slight changes in the spectrum were observed after 1 h of exposure to air. After 4 h, the peak at 250 nm became a shoulder and decreased (7 h of exposure) until it disappeared (17 h of exposure). A blue shift of about 7 nm at 287 nm was also observed with a decrease in the extinction coefficient. A red shift of 7 nm at 220 nm was also recorded with an increase in the extinction coefficient. Throughout the experiment, an increase in the absorbance at 330 nm was noticed. H,MPT exposed to air for 17 h was no longer active in the spectrophotometric assay. When a 1.5 x 10^3-fold excess of HCHO over H,MPT was used for the experiment, the spectral changes observed were much less dramatic and after 24 h of exposure to air the sample still retained H,MPT activity (data not shown).

**Biosynthesis and Characterization of Methenyl-H,MPT**

In the presence of Sephadex G-25-treated extract [methylene-\(^{13}\)C]methylene-H,MPT was oxidized to [methenyl-\(^{13}\)C]methenyl-H,MPT by a methylene-H,MPT oxidoreductase present in the extract. The \(^{13}\)C NMR spectrum contained four signals in addition to dioxane (66.5 ppm): one at 69.87 ppm corresponding to a nonsodiated molecular formula of C_{31}H_{42}N_{6}O_{16}P. The difference between H,MPT, C_{30}H_{42}N_{6}O_{16}P (M = 776) (15), and this \(C_1\)-derivative of H,MPT is 12 atomic mass units, i.e. one carbon, a methylene group bound to H,MPT. We refer to this derivative of H,MPT as methylene-H,MPT.

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corresponding to unreacted \([\text{methylene}^{13}\text{C}]\)methylene-H$_4$MPT, and additional signals at 148.4, 162.69, and 165.34 ppm (Fig. 10B). Single frequency off-resonance proton-decoupling (Fig. 10A) showed that only one proton was bound to the carbon atoms resonating at 148.4, 162.69, and at 165.34 ppm as indicated by their multiplicity (doublets). The other two signals, dioxane and methylene-H$_4$MPT, showed single frequency off-resonance decoupling of triplets as expected in both cases.

The $^{13}$C$_1$ derivative of H$_4$MPT with a resonance of 148.4 ppm was purified by anoxic reversed phase high performance liquid chromatography at pH 4.5 with a linear gradient of methanol/water (20 to 60\% methanol, v/v). The compound eluted from the column with 45\% methanol. The single frequency off-resonance spectrum of this compound showed the expected multiplicity of a doublet (spectrum not shown).

In a separate experiment, H$_4$MPT (2 \(\mu\)mol) reacted with HCHO (20 \(\mu\)mol) to form methylene-H$_4$MPT, which was enzymically oxidized to methenyl-H$_4$MPT$^+$ as described above. The sample was subjected to anoxic reversed phase HPLC at pH 4.5 as described above. The fraction obtained with 10\% methanol contained well resolved, isolated material which had a UV spectrum identical to that shown in Fig. 11. The molecular ion region of the FAB mass spectrum of this fraction is shown in Fig. 8B. The molecular ion (M) was observed at \(m/z\) 787. Signals observed at \(m/z\) 809, 825, 831, 847, 853, 863, and 869 represented M − H + Na, M − H + K, M − 2H + 2Na, M − 2H + Na + K, M − 3H + 3Na, M − 2H + 2K, and M − 3H + 2Na + K, respectively. A high resolution measurement performed on the signal at \(m/z\) 847 gave the value of 847.1881 in agreement with the formula C$_{31}$H$_{42}$N$_6$O$_{16}$PKNa corresponding to a molecular metal-free formula of [C$_{31}$H$_{44}$N$_6$O$_{16}$P]$^+$, a cationic species. The difference between H$_4$MPT (15) and this $^{13}$C$_1$ derivative of H$_4$MPT is 11 atomic mass units, i.e. a methine group bound to H$_4$MPT as a methenyl group.

