Formaldehyde activation factor, tetrahydromethanopterin, a coenzyme of methanogenesis

(methanopterin/*Methanobacterium*)

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ABSTRACT An oxygen-labile formaldehyde activation factor (FAF) was isolated in highly purified form by use of anoxic fractionation procedures. The molecular weight of FAF was determined to be 776 and that of methanopterin (MPT) 772 by fast-atom-bombardment mass spectrometry (FABMS). High-resolution FABMS measurements on MPT and FAF indicated molecular formulas of $C_{30}H_{41}N_6O_{16}P$ and $C_{30}H_{45}N_6O_{16}P$, respectively. The presence of phosphorus was confirmed by 100-MHz ^{31}P NMR. The 360-MHz 1H NMR spectrum of FAF in deuterium oxide was similar to that of MPT. A functional relationship between MPT and FAF was documented; both compounds stimulated the reductive demethylation of 2-(methylthio)ethanesulfonic acid (CH_3 -S-CoM) to CH_4 when formaldehyde oxidation provided a source of electrons, and FAF replaced MPT in the CH_3 -S-CoM-stimulated conversion of CO_2 to CH_4 under H_2 (the RPG effect). MPT was enzymically converted to FAF during the reduction of CH_3 -S-CoM, and $HCHO$ to CH_4 under H_2 . Evidence indicates that FAF is tetrahydromethanopterin.

A number of new coenzymes involved in methanogenesis from $H_2 + CO_2$ have been studied recently. 2-Mercaptoethanesulfonic acid (HS-CoM) has been shown to be involved as a methyl carrier, 2-(methylthio)ethanesulfonic acid (CH_3 -S-CoM) (1, 2). Coenzyme F_{420} [the *N*-(*N*-L-lactyl- γ -L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5'-phosphate] is involved in oxidation-reduction events (3, 4). Component B of the CH_3 -S-CoM methylreductase is required for the conversion of CH_3 -S-CoM to CH_4 (2). Methanopterin (MPT) (5, 6) and the carbon dioxide reduction (CDR) factor (7) have been shown to be involved in methanogenesis from CO_2 (8). The nickel tetrapyrrole F_{430} (9) has been shown to be the chromophore of the CH_3 -S-CoM methylreductase (10). In the present study a low-molecular-weight factor was found in boiled extracts of *Methanobacterium thermoautotrophicum*, and was shown to be involved in the conversion of CH_3 -S-CoM or $HCHO$ to CH_4 , with $HCHO$ as the source of reducing equivalents; this factor was named the formaldehyde activation factor, FAF (11). We present here the purification of the new coenzyme and document a functional-structural relationship between FAF and MPT, indicating that FAF is tetrahydromethanopterin (H_4 MPT).

MATERIALS AND METHODS

Purification. In the procedures outlined below we use the term anoxic to indicate that nitrogen scrubbed over reduced copper at 350°C was used to sparge oxygen from solutions (40 min gassing time at 20 liters of N_2 per liter of solution), from glassware and syringes, and to provide a blanket of gas over fractions and extract preparations. Where indicated, procedures were carried out in an anaerobic chamber (Coy

Scientific Products, Ann Arbor, MI) that contained an atmosphere of 95% N_2 /5% H_2 (12). Boiled cell extract was prepared as described (2), using 250 g of cell paste. This crude preparation of cofactors was acidified with 6 M HCl to pH 4.8 under anoxic conditions. Acid-insoluble material was removed by centrifugation at $48,000 \times g$ for 30 min at room temperature under anoxic conditions. The acidified boiled cell extract was loaded onto an anoxic DEAE-Sephadex A25 anion exchange column (5×18 cm; 350 ml) previously equilibrated with 30 mM sodium acetate buffer at pH 4.8 that contained 10 mM 2-mercaptoethanol. A spectrophotometric assay for FAF has been described (11), and an extinction coefficient of $20.8 \text{ mM}^{-1} \text{ cm}^{-1}$ was used. Anoxic fractions containing FAF were pooled and lyophilized. Inside an anaerobic chamber, dry FAF was suspended in a portion of anoxic buffer that contained 17% (vol/vol) methanol and 1% sodium formate (wt/vol) in 10 mM 2-mercaptoethanol adjusted to pH 3.0 with HCl. This buffer was used for anoxic reversed-phase high-performance liquid chromatography (HPLC) of this fraction on a 25 cm $\times$ 10 mm i.d. octadecyl Hi-Chrom preparative HPLC column (Regis, Morton Grove, IL). The anoxic buffer was degassed under reduced pressure in the airlock of the anaerobic chamber prior to use. Prior to injection of each 1 ml sample, the sampling syringe was flushed with anoxic buffer. The eluate was collected under a stream of N_2 . A pressure of 1.722×10^4 kPa was maintained on the column with a Waters Associates, Inc. Model 6000A HPLC pump, and the flow rate was 4 ml min^{-1} . The elution of the material from the column was monitored with a differential refractometer (Waters Associates, model R401). Each tube containing FAF was sealed with a butyl rubber stopper and kept on ice until transferred into an anaerobic chamber to be pooled. The material was then flash-evaporated to remove CH_3OH , lyophilized, and resuspended in anoxic glass distilled H_2O containing 1 mM 2-mercaptoethanol. The sample was applied to a 2.5 cm $\times$ 102 cm bed volume of anoxic Sephadex G-15 equilibrated with aqueous 1 mM 2-mercaptoethanol. The eluate was collected inside an anaerobic chamber at a flow rate of 30 ml min^{-1} . Fractions containing FAF activity were pooled and lyophilized. The final product was transferred into an anaerobic chamber and dispensed into serum vials. Each vial was sealed with a butyl rubber stopper and the contents were pressurized with N_2 prior to storage at -20°C .

