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Carbon Monoxide-Dependent Methyl Coenzyme M Methylreductase in Acetotrophic *Methosarcina* spp.

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Cell extracts of acetate-grown *Methanosarcina* strain TM-1 and *Methanosarcina acetivorans* both contained CH_3-S-CoM methylreductase activity. The methylreductase activity was supported by CO and H_2 but not by formate as electron donors. The CO-dependent activity was equivalent to the H_2-dependent activity in strain TM-1 and was fivefold higher than the H_2-dependent activity of *M. acetivorans*. When strain TM-1 was cultured on methanol, the CO-dependent activity was reduced to 5% of the activity in acetate-grown cells. *Methanobacterium formicicum* grown on H_2-CO_2 contained no CO-dependent methylreductase activity. The CO-dependent methylreductase of strain TM-1 had a pH optimum of 5.5 and a temperature optimum of 60°C. The activity was stimulated by the addition of $MgCl_2$ and ATP. Both acetate-grown strain TM-1 and acetate-grown *M. acetivorans* contained CO dehydrogenase activities of 9.1 and 3.8 U/mg, respectively, when assayed with methyl viologen. The CO dehydrogenase of acetate-grown cells rapidly reduced FMN and FAD, but coenzyme F_{420} and $NADP^+$ were poor electron acceptors. No formate dehydrogenase was detected in either organism when grown on acetate. The results suggest that a CO-dependent CH_3-S-CoM methylreductase system is involved in the pathway of the conversion of acetate to methane and that free formate is not an intermediate in the pathway.

The acetotrophic methane-producing bacteria metabolize acetate as follows:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

Studies with deuterated acetate have shown that the methyl group is transferred intact to CH_4 (19). It has been hypothesized (12) that acetate is cleaved by a mechanism analogous to a reversal of the pathway proposed for the biosynthesis of acetate in *Clostridium thermoaceticum*. In the biosynthetic pathway, a protein with carbon monoxide dehydrogenase activity is thought to be involved in the reduction of CO_2 to an enzyme-bound, reduced C_1 intermediate which then reacts with a methyl donor to form acetate (10, 14, 18). A similar mechanism may be utilized for the synthesis of acetate for cellular carbon by the H_2-CO_2-utilizing methanogen *Methanobacterium thermoautotrophicum* (25).

Recent reports are consistent with an involvement of CO dehydrogenase in the pathway of methanogenesis from acetate. *Methanosarcina barkeri* MS, adapted to grow on acetate, contained fivefold more CO dehydrogenase activity than cells grown on methanol or H_2-CO_2 (12); this enzyme may constitute up to 5% of the total protein in acetate-grown cells (13). High levels of CO dehydrogenase are also present in *Methanothrix soehngenii* (11), an acetotroph unable to utilize H_2-CO_2 for methanogenesis.

Coenzyme M (2-mercaptoethanesulfonate; HS-CoM) is the terminal methyl carrier for the formation of methane from methanol and CO_2 (16, 23, 26). Methanogenic organisms grown on either substrate contain H_2-dependent 2-(methylthio)ethanesulfonate (CH_3-S-CoM) methylreductase (H_2-methylreductase), which reductively demethylates CH_3-S-CoM to form methane, with H_2 as the electron donor (1, 4, 5, 7, 8). The H_2-methylreductase of *M. thermoautotrophicum* is a complex system composed of several separable protein and nonprotein components (17). Acetate-grown *M. barkeri* 227 also contains a H_2-methylreductase system; however, methanol-grown or H_2-CO_2-grown cells contain similar levels of H_2-methylreductase activity (1). Acetate-grown *M. barkeri* 227 is able to metabolize H_2-CO_2 (6); thus, the H_2-methylreductase system of this organism may only be present as a component of the H_2-CO_2 pathway.

Two acetotrophic methane-producing organisms, *Methanosarcina* strain TM-1 (30) and *Methanosarcina acetivorans* (24), were chosen by our laboratory for biochemical studies of the dissimilation of acetate to methane. When grown on acetate, these organisms are unable to utilize H_2-CO_2 for methanogenesis, simplifying the study of acetate metabolism. It was recently shown that the methyl group of acetate is transferred to HS-CoM during methanogenesis from acetate by *Methanosarcina* strain TM-1 (15), implicating CH_3-S-CoM methylreductase in the formation of methane from acetate. Here we report on the CH_3-S-CoM methylreductase systems of *Methanosarcina* strain TM-1 and *M. acetivorans*. The results show that CO serves as a novel electron donor for the CH_3-S-CoM methylreductase systems of these organisms when grown on acetate.

