JOURNAL OF BACTERIOLOGY, July 1985, p. 126–131
0021-9193/85/070126-06$02.00/0

Vol. 163, No. 1

Evidence of a Common Pathway of Carbon Dioxide Reduction to Methane in Methanogens

W. JACK JONES,† MARK I. DONNELLY, AND RALPH S. WOLFE*
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 4 February 1985/Accepted 3 April 1985

The roles of methanofuran and tetrahydromethanopterin as carriers of C_1 moieties in the reduction of carbon dioxide to methane were studied in representatives of diverse groups of methanogens, confirming that these roles, first reported for *Methanobacterium thermoautotrophicum*, are common for methanogenesis in general. Extracts of the methanogens tested converted formyl-methanofuran and methyl-tetrahydromethanopterin to methane; the extractable cofactors derived from the same methanogens, with one exception, complemented a methanofuran- and tetrahydromethanopterin-deficient enzyme system from *M. thermoautotrophicum*. The amounts of extractable methanofuran and tetrahydromethanopterin were determined for each representative methanogen.

The pioneering investigations into the biosynthesis of methane from CO_2, performed by H. A. Barker and colleagues, established that free formate, formaldehyde, and methanol were not intermediates in the reductive pathway, and Barker proposed that C_1 units at intermediate oxidation states were carried as derivatives of an enzymic cofactor or cofactors (3). One of these cofactors, 2-mercaptoethanesulfonic acid (HS-CoM), was identified in the early 1970s as a carrier of C_1 units at the methanol level of oxidation (17, 25). The methyl derivative of this cofactor, 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM), is the substrate for the methane-forming reaction which is catalyzed by the complex methylreductase enzyme system (11). Recently, two additional cofactors have been isolated from *Methanobacterium thermoautotrophicum* which, in conjunction with HS-CoM, serve as carriers for C_1 units in methane production from CO_2. Tetrahydromethanopterin (H_4MPT), an analog of tetrahydrofolate (27), carries C_1 units at the methine, methylene, and methyl levels of oxidation (7, 26). Methanofuran (MFR), formerly the carbon dioxide reduction factor (16, 21), a novel furan-containing cofactor (15), carries C_1 units at the formyl and possibly the carboxyl levels of oxidation (15a). Addition of these two newly described cofactors, CH_3-S-CoM, and component B to cell-free extracts of *M. thermoautotrophicum* which have been depleted of low-molecular-weight cofactors restores the ability of these extracts to convert CO_2 to methane (16). In addition, cell extracts of *M. thermoautotrophicum* readily convert the formyl derivative of MFR and the methenyl, methylene, and methyl derivatives of H_4MPT to methane under appropriate conditions (7, 15a). Accordingly, a model for the complete reduction of CO_2 to methane has been proposed in which derivatives of these cofactors account for the carrier-bound C_1 units of intermediate oxidation states (6). A simplified version of this model is shown in Fig. 1.

Data to support this model were obtained from studies of *M. thermoautotrophicum*. Here we present results which indicate that the proposed pathway is the common mechanism of CO_2 reduction in methanogens. We assayed several representative methanogens for their ability to convert derivatives of MFR and H_4MPT to methane, and we assayed boiled cell extracts from a number of methanogenic and nonmethanogenic bacteria for the presence of MFR and H_4MPT.

MATERIALS AND METHODS

Analytical procedures. The presence of methane was determined with a Packard model 428 gas chromatograph equipped with a flame ionization detector and a Porapak Q (Supelco, Bellefonte, Pa.) column. The standard methanogenic assay (19) included 18 μmol of potassium PIPES (1,4-piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) buffer at pH 6.3 or 6.6, 2.5 μmol of magnesium acetate, 0.8 μmol of sodium ATP, and additional components as specified in a final volume of 200 μl. Protein was estimated by measuring the turbidity at 400 nm after precipitation in 20% trichloroacetic acid (13). Standards were prepared with bovine serum albumin.

Chemicals. All routinely used chemicals were reagent grade. CH_3-S-CoM was synthesized as previously described (20); HS-CoM was purchased from MC/B Manufacturing Chemist, Inc., Cincinnati, Ohio. MFR and [*formyl*-^{14}C]-formyl-MFR were purified and prepared as described (16) and were gifts of John Leigh. H_4MPT and methyl-H_4MPT were purified and prepared as described (5, 7) and were gifts of Jorge Escalante-Semerena.

