Purification and Properties of the 5,10-Methenyltetrahydromethanopterin Cyclohydrolase from *Methanobacterium thermoautotrophicum*

ANTHONY A. DiMARCO, MARK I. DONNELLY,[†] AND RALPH S. WOLFE*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 21 July 1986/Accepted 15 September 1986

The 5,10-methenyltetrahydromethanopterin cyclohydrolase of *Methanobacterium thermoautotrophicum* was purified 128-fold to homogeneity. The enzyme had a subunit M_r of 41,000 as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. From high-performance size exclusion chromatography of the native protein, an M_r of 82,000 was determined, suggesting a dimer of identical subunits. The enzyme was inhibited by 10-formyltetrahydromethanopterin and stimulated by Mg^{2+} . Evaluation of the reaction equilibrium indicated that the methenyl derivative was favored over 5-formyltetrahydromethanopterin, with a much higher equilibrium constant than for the analogous reaction of tetrahydrofolate derivatives. Folate derivatives did not serve as substrates for this enzyme.

Methanogenic bacteria have been shown to possess unique coenzymes specifically involved as single-carbon carriers (23). Of these, only one, 5,6,7,8-tetrahydromethanopterin (H_4MPT), a 7-methylpterin (22), has both structural and biochemical similarity to a known coenzyme, namely, 5,6,7,8-tetrahydrofolic acid. Like tetrahydrofolate, the methanogenic coenzyme is sensitive to air and reacts with formaldehyde to yield the 5,10-methylene derivative. The purification of a dehydrogenase from *Methanobacterium thermoautotrophicum* which oxidizes the methylene derivative to methenyl was recently reported (13). In addition, Donnelly et al. (5) have shown the presence of a cyclohydrolase activity in 70% ammonium sulfate supernatant preparations of cell extract from *M. thermoautotrophicum* which catalyzes the energy-independent hydrolysis of 5,10-methenyl- H_4MPT to 5-formyl- H_4MPT . The methenyl derivative has also been shown to undergo a slow nonenzymatic hydrolysis to yield the oxygen-sensitive 10-formyl derivative (5).

Here we describe the purification and partial characterization of the 5,10-methenyl- H_4MPT cyclohydrolase. Assigning the product of the enzymatic hydrolysis of the 5,10-methenyl derivative as the 5-formyl derivative distinguishes this enzyme from the cyclohydrolases of tetrahydrofolate metabolism which yield the 10-formyl derivative (3). In addition, the enzyme is monofunctional, distinct from the 5,10-methylene- H_4MPT dehydrogenase and from formylmethanofuran: H_4MPT formyltransferase (M. I. Donnelly and R. S. Wolfe. Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-56, p. 174). This is in contrast to the bifunctional (cyclohydrolase-dehydrogenase) tetrahydrofolate enzyme of many procaryotes (3, 4) and to the trifunctional (cyclohydrolase-dehydrogenase-formyl-tetrahydrofolate synthetase) tetrahydrofolate enzyme of all eucaryotes studied (3, 19).

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: CFE, cell extract; bicine, *N,N*-(bis-2-hydroxyethyl)glycine;

SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Growth of cells and extract preparation. *M. thermoautotrophicum* ΔH was grown in a 200-liter fermentor as described previously (11). CFE was prepared from a cell slurry as described previously (12).

Preparation of substrates. Purification of H_4MPT from boiled cell extract of *M. thermoautotrophicum* was performed as described previously (7). Methenyl- H_4MPT was synthesized by the enzymatic oxidation of methylene- H_4MPT formed by spontaneous reaction of purified H_4MPT with formaldehyde (5). Chemical synthesis of a functional derivative of methenyl- H_4MPT was performed by formylation and acidification of pure H_4MPT with large excesses of acetic acid and formic acid as described previously (5). Methenyl- H_4MPT and the synthetic derivative of methenyl- H_4MPT were purified aerobically by reverse-phase (octadecyl) high-performance liquid chromatography by using a 10-min linear gradient of 10 to 60% methanol in 20 mM ammonium formate (pH 4.0) at a flow rate of 1.5 ml/min. Methenyl- H_4MPT eluted 11 min after injection, and the functional derivative eluted at 13 min. Pooled eluates were diluted with water and lyophilized, and the amount of methenyl- H_4MPT was estimated by A_{335} , the molar extinction coefficient being 21,600 (8).