The UV spectrum of methenyl-H$_4$MPT$^+$ is shown in Fig. 11. Unlike methylene-H$_4$MPT, methenyl-H$_4$MPT$^+$ has major absorption maxima at 335, 287, and 225 nm. Upon exposure to air, the extinction coefficient at 335 nm did not change after 24 h of exposure, but increases in the extinction coefficients at 225 nm and 287 nm were noted.

\([\text{methenyl}^{13}\text{C}]\)Methenyl-H$_4$MPT$^+$ was located on the TLC plate by UV irradiation, a blue-green fluorescent spot (RF$_1$ = 0.66, RF$_2$ = 0.69) being readily visualized. The location of the fluorescent spot corresponded to the radioactive area that was detected by x-ray film.

**Synthesis and Characterization of Methyl-H$_4$MPT—[methylen-$^{13}\text{C}]$Methyl-H$_4$MPT (18 \(\mu\)mol) was chemically reduced with KBH$_4$ (185 \(\mu\)mol) to \([\text{methyl}^{13}\text{C}]\)methyl-H$_4$MPT. Excess KBH$_4$ was quenched with an excess of HCHO.**
Derivatives of Tetrahydromethanopterin

Fig. 11. Changes in the UV spectrum of methenyl-H₄MPT⁺ in O₂ with time. Methenyl-H₄MPT⁺ was enzymically synthesized from H₄MPT, 2 μmol and HCHO, 20 μmol by use of Sephadex G-25-treated extract, 1 mg of protein. Other components and conditions are described under "Experimental Procedures." UV spectrum under anoxic conditions (---), and after 24 h of exposure to air (---).

Fig. 12. ¹³C NMR spectra of [methyl-¹³C]methyl-H₄MPT. A, single frequency off-resonance decoupling spectrum in ¹H₂O; B, broad band proton-decoupled spectrum. The number of acquisitions was 500. C, broad band proton-decoupled spectrum of the sample + CH₃OH (30 pmol); D, single frequency off-resonance spectrum of C; the number of acquisitions was 500. Dioxane (227 μmol, 66.5 ppm) was used to establish the chemical shifts in the spectra.

Fluorescence Spectroscopy Studies—The fluorescence emission maxima (λₑᵣᵦ, in nm) of H₄MPT, and its C₁ derivatives are presented as follows (derivative, maximum excitation, emission λₑᵣᵦ): H₄MPT, 302, 420; methenyl-H₄MPT⁺, 328, 434; methylene-H₄MPT, 285, 352; methyl-H₄MPT, 295, 360.

Methane Formation from C₁ Derivatives of H₄MPT—All three C₁ derivatives of H₄MPT were rapidly and stoichiometrically converted to CH₄. Fig. 14 shows the kinetics of CH₄ evolution when methyl-H₄MPT, methylene-H₄MPT, or methenyl-H₄MPT⁺ was used as substrates under a hydrogen atmosphere. The initial rate of methane formation from 1-, 2-, and 3-fold amounts of substrate were, in nanomoles of CH₄/min: 13.3, 20, and 20 for methenyl-H₄MPT⁺; 21.3, 25.4, and 24.1 for methylene-H₄MPT; and 5, 8.6, and 11.3 for methenyl-H₄MPT⁺. In all three cases, the rate of CH₄ formation from the 3-fold amount of the C₁ derivative of H₄MPT was at least 93% of the rate of CH₄ formation from an equal amount of CH₃-S-CoM. Yields of CH₄ in each instance were at least 90% of stoichiometric. Further, each C₁ derivative of
Derivatives of Tetrahydromethanopterin

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C1 Derivatives of Tetrahydromethanopterin

H4MPT stimulated the conversion of CO2 to CH4 as previously described for CH3-S-CoM (21). The results obtained for each C1 donor tested in a standard reaction mixture that contained 3 mg of protein and 50 μl of boiled cell extract are expressed as C1 donor (nanomoles), nanomoles of CH4 formed under HP, and nanomoles of CH4 formed under 80% H2 + 20% CO2 as follows: CH3-S-CoM (200) 192, 401; methenyl-H4MPT* (280) 235, 839; methylene-H4MPT (260) 244, 792; methyl-H4MPT (310) 306, 896. The values have been corrected for background formation of CH4 in the absence of C1 donor. The stimulation on the reduction of CO2 may be estimated for each substrate by dividing the yield of CH4 obtained under H2 and CO2 by that obtained under H2.