Organisms and growth conditions. The strict anaerobic procedures described by Balch et al. (1) were used for cultivation of methanogens. *Methanobacterium bryantii* M.o.H., *Methanobacterium ruminantium* M1, *Methanobrevibacter smithii* PS, and *Methanospirillum hungatei* JF1 were cultured in medium 1 of Balch et al. (1) under an atmosphere of H_2 and CO_2. *Methanobacterium thermoautotrophicum* ΔH was cultured in medium 2 of Balch et al. (1) under an atmosphere of H_2 and CO_2. *Methanococcus voltae* PS was cultured in defined medium under an atmosphere of H_2 and CO_2 as described by Whitman et al. (28). *Methanococcus jannaschii* JAL-1 was cultured in defined medium under an atmosphere of H_2 and CO_2 as described by Jones et al. (12). *Methanosarcina barkeri* 227 was cultured in the following medium (grams per liter except as noted): K_2HPO_4, 0.3; KH_2PO_4, 0.3; $(NH_4)_2SO_4$, 0.3; NaCl, 0.6; $MgSO_4 \cdot 7H_2O$, 0.13; $CaCl_2 \cdot 2H_2O$, 0.0008; $FeSO_4 \cdot 7H_2O$, 0.0003; $NiCl_2 \cdot 6H_2O$, 0.0005; Na_2SeO_4,

* Corresponding author.
† Present address: School of Applied Biology, Georgia Institute of Technology, Atlanta, GA 30332.

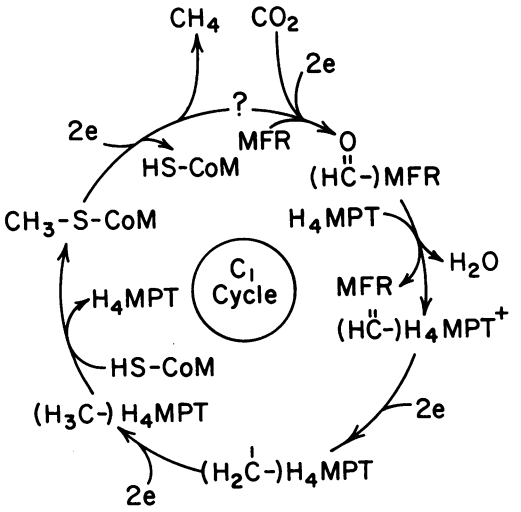

FIG. 1. Scheme showing the role of cofactors in the C_1 cycle of methanogenesis from CO_2. The (?) indicates that the coupling mechanism between the methylreductase system and CO_2 activation (the RPG effect, see reference 10) is unknown. C_1 carriers are labeled as follows: (HCO-)MFR, formyl-methanofuran; (HC≡)H_4MPT^+, methenyl-H_4MPT; (H_2C=)H_4MPT, methylene-H_4MPT; (H_3C—)H_4MPT, methyl-H_4MPT.

0.004; $NaHCO_3$, 4.0; cysteine hydrochloride · H_2O, 0.5; Na_2S · $9H_2O$, 0.5; yeast extract (Difco Laboratories, Detroit, Mich.), 1.0; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1.0; trace vitamin solution (1), 10 ml; and trace mineral solution (1), 10 ml. The final gas atmosphere was H_2 and CO_2 (4:1). In some experiments, methanol (50 mM) or sodium acetate (100 mM) was added as a sterile solution, and the final gas atmosphere was N_2 and CO_2 (4:1). *Methanogenium marisnigri* was cultured in medium 3 of Balch et al. (1) under an atmosphere of H_2 and CO_2.

Halobacterium volcanii D52 was cultivated in a tryptone-yeast extract-salt medium as described by Mullakhanbhai and Larsen (18). Cells of *Thermoproteus tenax* DSM2078 were a gift of C. R. Woese (University of Illinois at Urbana-Champaign) and were cultivated as described by Zillig et al. (30). *Rhodopseudomonas sphaeroides* 2.4.1 was cultivated photoautotrophically in the medium of Sistrom (24), without succinic acid, glutamic acid, and aspartic acid, and supplemented with 4.0 g of $NaHCO_3$ per liter under H_2 and CO_2. Cells of photoautotrophically grown *Rhodospirillum rubrum* (23) were a gift of F. C. Hartman (Oak Ridge National Laboratory, Oak Ridge, Tenn.). *Escherichia coli* UB1005 was grown on tryptone-yeast extract.