5-Formyl-tetrahydrofolic acid was purchased as a powder from Aldrich Chemical Co., Milwaukee, Wis. Methenyl-tetrahydrofolate was prepared by acidification of 5-formyl-tetrahydrofolate as described previously (20).

Enzyme assays. Cyclohydrolase activity was measured in 0.5-ml stoppered quartz cuvettes (Precision Cells, Inc., Hicksville, N.Y.) capped with 13-mm gray butyl serum vial stoppers (West Co., Phoenixville, Pa.). Assay mixtures were prepared with anoxic 100 mM bicine buffer (pH 8.2) in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). For assay of preparations after ammonium sulfate treatment, cuvettes were prepared aerobically. The cuvettes were incubated at 60°C for 10 min in a temperature-regulated cell holder of a Gilford model 252 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Methenyl- H_4MPT (20 nmol) or the synthetic derivative of methenyl- H_4MPT (15 nmol) was added by injecting 5 to 10 μ l

* Corresponding author.

[†] Present address: Amoco Research Center, Naperville, IL 60566.

TABLE 1. Purification of cyclohydrolase

Purification step	Protein (mg)	Cyclohydrolase				Dehydrogenase total activity (U)
		Sp act (U/mg)	Total activity (U)	Purification (fold)	% Recovery	
CFE	116	1.06	123	1	100	731
Ammonium sulfate	89	1.23	109	1.2	89	490
Phenyl-Sepharose	6.5	7.8	51	7.7	42	78
Methotrexate resin	2.1	63	132	62	109	0
FPLC Superose 12	0.72 ^a	130	94	128	78	

^a Represents theoretical yield for step performed on a portion of the methotrexate-purified enzyme.

of a concentrated solution, and the reaction was initiated with the addition of 5 to 20 μ l of enzyme, bringing the total volume in the cuvette to 0.5 ml. The disappearance of methenyl- H_4 MPT was monitored by the decrease in A_{335} . The slope of the initial decrease was used for rate calculations. For purification table activities, 25 nmol of methenyl- H_4 MPT was used. Dehydrogenase activity was measured as described previously (13) by using the following amounts: 20 nmol of H_4 MPT, 60 nmol of (*N*-L-lactyl-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5-phosphate, and 3 μ mol of formaldehyde. The assay for formylmethanofuran: H_4 MPT formyltransferase was performed by adding enzyme to a quartz spectrophotometric cuvette containing 25 nmol of H_4 MPT and 60 nmol of formylmethanofuran in 1.0 ml of anoxic 20 mM potassium phosphate (pH 7.0) (M. I. Donnelly and R. S. Wolfe, submitted for publication).

Effectors of cyclohydrolase activity. To study the effects of various compounds on the cyclohydrolase reaction, each of the following compounds was included in a separate standard assay mixture containing methenyl- H_4 MPT (50 μ M) before the addition of enzyme: amethopterin, methylene- H_4 MPT, NAD, NADP, ATP, magnesium acetate, magnesium chloride, manganese acetate, potassium acetate, and EDTA. To observe the effect of 10-formyl- H_4 MPT, an assay mixture was prepared in a 1.0-ml cuvette under anoxic conditions with 8.5 nmol of methenyl- H_4 MPT and maintained at room temperature. After 24 h, the nonenzymatic interconversion of the 10-formyl and methenyl derivatives achieved equilibrium, and the concentration of methenyl was estimated by A_{335} . The reaction was initiated by the addition of 0.3 μ g of partially purified anoxic enzyme (methotrexate affinity column eluate described below). The standard assay mixture containing methenyl- H_4 MPT without further addition of an effector served as the control.