UV Spectroscopy. The UV spectrum of FAF was obtained with a Cary 14 spectrophotometer; the samples were sealed into quartz cuvettes inside an anaerobic chamber.

Chemical Reduction of Air-Exposed FAF. Inside an anaerobic chamber, about 5 mg of reductant was added to a 200- μ l aliquot of a solution containing 2 mg of air-exposed FAF

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Abbreviations: FABMS, fast-atom-bombardment mass spectrometry; MPT, methanopterin; H_4 MPT, tetrahydromethanopterin; CDR factor, carbon dioxide reduction factor; FAF, formaldehyde activation factor; CH_3 -S-CoM, 2-(methylthio)ethanesulfonic acid; HS-CoM, 2-mercaptoethanesulfonic acid.

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ml⁻¹. The reaction was allowed to proceed for 30 min at room temperature. Excess KBH₄ was quenched by addition of 50 μ l of anoxic acetone. The reaction mixture containing Na₂S₂O₄ was not quenched. For reduction with palladium under hydrogen, about 0.5 mg of finely ground (surface area = 11.37 m²·g⁻¹) palladium black (Sigma) was added to a 500- μ l aliquot of air-exposed FAF (2 mg·ml⁻¹), and the solution was sparged with H₂ for 30 min at room temperature. Anoxic FAF was subjected to the same treatments as control.

Enzymic Conversion of MPT into FAF. The reaction mixture contained, unless indicated otherwise: Sephadex G-25-treated extract, 1.0 mg of protein; CH₃-S-CoM, 475 nmol; HCHO, 350 nmol; MPT, 162 nmol; ATP, 0.8 μ mol; magnesium acetate, 4 μ mol; Pipes (K⁺) buffer at pH 6.3, 19 μ mol. The headspace in the vials was exchanged with oxygen-free hydrogen. The reaction was started by transferring the reaction vials into a 60°C water bath. When methane evolution ceased (reaction time, 58 min), the vials were transferred into an anaerobic chamber, and 0.5 ml of degassed CH₃OH (HPLC grade) was added to each vial to precipitate most of the protein. Each sample was centrifuged under anoxic conditions, and aliquots were assayed for FAF as described (11).

Proton Magnetic Resonance Spectra of Anoxic Specimens. ¹H NMR spectra were obtained in 100.00 atom % ²H₂O (Aldrich) with a Nicolet 360-MHz Fourier transform NMR instrument. The internal standard was 3-(trimethylsilyl)propionic acid, sodium salt (Wilma Glass, Buena, NJ), and was set at 0 ppm (the powder was stored in an anaerobic chamber). Exchangeable protons present in FAF were replaced by resuspending FAF powder in anoxic ²H₂O followed by lyophilization. This procedure was repeated 3 times. FAF was then dispensed into an NMR tube (5 mm, i.d.) inside an anaerobic chamber, and the tube was sealed with a butyl rubber stopper.