Preparation of cell-free extracts and partially purified enzymes. Cell-free extracts of methanogens were prepared by the anaerobic procedures described previously (29). Harvested cells of *M. thermoautotrophicum* and *Methanosarcina barkeri* were washed and suspended in equal volumes of anoxic 30 mM TES buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Ultrol brand, Calbiochem-Behring, La Jolla, Calif.] at pH 6.8, containing 10 mM 2-mercaptoethanol. Cells were broken by passage through a French pressure cell (1×10^5 kPa), and the resulting suspension was treated with approximately 0.1 mg of DNase per g of cells. After removal of cell debris by centrifugation at 40,000 × *g* for 20 min, the supernatant solution was stored at 4°C under N_2 (100 kPa). Cell-free extracts of *Methanococcus jannaschii*, *Methanococcus voltae*, and *Methanogenium marisnigri* were prepared similarly except that cells were broken by osmotic shock in 30 mM TES buffer (pH 6.8) plus 10 mM 2-mercaptoethanol.

Certain extracts were freed of MFR and H_4MPT by passage through a column (2.5 by 95 cm) of Sephadex G-25 (superfine) equilibrated with anoxic buffer that contained 50 mM Tris-hydrochloride (pH 7.2), 20 mM magnesium acetate, 10 mM 2-mercaptoethanol, 1 mM dithiothreitol, and 10% glycerol. Fractions of the eluate were collected anaerobically (9), and those determined to be free of MFR and H_4MPT, based on their inability to reduce CO_2 to methane without the addition of both cofactors, were pooled and stored at −20°C under an atmosphere of N_2 (100 kPa). Extract prepared in this manner is referred to as G-25-treated extract. Partially purified methenyl-H_4MPT oxidoreductase was prepared by ammonium sulfate fractionation as described previously (7).

Preparation of anaerobic boiled cell-free extracts. Harvested cells of each bacterium tested were suspended in anoxic 20 mM KH_2PO_4 buffer that contained 10 mM 2-mercaptoethanol at pH 6.8; the ratio of packed wet cells to buffer was 1.0. In most cases, approximately 10 to 20 g (wet weight) of cell paste was used. Cells were extracted under anaerobic conditions by heating for approximately 30 min in a boiling water bath while passing a stream of O_2-free N_2 over the slurry with gentle mixing. Cell debris was removed by centrifugation at 40,000 × *g* at 4°C for 25 min. The anoxic supernatant solution was stored anaerobically in butyl-rubber-stoppered serum vials under N_2 (100 kPa) at −20°C. Deproteinated cell extracts of *Methanococcus jannaschii* were prepared by autoclaving the anoxic cell-buffer slurry at 121°C for 5 min followed by centrifugation or by methanol (60% final concentration) precipitation after cell breakage with a French pressure cell (10^5 kPa); the anoxic supernatant solution remaining after centrifugation of the methanol-precipitated cell extract was lyophilized to dryness, suspended in anoxic H_2O, and stored at −20°C under N_2 (100 kPa).

Methane formation from radiolabeled formyl-MFR. In the presence of H_4MPT and CH_3-S-CoM, G-25-treated extracts of *M. thermoautotrophicum* convert [*formyl*-^{14}C]formyl-MFR to [^{14}C]methane (15a). Cell extracts of other methanogens were tested for their ability to produce radiolabeled methane from this derivative. The standard methanogenic assay described above was prepared on ice and contained 200 nmol of CH_3-S-CoM and 1 to 3 mg of extract protein under an atmosphere of hydrogen. [*formyl*-^{14}C]formyl-MFR (4,160 dpm; specific activity, approximately 2 mCi per mmol) was added, and the assays were initiated by incubation at 37°C or, in the case of *Methanococcus jannaschii*, at 60°C. Upon completion of methane formation, the reactions were terminated by transferring the vials to ice, and 60 μl of 4 M sodium hydroxide was added. After shaking gently for 20 min to allow any radioactive CO_2 to be trapped in the aqueous phase, the gas phase was assayed for radioactivity with a Packard model 894 gas proportional counter linked to a gas chromatograph as described earlier (15a). Duplicate 0.5-ml samples were taken, and in the case of the second sample, the measured radioactivity was adjusted for the volume of gas removed. Radioactivity in the aqueous phase was determined by liquid scintillation counting with toluene–Triton X-100 (3:1) containing 0.6% 2,5-diphenyloxazole.