Enzyme purification. Manipulations of enzyme preparations during purification were performed in an anaerobic chamber unless otherwise stated. Solid ammonium sulfate (2.1 g) was slowly added with stirring to a volume of 5.8 ml of CFE to a final concentration of 60% saturation. This solution was stirred for 2 h at 4°C, transferred to a stainless steel centrifuge tube, and centrifuged at $30,000 \times g$ for 20 min at 4°C (Sorvall; Du Pont Co., Wilmington, Del.). The supernatant was then applied to a column (2.5 by 5 cm) of phenyl-Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) equilibrated in buffer A (20 mM potassium phosphate, 10 mM 2-mercaptoethanol [pH 7.0]) with 2 M potassium acetate. The column was washed with 2 bed volumes of the equilibration buffer and developed with a nonlinear gradient of 1 to 0 M potassium acetate in buffer A. Additional protein was eluted stepwise with 40% ethylene glycol in buffer A. The pool of active fractions was then loaded onto a 20-ml column of immobilized methotrexate (amethopterin linked to

Sepharose by a six-carbon spacer; Pierce Chemical Co., Rockford, Ill.) equilibrated in buffer A. The column was washed with 2 bed volumes of equilibration buffer and developed with a linear gradient of 0 to 1 M potassium acetate in buffer A. The fractions containing cyclohydrolase activity but not dehydrogenase activity were pooled and concentrated by ultrafiltration on a PM10 membrane filter (Amicon Corp., Lexington, Mass.). Further purification was performed by using the Pharmacia FPLC system. Samples (200 μ l) of the concentrated methotrexate column eluate were loaded onto a Superose 12 10/30 column (Pharmacia) equilibrated in 2 M potassium acetate in buffer A. The eluate was monitored at 280 nm. A symmetrical peak of protein containing cyclohydrolase activity was observed and collected. Active fractions from several fractionations were pooled.

Analytical PAGE. Denaturing and nondenaturing analytical discontinuous PAGE was performed in slab gels with a pH system of 6.8 to 8.8 (17) as described previously (21). The Coomassie brilliant blue R-250 solution described by Fairbanks et al. (9) was used to stain the proteins.

Molecular weight determinations. The relative molecular mass of the native cyclohydrolase was determined by high-performance size-exclusion chromatography (14) with a TSK SW3000 column (0.75 by 30 cm; Toyo Soda; Beckman Instruments, Inc., Fullerton, Calif.) connected to a model 501 pump (Waters Associates, Inc., Milford, Mass.) and a UV detector with a variable wavelength monitor set at 280 nm (Varian Inc.). The buffer contained 10 mM potassium phosphate (pH 7.0), 300 mM potassium acetate, and 5% (vol/vol) ethylene glycol, as modified by Rouvière et al. (21). Back pressure was maintained below 10 kPa with a flow rate of 0.5 ml/min. Samples of the following standards (20 μ l, 2 mg/ml) were used: blue dextran (M_r 2,000,000), bovine serum albumin (M_r s 68,000 and 136,000), ovalbumin (M_r 45,000), and lactalbumin (M_r 36,000). Cyclohydrolase (50 μ l, 0.5 mg/ml) was detected by the assay described earlier, and its molecular size was calculated from its relative elution volume.

The subunit molecular mass of the denatured protein was determined by SDS-PAGE at 8 and 10% acrylamide (17). The following standards were used: bovine serum albumin, ovalbumin, methylcoenzyme M methylreductase component C (M_r s: γ , 38,500; β , 45,000; α , 68,000) (6), and phosphorylase b (M_r 92,500). Subunit size was determined by calculating the relative mobility of the denatured cyclohydrolase.

Equilibria of formyl isomers of H_4 MPT. The equilibrium of the nonenzymatic hydrolysis of methenyl- H_4 MPT to 10-formyl- H_4 MPT was investigated by measuring the initial and final A_{335} in a cuvette containing 8.5 nmol of 5,10-methenyl- H_4 MPT and 100 mM bicine buffer (pH 8.2) in a 1.0-ml volume. A similar reaction at pH 9.0 also was monitored. The cuvettes were prepared in an anaerobic chamber to

FIG. 1. Native PAGE of homogeneous cyclohydrolase (6 μ g) obtained by chromatography on a Superose 12 column. Conditions of PAGE are described in Materials and Methods.

prevent possible oxidation of the hydrolysis product. Equilibrium was reached after 24 h at room temperature. The equilibrium of the enzymatic hydrolysis of methenyl- H_4 MPT to 5-formyl- H_4 MPT was calculated from published results of the extent of the enzymatic reaction (5). Because enzymatic equilibrium was reached within 30 s after the addition of enzyme, the accumulation of the nonenzymatic product was negligible and therefore not considered in the calculations. The equilibrium constant (K_{eq}) is defined as the concentration of methenyl- H_4 MPT divided by the product of the formyl- H_4 MPT and proton concentrations. The concentration of the methenyl derivative was determined from the A_{335} at equilibrium, the λ_{max} of this derivative. Concentrations of the formyl derivatives were determined from the difference in the methenyl concentration (initial minus final) at equilibrium, assuming the simple interconversion previously reported (5).