Miscellaneous. Preparation of MPT and CDR factor from *M. thermoautotrophicum* has been described (8). Sephadex G-25-treated extract was prepared as described (7). For the experiment shown in Fig. 8, Sephadex G-25-treated extract was prepared as described (8). CH₃-S-CoM was synthesized as reported (13). ¹⁴CH₃I (Amersham/Searle, Arlington Heights, IL) was used to synthesize [methyl-¹⁴C]-CH₃-S-CoM. The specific activity obtained was 7.2 $\times$ 10⁴ dpm mol⁻¹. Purified component B was obtained as described (14). Fast-atom-bombardment mass spectra were obtained on a VG Analytical ZAB HF mass spectrometer. Samples for fast-atom-bombardment mass spectrometry (FABMS) were dissolved in water and suspended in a thioglycerol matrix. ¹⁴CH₄ was measured as described (8).

RESULTS

During purification steps, FAF was found to be acid stable. In anion exchange chromatography, 71 μ mol of FAF (93%) was recovered from the column with buffer containing 200 mM NaCl. With reversed-phase HPLC, FAF was isocratically eluted from the column 8 min after the injection was made (Fig. 1), and 45 μ mol (63%) was recovered. The coenzyme eluted as a sharp, symmetrical peak, well resolved from other compounds. The FAF fraction desalted on a Sephadex G-15 column was free of any formate. During desalting, the presence of an intensely blue fluorescent compound of small molecular weight (200) was detected; this compound had no FAF activity. The fractions containing the blue fluorescent compound were pooled and lyophilized; however, the amount of dry material recovered was small (<1 mg), and it was not studied further. A second pass of FAF on Sephadex G-15 generated an additional fraction of the blue fluorescent compound. The final recovery of FAF activity was 21 μ mol, 28% of the activity originally present in boiled cell extract. The amount of FAF obtained per kg (wet

FIG. 1. Anoxic HPLC of FAF.

weight) of cells was 65 mg. Analysis by ¹H NMR spectrometry showed that the integrals of areas under the peaks in the spectrum were multiples of one another. The final product was a hygroscopic yellow powder.

The UV spectrum of anoxic FAF at pH 7.0 showed absorption maxima at 302 nm, 247 nm, and 220 nm, and on exposure to O₂ the UV spectrum of the coenzyme showed absorption maxima at 325 nm, 280 nm, and 230 nm (Fig. 2). Changes in the UV spectrum were evident after 2 hr of exposure to air. Drastic changes in the UV spectrum of FAF were recorded at different pH values. At pH 1.0, only one absorption maximum was present (292 nm), whereas at pH 13.0 absorption peaks were at 290 nm and 247 nm (Fig. 3).

We studied the effect of FAF and MPT on the reduction of [methyl-¹⁴C]-CH₃-S-CoM to ¹⁴CH₄ under N₂ with HCHO as the source of reducing power (Fig. 4). The rate of methanogenesis from [methyl-¹⁴C]CH₃-S-CoM was 2-fold faster with FAF (10 nmol of CH₄ formed per min) than with MPT (5 nmol of CH₄ formed per min). No lag was observed when FAF was used, whereas a 60-min lag was observed with MPT in the reaction mixture. The radioactivity in the gas phase and in the liquid phase was measured after 3 hr of

FIG. 2. Spectral changes of FAF on exposure to O₂. An aliquot containing 0.03 μ mol of FAF was diluted to 3.0 ml with degassed 20 mM phosphate buffer (pH 7.0) inside an anaerobic chamber and sealed in a cuvette. The solution was scanned and the stopper was then removed; the contents were exposed to air by periodic mixing. The solution was then scanned after 2 hr (—) and 13 hr (— · —) of exposure to air. ----, Anoxic.

FIG. 3. UV absorption spectra of FAF at different pH values. ---, pH 1; —, pH 7; ···, pH 13.

reaction time, when CH_4 evolution had ceased. In all instances, a total of 2.13×10^4 dpm was added to the reaction mixture. In the absence of any added cofactors, 2.3×10^4 dpm was found in the liquid phase, and no radioactivity was detected in the gas phase. In the presence of FAF, 1×10^4 dpm was found in the liquid phase, and 1.04×10^4 was detected in the gas phase. In the presence of MPT, 1.24×10^4 dpm was found in the liquid phase and 0.94×10^4 dpm was detected in the gas phase. FAF replaced MPT in the assay for CH_4 formation from H_2 and CO_2 (Fig. 5); the reaction was CDR-factor dependent (8). FAF that had been exposed to air for 20 hr no longer stimulated CH_4 production from CO_2 (10 and 50 μg of FAF tested), whereas FAF kept anaerobic under the same conditions was fully active. When an assay mixture containing 16 μg of air-exposed FAF was preincubated at 60°C under H_2/CO_2 (80:20) for 25 min before the addition of $\text{CH}_3\text{-S-CoM}$, no production of CH_4 occurred; a preincubated positive control containing 22 μg of anaerobic FAF produced 405 nmol of CH_4 (200 nmol of $\text{CH}_3\text{-S-CoM}$ added). These results indicated that MPT was not simply air-exposed FAF. MPT was converted into FAF by cell extracts under a hydrogen atmosphere when methanogenesis from $\text{CH}_3\text{-S-CoM}$ and HCHO occurred. The amount of FAF formed was measured spectrophotometrically (11) after quantitative conversion of the substrates to CH_4 (58 min). MPT was converted into FAF (23 nmol) only when $\text{CH}_3\text{-S-CoM}$ and HCHO were present in the reaction mixture. FAF was not formed when either $\text{CH}_3\text{-S-CoM}$ or HCHO was added as a single substrate. MPT was not converted into FAF in