Methane formation from methyl-H_4MPT. Conversion of methyl-H_4MPT to methane was assayed by incubating cell

extracts of methanogens (3 mg of protein) in the standard methanogenic assay at pH 6.3 supplemented with 50 μl of anoxic boiled cell extract of *M. thermoautotrophicum* and various amounts of either CH_3-S-CoM or methyl-H_4MPT. Reaction mixtures were incubated at 37°C for mesophilic methanogens and 60°C for thermophiles.

Assay for MFR. The amount of MFR in anoxic boiled cell-free extracts was estimated by evaluating the dependence of the yield of methane production from CO_2 on the amount of boiled cell-free extract added to a methanogenic assay (16). The standard methanogenic assay was prepared as described above at pH 6.6 and included 0.2 μmol of CH_3-S-CoM, 52 nmol of of H_4MPT, and 0.6 to 0.8 mg of protein of Sephadex G-25-treated cell extract of *M. thermoautotrophicum*. MFR or anaerobic boiled cell-free extract of various organisms was added as desired. Assay vials were gassed with H_2 and CO_2 (4:1), and assays were initiated by incubating the vials at 60°C. CH_3-S-CoM rather than HS-CoM was included in the assay due to the tight coupling of the reduction of CO_2 to CH_4 with the reduction of CH_3-S-CoM; in the absence of CH_3-S-CoM, no reduction of CO_2 to CH_4 was observed, whereas in its presence additional CH_4 was formed above the amount attributable to the reduction of CH_3-S-CoM (10, 21, 22). The additional methane formed from the reduction of CO_2 reflected the amount of MFR added. In the presence of saturating amounts of H_4MPT, the yield of methane from CO_2 was hyperbolically dependent upon the amount of MFR present (16). This saturable phenomenon is amenable to analysis by linear regression of the reciprocal of the yield of methane from CO_2 against the reciprocal of added MFR or anoxic boiled cell extract. Analysis of the response to purified MFR of G-25-treated extract of *M. thermoautotrophicum* used in our experiments indicated that 0.55 μg of MFR produced one-half of the maximal stimulation of the conversion of CO_2 to methane. As an estimate of the amount of MFR in extracts, the volume of boiled cell-free extract which produced one-half the maximal stimulation was assumed to contain 0.55 μg of MFR.

TABLE 1. Methanogenesis from [*formyl*-^{14}C]formyl-MFR by cell-free extracts of methanogens[a]

Organism[b]	Disintegrations per minute		% CH_4 (gaseous)
	Gaseous	Aqueous	
Methanobacterium thermoautotrophicum	3,403	773	81
Methanobacterium bryantii	3,532	414	90
Methanococcus jannaschii	3,225	474	87
Methanospirillum hungatei	3,169	1,098	74
Methanogenium marisnigri	3,383	343	91
Methanosarcina barkeri	3,593	1,209	75

[a] Methanogenic assays included 18 μmol of potassium PIPES (pH 6.6), 2.5 μmol of magnesium acetate, 0.8 μmol of sodium ATP, 20 μl of anoxic boiled cell extract of *M. thermoautotrophicum*, 0.2 μmol of CH_3-S-CoM, and 1 to 3 mg of cell extract protein in 220 μl under an atmosphere of H_2. Methane formed from [*formyl*-^{14}C]formyl-MFR was determined as gaseous radioactivity after the injection of 60 μl of 4 M sodium hydroxide as described in the text. In the absence of cell extract, no radioactive gas was produced.

[b] All organisms were grown on H_2 and CO_2.