Folate derivatives as substrates for cyclohydrolase. Quartz cuvettes (1.0 ml) prepared under anoxic conditions contained either 86 nmol of 5-formyl-tetrahydrofolate in 1.0 ml

of 50 mM potassium phosphate buffer (pH 6.4) or 30 nmol of 5,10-methenyl-tetrahydrofolate in 1.0 ml of 50 mM bicine buffer (pH 8.2). The assay was initiated by the addition of 10 μ l of partially purified enzyme (methotrexate affinity column eluate) containing 0.6 μ g of protein, and monitored for an increase (5-formyl-tetrahydrofolate) or decrease (methenyl-tetrahydrofolate) in A_{340} .

Protein determinations. Protein concentrations were determined by the method of Bradford (2) for elution profiles and by measuring the turbidity of samples in 20% trichloroacetic acid (16) for all other samples.

RESULTS

Purification of the 5,10-methenyl- H_4 MPT cyclohydrolase.

The 5,10-methenyl- H_4 MPT cyclohydrolase was purified 128-fold (Table 1) to electrophoretic homogeneity (Fig. 1). After ammonium sulfate treatment, the enzyme could be handled aerobically without loss of activity. In the first chromatographic step, phenyl-Sepharose (Fig. 2), a majority of the activity eluted at a potassium acetate concentration of 350 mM. This step removed much of the methylcoenzyme M methylreductase component C from the resulting pool, as determined by native PAGE (Fig. 3, lane 3). Formylmethanofuran- H_4 MPT formyltransferase activity was entirely resolved from the pool of cyclohydrolase activity and was eluted only after the column was washed with 40% ethylene glycol (Fig. 2). Chromatography on immobilized methotrexate completely separated the methylene- H_4 MPT dehydrogenase activity from the cyclohydrolase (Table 1), with the latter eluting at a concentration of 800 mM potassium acetate (Fig. 4). The resulting preparation exhibited a twofold increase in total activity. At this stage in the purification, the preparation still contained methylreductase component C, as identified by native PAGE (Fig. 2, lane 4) and SDS-PAGE (data not shown). Small amounts of homogeneous cyclohydrolase could be obtained by passing this preparation through a Superose 12 column equilibrated in 2 M potassium acetate. High salt concentrations were required to separate the cyclohydrolase from methylreductase compo-

FIG. 2. Separation of formyltransferase activity from cyclohydrolase and dehydrogenase activities by phenyl-Sepharose chromatography; 2.0-ml fractions were collected. EG, Ethylene glycol.

FIG. 3. Native PAGE of active cyclohydrolase fractions at consecutive steps. Lanes: 1, CFE (20 μ g); 2, 60% ammonium sulfate supernatant (38 μ g); 3, phenyl-Sepharose column eluate (10 μ g); 4, immobilized methotrexate affinity column eluate (7 μ g). Open arrow corresponds to methylcoenzyme M methylreductase system component C. Closed arrow indicates the position of cyclohydrolase.

nent C. In buffer A alone, the two proteins comigrated despite their large size differences.

Subunits and molecular size. SDS-PAGE of homogeneous cyclohydrolase at 8 and 10% acrylamide showed a single protein band. The relative molecular mass of the denatured enzyme was estimated as 41,000 (Fig. 5A). High-performance size-exclusion chromatography of the partially purified methotrexate eluate and homogeneous enzyme each yielded one active protein peak with an elution time corresponding to a relative molecular mass of the native enzyme of 82,000 (Fig. 5B).