FIG. 4. Methane formation from $[\text{methyl-}^{14}\text{C}]\text{CH}_3\text{-S-CoM}$ under N_2 with HCHO as the source of reducing equivalents. The reaction mixture contained the following: Sephadex G-25-treated extract, 1.0 mg of protein; HCHO , 630 nmol; $[\text{methyl-}^{14}\text{C}]\text{CH}_3\text{-S-CoM}$, 296 nmol (specific activity, 72 dpm nmol $^{-1}$); FAF (●), 200 nmol; or MPT (○), 195 nmol. Δ, Background. Radioactive measurements were made after 3 hr of reaction time.

FIG. 5. Stimulation of methane production from $\text{H}_2 + \text{CO}_2$ by FAF. Each reaction mixture contained standard reaction buffer C (8), 50 μl (0.7 mg of protein) of Sephadex G-25-treated cell extract with Mg^{2+} , 400 nmol of $\text{CH}_3\text{-S-CoM}$, 5 μl of component B, 0.8 μg of CDR, and FAF as indicated. The horizontal dotted line represents the amount of methane produced from $\text{CH}_3\text{-S-CoM}$. ●, Total methane produced; ○, rate of methane production. The reaction was complete after 80 min.

the absence of substrate or in the absence of added enzyme. The positive ion fast-atom-bombardment mass spectrum of FAF in a matrix of thioglycerol showed the molecular weight of FAF to be 776 (Fig. 6A). The molecular ion plus H^+ ($\text{M} + \text{H}^+$) was shown at m/z 777. The peaks at m/z 799, m/z 821, and m/z 843 corresponded to the molecular ion plus Na^+ ($\text{M} + \text{Na}^+$)

FIG. 6. (A) Positive ion fast-atom bombardment mass spectrum of H_4MPT (FAF) in a thioglycerol matrix. The acceleration potential was 8 kV, the source temperature was 30°C , and the resolution was 1000. For high-resolution measurement of the peak at m/z 843 the resolution was 15,000. (B) Positive ion fast-atom bombardment mass spectrum of methanopterin in a thioglycerol matrix. The acceleration potential was 8 kV, the source temperature was 50°C , and the resolution was 1000. For high-resolution measurement of the peak at m/z 773 the resolution was 15,000.

+ Na)⁺, the molecular ion plus 2Na⁺ (M-H + 2Na)⁺, and the molecular ion plus 3Na⁺ (M-2H + 3Na)⁺, respectively. A high-resolution measurement was performed on the signal at *m/z* 843 giving a value of 843.21142. A molecular formula for sodiated FAF agreeing with this measurement was C₃₀H₄₃N₆O₁₆PNa₃, with an error of 5 millimass units. The nonsodiated formula would then be C₃₀H₄₅N₆O₁₆P. The molecular weight of MPT also was determined by FABMS to be 772. The positive ion fast-atom-bombardment mass spectrum of MPT in a thioglycerol matrix is shown in Fig. 6B. The (M + H)⁺ was shown at *m/z* 773. Peaks at *m/z* 795 and *m/z* 817 represented (M + Na)⁺ and (M-H + 2Na)⁺, respectively. A high-resolution measurement was performed on the signal at *m/z* 773, giving a value of 773.2398 with an error of -0.3 millimass units, corresponding to a molecular formula for MPT of C₃₀H₄₁N₆O₁₆P. Negative ion FABMS spectra of FAF and MPT confirmed the assigned molecular weights. The molecular ions minus H, (M - H), were observed at *m/z* 775 in FAF, and at *m/z* 771 in MPT (data not shown). The presence of 1 mol of phosphorus per mol of FAF or MPT was suggested by elemental analyses performed on the coenzymes (data not shown). In the case of FAF, the presence of phosphorus was confirmed by its 100-MHz ³¹P NMR spectrum (Fig. 7). The chemical shift of the observed signal was -0.383 ppm relative to an 85% H₃PO₄ external standard. No evidence of sulfur was found by elemental analysis.