TABLE 2. Methanogenesis from methyl-H_4MPT by cell-free extracts of methanogens

Organism[a]	Substrate added (nmol)		CH_4 formed (nmol)[b]
	CH_3-S-CoM	methyl-H_4MPT	
Methanobacterium thermoautotrophicum	200	0	213
	400	0	389
	0	60	45
	0	120	93
Methanococcus voltae	100	0	92
	200	0	180
	0	50	46
	0	100	80
Methanococcus jannaschii	200	0	180
	0	100	91
Methanosarcina barkeri			
Acetate grown	200	0	176
	0	50	42
	0	100	85
Methanol grown	200	0	181
	0	50	44
	0	100	91
	400	200	525

[a] Methanogens were cultivated under H_2 and CO_2 unless otherwise indicated. Cell-free extracts were prepared as described in the text.

[b] Final yields of CH_4 were obtained after 2 h of incubation. The assay mixture (200 μl) was prepared as described (7) and contained 18 μmol of potassium PIPES buffer (pH 6.3), 2.5 μmol of magnesium acetate, 0.8 μmol of sodium ATP, 50 μl of boiled cell extract of *M. thermoautotrophicum*, 4 mg of cell extract, and CH_3-S-CoM or methyl-H_4MPT as indicated under an atmosphere of H_2. The assay temperature was 37°C; for *M. thermoautotrophicum* and *Methanococcus jannaschii*, the assay temperature was 60°C. Values are corrected for amount of CH_4 formed in the absence of added substrates.

Assay for H_4MPT. The spectrophotometric assay described by Escalante-Semerena et al. (7) was used in most experiments to estimate the amount of H_4MPT in anoxic boiled cell-free extracts from various bacteria. The assay involves the chemical reaction of H_4MPT with formaldehyde to generate methylene-H_4MPT, which is enzymatically oxidized to methenyl-H_4MPT, with a consequent increase in absorbance at 340 nm. Dialyzed supernatant solution of a 70% ammonium sulfate fractionation of cell-free extract of *M. thermoautotrophicum* was used as a source of enzyme for the conversion of methylene-H_4MPT to methenyl-H_4MPT. Methenyl-H_4MPT has an extinction coefficient at 340 nm of 20,800 M^{-1} cm^{-1} (7). Reactions were performed at 60°C.

Boiled cell-free extracts of certain methanogens were tested as sources of H_4MPT by a modification of the enzymatic assay described above for measuring MFR. H_4MPT was excluded from the assay mixture, and a saturating amount of MFR (4.0 μg) was added. By use of purified H_4MPT, it was established that the half-maximal stimulation of the yield of methane from CO_2 occurred in the presence of 2.9 μg of H_4MPT.

RESULTS AND DISCUSSION

Cell extracts of all methanogens tested converted [*formyl*-^{14}C]formyl-MFR to radioactive methane in the presence of CH_3-S-CoM (Table 1). Thus, as established for *M. thermoautotrophicum*, the N-formyl derivative of MFR is a precursor of methane in all methanogens tested under the conditions normally required for the conversion of CO_2 to methane (10). In the case of *M. thermoautotrophicum*, Leigh et al. (15a) demonstrated that the conversion of formyl-MFR to methane by G-25-treated extract required the addition of methanopterin, and we showed that other methanogens

TABLE 3. Amounts of H_4MPT and MFR in methanogens, nonmethanogenic archaebacteria, and eubacteria

Organism (growth substrate)[a]	Concn (nmol per mg [dry weight] of cells) of:	
	H_4MPT[b]	MFR[c]
Methanogens		
Methanobacteriaceae		
Methanobacterium thermoautotrophicum ΔH	3.0	1.8
Methanobacterium bryantii M.o.H.	1.3	1.8
Methanobacterium ruminantium M1	2.2	3.1
Methanobrevibacter smithii PS	1.9	1.4
Methanococcaceae		
Methanococcus voltae PS	1.3	0.7
Methanococcus jannaschii JAL-1	9.6	2.7
Methanomicrobiaceae		
Methanospirillum hungatei JF1	0	0.7
Methanogenium marisnigri	1.7	0.8
Methanosarcinaceae		
Methanosarcina barkeri 227 (H_2 + CO_2)	2.8	1.7
(acetate)	2.1	2.5
(methanol)	7.4	2.1
Nonmethanogenic archaebacteria		
Thermoproteus tenax DSM2078 (glucose + yeast extract)	0	0
Halobacterium volcanii D52 (tryptone + yeast extract)	0	0
Eubacteria		
Rhodopseudomonas sphaeroides 2.4.1	0	0
Rhodospirillum rubrum	0	0
Escherichia coli (tryptone + yeast extract)	0	0