Equilibria of the formyl isomers. The equilibrium constants for the interconversion of the H_4 MPT derivatives, methenyl/10-formyl and methenyl/5-formyl, were determined over a range of pH values (Table 2). The average value for the enzymatic reaction methenyl- H_4 MPT $\rightleftharpoons$ 5 formyl- H_4 MPT + H^+ was $5.4 \times 10^7 M^{-1}$, but it showed a consistent increase as pH increased. The value for the

FIG. 5. Molecular size determination of cyclohydrolase. (A) Estimation of cyclohydrolase subunit size ($\circ$) by SDS-PAGE at 8 and 10% acrylamide. Standards at 8% ($\bullet$): lactalbumin, ovalbumin, γ subunit of methylcoenzyme M methylreductase system component C, and phosphorylase *b*. Standards at 10% ($\blacksquare$): γ , β , and α subunits of component C and phosphorylase *b*. M_r s of standards are given in Materials and Methods. (B) Estimation of native cyclohydrolase ($\circ$) molecular size by gel filtration. Standards ($\bullet$) are bovine serum albumin (monomer and dimer), ovalbumin, and lactalbumin. Conditions are described in Materials and Methods.

nonenzymatic reaction methenyl- H_4 MPT $\rightleftharpoons$ 10 formyl- H_4 MPT + H^+ was $7.84 \times 10^8 M^{-1}$.

Sensitivity of cyclohydrolase to effectors. Cyclohydrolase activity in the presence of 50 μ M methenyl- H_4 MPT was unaffected by the addition of methylene- H_4 MPT (100 μ M), NAD (10 μ M), and NADP (10 μ M). The specific activity of the methenyl- H_4 MPT cyclohydrolase in the presence of amethopterin (100 μ M), an analog of tetrahydrofolate, was decreased to 74% of the control. The presence of 10-formyl-

FIG. 4. Separation of dehydrogenase activity from cyclohydrolase activity by immobilized methotrexate chromatography.

TABLE 2. Equilibria of interconversion of formyl- and methenyl-H₄MPT derivatives

Reaction and expt ^a	Concn (M [10 ⁶]) of:			H ⁺ (M)	K _{eq} (M [10 ⁻⁸])
	10-Formyl-H ₄ MPT	5-Formyl-H ₄ MPT	5,10-Methenyl-H ₄ MPT		
1					
1	2.20		11.6	6.3 × 10 ⁻⁹	8.37
2	2.23		11.1	6.3 × 10 ⁻⁹	7.56
3	9.03		6.25	9.1 × 10 ⁻¹⁰	7.60
2					
4		8.4	21.6	1.78 × 10 ⁻⁷	0.14
5		13.5	16.5	7.08 × 10 ⁻⁸	0.17
6		18.0	12.0	2.82 × 10 ⁻⁸	0.24
7		21.0	9.0	1.12 × 10 ⁻⁸	0.38
8		22.5	7.5	4.46 × 10 ⁻⁹	0.75
9		23.4	6.6	1.78 × 10 ⁻⁹	1.58

^a Reaction 1: 10-formyl-H₄MPT + H⁺ ⇌ 5,10-methenyl-H₄MPT [$K_{eq} = (5,10\text{-methenyl-H}_4\text{MPT})/(10\text{-formyl-H}_4\text{MPT})(\text{H}^+)$]. Reaction 2: 5-formyl-H₄MPT + H⁺ ⇌ 5,10-methenyl-H₄MPT [$K_{eq} = (5,10\text{-methenyl-H}_4\text{MPT})/(5\text{-formyl-H}_4\text{MPT})(\text{H}^+)$]. The average K_{eq} for reaction 1 was 7.84; for reaction 2 it was 0.54.

H₄MPT (5.8 μM) lowered the specific activity of the cyclohydrolase for methenyl-H₄MPT (11.2 μM) by 54%. The divalent cation Mg²⁺ enhanced activity markedly; magnesium acetate (20 mM) caused a 2.2-fold increase, and magnesium chloride (20 mM) caused a 2.4-fold increase. The addition of potassium acetate (20 mM) caused no increase, and manganese acetate inhibited activity to 36% of the control at 20 mM and to 24% of the control at 40 mM (final concentrations). The addition of EDTA (20 mM) just before the addition of enzyme or the preincubation of EDTA with enzyme decreased activity to 82 and 80% of the control, respectively. When the synthetic derivative of methenyl-H₄MPT served as the substrate, the addition of 20 mM magnesium acetate decreased the specific activity of the cyclohydrolase to 63% of the control.

Activity of folate derivatives as substrates. No increase in the rate of hydrolysis of the methenyl derivative of tetrahydrofolic acid was detected after the addition of enzyme. 5-Formyl-tetrahydrofolate showed no cyclization, either in the absence or in the presence of enzyme, and cyclization was not stimulated by the addition of 27 nmol of ATP and 1 μmol of Mg²⁺.