A comparison of the 360-MHz ¹H NMR spectra of H₄MPT (FAF) and MPT in ²H₂O is presented in Fig. 8. H₄MPT (Fig. 8A) and MPT (Fig. 8B) were remarkably similar. Signals present in H₄MPT but not present in MPT were two overlapping doublets at 1.24 ppm, a doublet at 2.85 ppm, and a multiplet at 3.52 ppm. Signals present in MPT but not in H₄MPT were a quartet at 5.10 ppm, a singlet at 2.72 ppm, and a doublet at 1.57 ppm. Homonuclear proton spin-decoupling experiments performed on MPT indicated that the methyl protons at 1.57 ppm were coupled to the methine proton at 5.10 ppm. Irradiation of the signal in H₄MPT at 1.24 ppm resulted in simplified signals at 3.52 ppm and 3.71 ppm. All remaining resonances in both molecules revealed similar coupling patterns.

DISCUSSION

Although a well documented chemical structure of MPT has not appeared, evidence presented in this communication in-

FIG. 7. The 100-MHz ³¹P NMR spectrum of H₄MPT (FAF). FAF (5 mg) dissolved in anoxic ²H₂O (99.8 atom % ²H), was scanned under the following conditions: pulse width, 25 μsec; post-acquisition delay, 1 sec; number of acquisitions, 12,174; sweep width, 5000 Hz; spectrometer frequency, 40.5 MHz; temperature, 25°C.

FIG. 8. (A) The proton NMR spectrum of H₄MPT (FAF). The 360-MHz ¹H NMR spectrum of a solution of H₄MPT (2 mg of H₄MPT in 0.5 ml of ²H₂O) was acquired under the following conditions: pulse width, 3 μsec; sweep width, 2000 Hz; post-acquisition delay, 250 μsec; number of scans, 200; line broadening, 0.2 Hz; spectrometer frequency, 360.061 MHz; temperature, 25°C. The ¹H NMR spectrum was obtained under anoxic conditions. (B) The 360-MHz ¹H NMR spectrum of MPT in ²H₂O (5 mg of MPT in 0.5 ml of ²H₂O). The number of scans was 80. TSP, 3-(trimethylsilyl)propionic acid.

icates that FAF is H₄MPT (5, 6), there being only a 4 atomic mass unit difference between the two molecules. High-resolution FABMS measurements on both molecules indicated the molecular formulas of C₃₀H₄₁N₆O₁₆P for MPT and C₃₀H₄₅N₆O₁₆P for FAF. The differences between the ¹H NMR spectra of H₄MPT and MPT are consistent with the reduction of two double bonds in MPT to yield H₄MPT. The signals for two of the additional protons could be observed in the spectrum of H₄MPT at 3.52 ppm and 2.85 ppm; these are absent in MPT. The other two protons could be bound to atoms that exchange protons for deuterons in ²H₂O. The signal at 1.24 ppm in H₄MPT represents two methyl groups; these have shifted from 2.72 ppm and 1.57 ppm in MPT as a result of the reduction to its tetrahydro form, suggesting that these methyl groups are in proximity to carbons that have become hydrogenated, resulting in two magnetically identical environments. The methine at 5.10 ppm in MPT could be shifted to a new location in H₄MPT. Homonuclear proton spin decoupling of MPT indicated that the methyl at 1.57 ppm was coupled to the methine at 5.10 ppm. Since this methyl and the methyl at 2.72 ppm are observed in H₄MPT at 1.24 ppm, and irradiation at the latter resulted in the simplification of the signals at 3.52 ppm and 3.71 ppm, it follows that the chemical shift of the methine at 5.10 ppm in MPT is either the signal at 3.52 ppm or the one at 3.71 ppm in H₄MPT. This methine, then, is also in proximity to carbons that become hydrogenated in MPT to yield H₄MPT. The oxi-

dation of H₄MPT does not produce MPT but a compound that is enzymically refractory, probably dihydro MPT. Until the exact chemical structure of MPT is known, it will be difficult to interpret these latter observations. The enzymic conversion of MPT into FAF indicates the H₄MPT is the physiologically important form of this coenzyme, and evidence has been obtained (11) that H₄MPT may carry C₁ precursors of methane at the methine, methylene, and methyl levels of oxidation.

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