[a] Cells were cultivated with H_2 and CO_2 (4:1) unless otherwise stated.
[b] Estimated spectrophotometrically by enzymatic conversion to methenyl-H_4MPT (see the text).
[c] Estimated by the stimulation of CO_2 conversion to methane as described in the text.

contain H_4MPT, as described below. Cell extracts of representative methanogens also converted methyl-H_4MPT to methane under a hydrogen atmosphere (Table 2). Near stoichiometric conversion of this derivative to methane was catalyzed by members of each order of methanogenic bacteria. Thus, the enzyme systems of other methanogens recognize and interconvert C_1 derivatives of the cofactors isolated from *M. thermoautotrophicum*, providing yields of methane comparable to those produced by extracts of *M. thermoautotrophicum* (7, 15a).

The model (Fig. 1) is further supported by our observation that boiled cell extracts of all methanogens tested, with one exception, were able to complement an enzyme system from *M. thermoautotrophicum* which required MFR or H_4MPT. Thus, these extracts contain MFR and H_4MPT or, possibly, modified forms of the cofactors which are recognized by the enzymes of *M. thermoautotrophicum* that are involved in the conversion of CO_2 to methane. We calculated that the concentrations of these cofactors were comparable in all methanogens tested (Table 3). H_4MPT levels in eight strains ranged from 1.3 to 3.4 nmol of H_4MPT per mg (dry weight) of cells, with two strains, *Methanosarcina barkeri* grown on methanol and *Methanococcus jannaschii*, possessing significantly higher levels of the cofactor, 7.4 and 9.6 nmol per mg (dry weight) of cells, respectively. MFR occurred at slightly lower concentrations, ranging from 0.7 to 3.1 nmol per mg (dry weight) of cells. However, H_4MPT was present at higher levels in 7 out of the 10 strains tested. No H_4MPT was detected in *Methanospirillum hungatei*, although this organism produces methane from CO_2 and converts formyl-MFR to methane (Table 1). We do not have a convincing explanation for the failure to detect this cofactor in boiled cell extract of this strain. Possibly H_4MPT is tightly bound to protein or the method of extract preparation failed to yield biologically active cofactor from these cells. However, H_4MPT was detected in normal levels in *Methanogenium marisnigri*, a member of the same family, *Methanomicrobiaceae*, as *Methanospirillum hungatei* (reference 1, Table 3).

The levels of the cofactors determined in this study approximate the yields reported for the isolation of the purified cofactors from *M. thermoautotrophicum* ΔH (5, 14; J. C. Escalante-Semerena, personal communication). In both cases about 50 to 70 mg of cofactor was isolated from 1 kg of wet cells. We determined that the dry weight of *M. thermoautotrophicum* was 27% of the wet weight. Thus, based on molecular weights of 776 for H_4MPT and 748 for MFR, 1 kg of wet cells of *M. thermoautotrophicum* would be expected to contain approximately 630 mg of H_4MPT and 360 mg of MFR. The reported purifications of cofactors therefore represent recoveries between 10 and 15%, based on the data of this study.

Consistent with the unique structures of the two cofactors and their role in a novel pathway of CO_2 fixation, we were unable to detect MFR or H_4MPT in extracts from several nonmethanogenic bacteria (Table 3). For both cofactors, amounts as low as 0.1 nmol per mg (dry weight) of cells would be readily detected by the assays employed. The strains tested included two nonmethanogenic archaebacteria, *T. tenax* and *H. volcanii*, two phototrophic organisms grown on H_2 plus CO_2, *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*, and the enteric *E. coli*.