DISCUSSION

The 5,10-methenyl-H₄MPT cyclohydrolase from *M. thermoautotrophicum* was purified by three chromatographic steps, including affinity chromatography on immobilized methotrexate. Through this stage of purification, the enzyme copurified with component C of the methylcoenzyme M methylreductase system. Resolution of these two proteins required gel filtration at high salt concentrations, conditions in which the presumed electrostatic interactions would be shielded and elution would depend on their respective molecular sizes. We have not yet ascertained whether this association is of physiological significance. The purified enzyme appears to exist as a dimer of identical subunits, as judged by results from SDS-PAGE, with an estimated native M_r of 82,000. In addition, the cyclohydrolase activity could be separated from both formyltransferase and dehydrogenase activities in consecutive chromatographic steps. Thus far, only two other organisms, *Clostridium formicoaceticum* (3) and *Acetobacterium woodii* (3), have been shown to possess a monofunctional cyclohydrolase. These enzymes stand in contrast to the bifunctional enzymes of many procaryotes (4) and the trifunctional enzymes of all eucaryotes studied (19).

The cyclohydrolase from *M. thermoautotrophicum* represents a unique enzyme involving an energy-independent interconversion of a 5-formyl-tetrahydropterin coenzyme and its methenyl derivative (5). Enzymes which convert 5-formyl-tetrahydrofolate to the methenyl (10) or 10-formyl (15) derivative have been purified in other organisms, but both are ATP-utilizing reactions. The role of the 10-formyl derivative in methanogens has not been evaluated, and speculation has arisen as to its possible involvement in biosynthetic pathways (5). The reportedly small amounts of folic acid in *M. thermoautotrophicum* (18) make this an attractive model for further research.

The equilibrium constants for the interconversions of the formyl isomers with methenyl-H₄MPT indicate that the methenyl derivative was favored over both formyl derivatives. The equilibrium for the methenyl derivative/5-formyl derivative was much greater for H₄MPT than for tetrahydrofolate, i.e., $5.4 \times 10^7 \text{ M}^{-1}$ versus $6.5 \times 10^2 \text{ M}^{-1}$, respectively (15), and may be related to the additional methyl substituents of the pterin of H₄MPT. There was, however, consistency with respect to the effect of increasing pH; the apparent K_{eq} for both enzymes increased with pH. This phenomenon has been postulated to be a result of complicating factors, such as the possible ionization of a functional group in the molecule (1).

Contrary to other cyclohydrolases studied, Mg²⁺ had a stimulatory effect on the activity of the methenyl-H₄MPT cyclohydrolase. Whether the divalent cation is necessary for enzymatic catalysis is unclear, since activity was virtually unaffected by the presence of EDTA. Proposed mechanisms for the enzymatic hydrolysis of the folate derivative indicate the involvement of general acid-base catalysis (20). We speculate that a chelation of the Mg²⁺ ion by phosphate of the phosphoglutarate moiety of H₄MPT caused an increase in affinity for the substrate. This is substantiated by the inability of Mg²⁺ to stimulate activity when a derivative lacking the α-hydroxyglutaryl-phosphate group served as the substrate. The inhibitory effects of Mn²⁺ with authentic substrate are not understood.

The 5,10-methenyl-H₄MPT cyclohydrolase from *M. thermoautotrophicum* appears to be distinct from other cyclohydrolases so far studied. The inability of the enzyme to utilize the analogous folate derivatives as substrates and the differences in equilibrium values of the H₄MPT derivatives suggest that this apparent uniqueness may be partly a reflection of the chemical differences between the two coenzymes.

ACKNOWLEDGMENTS

We thank Victor Gabriel for mass culture of the organism and Peter Rubinelli for assistance.

This work was supported by Public Health Service grant AI12277 from the National Institutes of Health.