DISCUSSION

We have reported here the first experimental evidence that H4MPT can function as a C1 carrier at three different oxidation states along the methanogenic pathway. HCHO and H4MPT react chemically to form an adduct documented by HRFABMS to be methylene-H4MPT. The methylene group is likely bonded to two nitrogen atoms as suggested by 13C NMR studies on methylene-H4MPT and hexamine, a model compound with methylene groups bound to two nitrogen atoms. The spontaneous formation of methylene-H4MPT resembles that of methylene-H4folate (22). The methyl group in methyl-H4MPT has a chemical shift (2.99 ppm) which agrees with that of a methyl group bound to a nitrogen atom. This is supported by the chemical shift and the multiplicity of the signal of the methyl group (singlet) in the 1H NMR spectrum of methyl-H4MPT. The enzymic oxidation of methylene-H4MPT resulted in the formation of several C1 derivatives of H4MPT, all at the methine level of oxidation as documented by 13C NMR spectroscopy. The addition of C1 units at the methine level of oxidation to other pterins like H4folate can result in N-5-, or N-10-formyl, or 5,10-methenyl-H4folate. Interconversion of these compounds is pH-dependent with the methenyl derivative being stable at acidic pH and the formyl derivatives stable at alkaline pH (23). Of all these compounds only 5,10-methenyl-H4folate has an absorption maximum of 350 nm with an extinction coefficient of 25,100 (24), this intense absorption at 350 nm being due to the formation of a new cycle within the structure of H4folate with an additional conjugated bond. Similar spectral properties were observed when methylene-H4MPT was enzymically oxidized to the methine level of oxidation. This reaction was monitored by the increase in the absorption at 340 nm and the apparent molar extinction coefficient of the product of the reaction at 340 nm was estimated to be 20,800. Our results obtained by 13C NMR spectroscopy are consistent with potential for interconversion among these derivatives of H4MPT. The resonance signal in the spectrum of methine-H4MPT at 165.34 and 162.69 ppm are very similar to those reported for the two conformations of the formyl group of N-5-formyl-H4folate, 165.2 and 163.2 ppm (27). The signal at 162.69 ppm is close to that reported for the methenyl group of 5,10-methenyl-H4folate, 146.3 ppm (28). Purification of the 340-nm-absorbing derivative of H4MPT at pH 4.5 and low and high resolution FABMS clearly document that this C1 derivative is methyl-H4MPT, a cationic species, and not formyl-H4MPT. The interconversion among these compounds can be of physiological significance in vivo as formyl groups are...
Derivatives of Tetr... required for cell carbon biosynthesis. Our results are consistent with the proposed structure of methanopterin recently reported by van Beelen et al. (25). These authors also proposed a structure for methenyl-H4MPT+ and the ultraviolet spectrum reported for this compound is very similar to the one reported in this communication. Comparison of the kinetics and stoichiometry of the conversion of the derivatives of H4MPT to CH4 relative to CH3-S-CoM indicates that methenyl-, methylene-, and methyl-H4MPT are intermediates in methanogenesis. This role is further supported by their ability to stimulate the conversion of CO2 to CH4 at least as efficiently as CH3-S-CoM. Evidence presented here indicates that methylene-H4MPT can be formed enzymically under a hydrogen atmosphere from methenyl-H4MPT+. These findings indicate that the methylene-H4MPT oxidoreductase is part of the methanogenic pathway.

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