Differences in the levels of cofactors among the different methanogen strains are of dubious physiological significance; a more likely explanation is that these differences simply reflect variation in the extraction of the cofactors by boiling. Anaerobic boiled cell extract of methanol-grown *Methanosarcina barkeri* was previously reported to contain considerably higher than average amounts of HS-CoM (2),

and similarly, we found that it possessed high levels of H_4MPT, compared with other methanogens. H_4MPT was not detected in *Methanococcus jannaschii* with the spectrophotometric assay, but its presence could be quantified by assaying the ability of boiled cell-free extract to sustain H_4MPT-dependent methanogenesis from CO_2 with *M. thermoautotrophicum* G-25 enzyme. As *Methanococcus jannaschii* is an extreme thermophile (12), it is possible that some enzymes survived the 100°C deproteination procedure used to prepare the extract and that these enzymes interfered with the accumulation of methenyl-H_4MPT in the spectrophotometric assay.

The apparent presence of both H_4MPT and MFR in *Methanosarcina barkeri* grown on either acetate or methanol is of considerable interest. In neither case does the organism need to convert CO_2 to methane to grow, and the occurrence of both cofactors in normal or above normal amounts might be interpreted as being inconsistent with their proposed essential role in methanogenesis from CO_2. A simple explanation is that the biosynthesis of the cofactors may not be regulated. More interesting, however, is the possibility that, in addition to methanogenesis from CO_2, both cofactors participate in other essential metabolic processes. Tetrahydrofolate functions as a C_1 carrier in several important biosynthetic reactions in nonmethanogenic bacteria (4) and is not found in the methanogens (14). It is reasonable to anticipate that in methanogens H_4MPT functions analogously to tetrahydrofolate and mediates C_1 transfers involved in purine and thymine biosynthesis and in acetate formation, the principle mechanism of CO_2 assimilation in methanogens (8). Additionally, the cofactors may be directly involved in the acetoclastic reactions by which the methyl carbon of acetate is reduced to methane and the carboxyl group is converted to CO_2. Further studies are essential to determine the possible roles and importance of H_4MPT and MFR in these metabolic processes.

Regardless of the possible roles of H_4MPT and MFR in synthetic reactions in methanogens, our data strongly support their role in the conversion of CO_2 to methane. Enzymes from all methanogens tested converted derivatives of the cofactors isolated from *M. thermoautotrophicum* to methane. Conversely, cofactors present in extracts of all methanogens tested, with one exception, complemented MFR- and H_4MPT-dependent enzymes of *M. thermoautotrophicum*. Thus, the proposed pathway of CO_2 reduction to methane (Fig. 1) appears to be common to methanogens.

ACKNOWLEDGMENTS

This work was supported by grant DE-ACO2-80ER10681 from the U.S. Department of Energy.

We thank Victor Gabriel for assistance in culture of the organisms, J. Leigh and J. C. Escalante-Semerena for samples of MFR and H_4MPT as well as for helpful discussions, and F. C. Hartman and C. R. Woese for the cultures indicated.

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Balch, W. E., and R. S. Wolfe.** 1979. Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). J. Bacteriol. **137**:256–263.
3. **Barker, H. A.** 1956. Bacterial fermentations. John Wiley & Sons, Inc., New York.
4. **Blackley, R. L.** 1969. The biochemistry of folic acid and related pteridines. North-Holland Publishing Co., Amsterdam.
5. **Escalante-Semerena, J. C., J. A. Leigh, K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Formaldehyde activation factor, tetrahydromethanopterin, a coenzyme of methanogenesis. Proc. Natl. Acad. Sci. U.S.A. **81**:1976–1980.
6. **Escalante-Semerena, J. C., J. A. Leigh, and R. S. Wolfe.** 1984. New insights into the biochemistry of methanogenesis from H_2 and CO_2, p. 191–198. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, D.C.
7. **Escalante-Semerena, J. C., K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Tetrahydromethanopterin, a carbon carrier in methanogenesis. J. Biol. Chem. **259**:9447–9455.
8. **Fuchs, G., and E. Stupperich.** 1980. Acetyl CoA, a central intermediate of autotrophic CO_2 fixation in *Methanobacterium thermoautotrophicum*. Arch. Micobiol. **127**:267–272.
9. **Gunsalus, R. P., S. M. Tandon, and R. S. Wolfe.** 1980. A procedure for anaerobic column chromatography employing an anaerobic Freter-type chamber. Anal. Biochem. **101**:327–331.
10. **Gunsalus, R. P., and R. S. Wolfe.** 1977. Stimulation of CO_2 reduction to methane by methyl coenzyme M in extracts of *Methanobacterium*. Biochem. Biophys. Res. Commun. **76**:790–795.
11. **Gunsalus, R. P., and R. S. Wolfe.** 1980. Methyl coenzyme M reductase from *Methanobacterium thermoautotrophicum*. J. Biol. Chem. **255**:1891–1895.
12. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136**:254–261.
13. **Kunitz, M. J.** 1952. Crystalline inorganic pyrophosphatase isolated from baker's yeast. J. Gen. Physiol. **35**:423–450.
14. **Leigh, J. A.** 1983. Levels of water-soluble vitamins in methanogenic and non-methanogenic bacteria. Appl. Environ. Microbiol. **45**:800–803.
15. **Leigh, J. A., K. L. Rinehart, Jr., and R. S. Wolfe.** 1984. Structure of methanofuran, the carbon dioxide reduction factor of *Methanobacterium thermoautotrophicum*. J. Am. Chem. Soc. **106**:3636–3640.