LITERATURE CITED

1. Blakley, R. L. 1969. The biochemistry of folic acid and related pteridines. *Front. Biol.* **13**:58–106.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **86**:142–146.
3. Clark, J. E., and L. G. Ljungdahl. 1982. Purification and properties of 5,10-methenyltetrahydrofolate cyclohydrolase from *Clostridium formicoaceticum*. *J. Biol. Chem.* **257**:3833–3836.
4. Dev, I. K., and R. J. Harvey. 1978. A complex of 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methenyltetrahydrofolate cyclohydrolase in *Escherichia coli*. *J. Biol. Chem.* **253**:4245–4253.
5. Donnelly, M. I., J. C. Escalante-Semerena, K. L. Reinhart, Jr., and R. S. Wolfe. 1985. Methenyltetrahydromethanopterin cyclohydrolase in cell extracts of *Methanobacterium*. *Arch. Biochem. Biophys.* **242**:430–439.
6. Ellefson, R. P., and R. S. Wolfe. 1981. Component C of the methylreductase system of *Methanobacterium*. *J. Biol. Chem.* **256**:4259–4262.
7. Escalante-Semerena, J. C., J. A. Leigh, K. L. Reinhart, Jr., and R. S. Wolfe. 1984. Formaldehyde activation factor, a coenzyme of methanogenesis. *Proc. Natl. Acad. Sci. USA* **81**:1976–1980.
8. Escalante-Semerena, J. C., K. L. Reinhart, Jr., and R. S. Wolfe. 1984. Tetrahydromethanopterin, a carbon carrier in methanogenesis. *J. Biol. Chem.* **259**:9447–9455.
9. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. *Biochemistry* **12**:2606–2617.
10. Grimshaw, C. E., G. B. Henderson, G. G. Soppe, G. Hansen, E. J. Mathur, and F. M. Huennekens. 1984. Purification and properties of 5,10-methenyltetrahydrofolate synthetase from *Lactobacillus casei*. *J. Biol. Chem.* **259**:2728–2733.
11. Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe. 1978. Preparation of coenzyme M analogs and their activity in the methylcoenzyme M reductase system of *Methanobacterium thermoautotrophicum*. *Biochemistry* **17**:2374–2377.
12. Gunsalus, R. P., and R. S. Wolfe. 1980. Methyl coenzyme M reductase from *Methanobacterium thermoautotrophicum*. *J. Biol. Chem.* **255**:1891–1895.
13. Hartzell, P. L., G. Zvilius, J. C. Escalante-Semerena, and M. I. Donnelly. 1985. Coenzyme F₄₂₀ dependence of the methylene-tetrahydromethanopterin dehydrogenase of *Methanobacterium thermoautotrophicum*. *Biochem. Biophys. Res. Commun.* **133**:884–890.
14. Hefti, F. 1982. High performance size-exclusion chromatography: a buffer for the reliable determination of molecular weight of proteins. *Anal. Biochem.* **121**:378–381.
15. Kay, L. D., M. J. Osborn, Y. Hatefi, and F. M. Huennekens. 1960. The enzymatic conversion of 5-formyl tetrahydrofolic acid (folinic acid) to 10-formyl tetrahydrofolic acid. *J. Biol. Chem.* **235**:195–201.
16. Kunitz, M. J. 1952. Crystalline inorganic pyrophosphatase isolated from baker's yeast. *J. Gen. Physiol.* **35**:423–450.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
18. Leigh, J. A. 1983. Levels of water-soluble vitamins in methanogenic and nonmethanogenic bacteria. *Appl. Environ. Microbiol.* **45**:800–803.
19. Paukert, J. L., L. D. Straus, and J. C. Rabinowitz. 1976. Formyl-methenyl-methylenetetrahydrofolate synthetase (combined): an ovine protein with multiple catalytic activities. *J. Biol. Chem.* **251**:5104–5111.
20. Robinson, D. R., and W. P. Jencks. 1967. Mechanism and catalysis of the hydrolysis of methenyltetrahydrofolic acid. *J. Am. Chem. Soc.* **89**:7098–7103.
21. Rouvière, P. E., J. C. Escalante-Semerena, and R. S. Wolfe. 1985. Component A2 of the methylcoenzyme M methylreductase system from *Methanobacterium thermoautotrophicum*. *J. Bacteriol.* **162**:61–66.
22. Van Beelen, P., A. P. Stassen, J. W. Bosch, G. D. Vogels, W. Guijt, and C. A. Haasnoot. 1984. Elucidation of the structure of methanopterin, a coenzyme from *Methanobacterium thermoautotrophicum*, using two-dimensional nuclear-magnetic-resonance techniques. *Eur. J. Biochem.* **138**:563–571.
23. Wolfe, R. S. 1985. Unusual coenzymes of methanogenesis. *Trends Biochem. Sci.* **10**:396–399.