15a. **Leigh, J. A., K. L. Rinehart, Jr., and R. S. Wolfe.** 1985. Methanofuran (carbon dioxide reduction factor), a formyl carrier in methane production from carbon dioxide in *Methanobacterium*. Biochemistry **24**:995–999.

16. **Leigh, J. A., and R. S. Wolfe.** 1983. Carbon dioxide reduction factor and methanopterin, two coenzymes required for CO_2 reduction to methane by extracts of *Methanobacterium*. J. Biol. Chem. **258**:7536–7540.
17. **McBride, B. C., and R. S. Wolfe.** 1971. A new coenzyme of methyl transfer, coenzyme M. Biochemistry **10**:2317–2324.
18. **Mullakhanbhai, M. F., and H. Larsen.** 1975. *Halobacterium volcanii* spec. nov., a Dead Sea halobacterium with moderate salt requirement. Arch. Microbiol. **104**:207–214.
19. **Nagle, D. P., Jr., and R. S. Wolfe.** 1983. Component A of the methyl coenzyme M methylreductase system of *Methanobacterium*: resolution into four components. Proc. Natl. Acad. Sci. U.S.A. **80**:2151–2155.
20. **Romesser, J. A., and W. E. Balch.** 1980. Coenzyme M. Preparation and assay. Methods Enzymol. **67**:545–552.
21. **Romesser, J. A., and R. S. Wolfe.** 1982. CDR factor, a new coenzyme required for carbon dioxide reduction to methane by extracts of *Methanobacterium thermoautotrophicum*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C **3**:271–276.
22. **Romesser, J. A., and R. S. Wolfe.** 1982. Coupling of methyl coenzyme M reduction with carbon dioxide activation in extracts of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **152**:840–847.
23. **Schloss, J. V., E. F. Phares, M. V. Long, I. L. Norton, C. D. Stringer, and F. C. Hartman.** 1979. Isolation, characterization, and crystallization of ribulosebisphophate carboxylase from autotrophically grown *Rhodospirillum rubrum*. J. Bacteriol. **137**:490–501.
24. **Sistrom, W. R.** 1962. The kinetics of the synthesis of photopigments in *Rhodopseudomonas sphaeroides*. J. Gen. Microbiol.

28:607–616.

25. **Taylor, C. D., and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M. J. Biol. Chem. **249**:4879–4885.
26. **Van Beelen, P., R. M. deCock, W. Guijt, C. A. G. Haasnoot, and G. D. Vogels.** 1984. Isolation and identification of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin, a coenzyme involved in methanogenesis. FEMS Microbiol. Lett. **21**:159–163.
27. **Van Beelen, P., A. P. M. Stassen, J. W. G. Bosch, G. D. Vogels, W. Guijt, and C. A. G. 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.
28. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
29. **Whitman, W. B., and R. S. Wolfe.** 1983. Activation of the methylreductase system from *Methanobacterium bryantii* by ATP. J. Bacteriol. **154**:640–649.
30. **Zillig, W., K. O. Stetter, W. Schafer, D. Janekovic, S. Wunderl, I. Holz, and P. Palm.** 1981. *Thermoproteales*: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C **2**:205–227.