MATERIALS AND METHODS

Organisms and culture conditions. *Methanosarcina* strain TM-1 (30) was obtained from S. H. Zinder, and *M. acetivorans* ATCC 35395 (24) was obtained from K. R. Sowers. Both organisms were cultured on acetate as large-scale cultures (10 liters) with a pH auxostat system (K. R. Sowers, M. J. Nelson, and J. G. Ferry, Curr. Microbiol., in press) similar to that used for the cultivation of methanogenic bacteria on formate (21). Acetic acid (10 M) was automatically added to fermentors by a pH controller; this maintained both a constant pH (7.0) and a constant acetate concentration (50 mM). The basal medium for strain TM-1 contained the indicated concentrations (in grams per liter) of the following constituents: NH_4Cl, 1.44; K_2HPO_4, 1.13; KH_2PO_4, 1.13; NaCl, 0.45; $MgSO_4 \cdot 7H_2O$, 0.09; $CaCl_2 \cdot 2H_2O$, 0.06; yeast extract (Difco Laboratories), 0.5; Trypticase (BBL Microbiology Systems), 0.5; $Fe(NH_4)_2(SO_4)_2$, 0.01; cysteine · HCl, 0.27; $Na_2S \cdot 9H_2O$, 0.27; Antifoam C, 0.5; and resazurin,

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0.001. Trace elements and vitamin solutions (29) were each added at a final concentration of 1% (vol/vol). *M. acetivorans* was cultured in maintenance medium as previously described (24); the basal medium contained (concentrations in grams per liter) the following: NaCl, 23.4; $MgSO_4$, 6.3; yeast extract, 1.0; Trypticase, 1.0; NH_4Cl, 0.5; KCl, 0.8; $CaCl_2 \cdot 2H_2O$, 0.14; Na_2HPO_4, 0.6; cysteine · HCl, 0.25; $Na_2S \cdot 9H_2O$, 0.25; and resazurin, 0.001. Sodium acetate (50 mM) or methanol (100 mM) was added as the substrate. When methanol was utilized, additional methanol (2 g/liter) was added each day during growth. *Methanobacterium formicicum* JF-1 was cultured on H_2-CO_2 (80%:20%, gas phase) as previously described (21) with the basal medium described for strain TM-1 but without yeast extract and Trypticase and with sodium formate (6.1 g/liter). All cells were harvested in a continuous-flow centrifuge (Cepa type LE) under a stream of N_2, and the resulting cell paste was frozen and stored in liquid nitrogen.

Anaerobic procedures. The general anaerobic procedures for the preparation of cell extracts and for assays were as previously described (21). All containers and solutions used for anaerobic procedures were made O_2 free by repeated vacuum degassing and replacement with O_2-free gas (N_2, H_2, or CO). All gasses used were scrubbed for trace amounts of O_2 by passage through reduced BASF catalyst R3-11 (Chemical Dynamics) or a solution of 2 mM reduced methyl viologen.

Preparation of cell extracts. Cell extracts were prepared anaerobically under an H_2 atmosphere by suspension of frozen cells in twice their weight of anaerobic breakage buffer, passage of the suspension through a French pressure cell at 135 MPa (20,000 lb/in^2), and collection of the broken cell mixture directly in sealed, H_2-filled centrifuge tubes. The mixture was then centrifuged at 27,000 × *g* (4°C) for 20 min; the resulting supernatant solution (15 to 35 mg of protein per ml) was removed from the tubes and stored in liquid nitrogen until use. Breakage buffer consisted of 50 mM potassium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5% (vol/vol) glycerol, and 0.01 mg of DNase I (Sigma Chemical Co.) per ml.

Enzyme assays. CH_3-S-CoM methylreductase assays were performed anaerobically in sealed, calibrated, 5-ml serum vials (Wheaton Scientific) that contained the appropriate gas at 1 atm (101 KPa) of pressure and were placed in a reciprocating (60-rpm) shaker water bath at 40°C, except as otherwise indicated. The standard assay (0.5 ml) contained 100 mM potassium 2-(*N*-morpholino)ethanesulfonate (pH 6.0), 2 mM ATP, 20 mM $MgCl_2$, 5 mM CH_3-S-CoM, cell extract, and the appropriate electron donor. The CO utilized (99.5% CO; Matheson Gas) contained 0.05% H_2 as a contaminant. Control assays that contained N_2 with up to 0.25% H_2 showed no significant CH_3-S-CoM methylreductase activity. For formate-dependent reactions, 2 to 20 mM sodium formate was included as indicated in the reaction mixture, which was equilibrated with N_2. An N_2 gas phase was also used for control assays with no electron donor. Cell extracts were equilibrated with H_2 for 10 min before H_2-dependent reactions were initiated. Cell extracts for other assays were equilibrated with N_2 in the same manner. Reactions were initiated by the addition of the cell extract. Gas samples (50 μl) were taken with a gas-tight syringe at several time intervals, CH_4 was determined, and assay rates were calculated from the linear portion of the time course obtained. The reaction rates are reported as net rates after subtraction of the reaction rates of N_2 controls. Methane was analyzed by gas chromatography with a Varian 3700 gas chromatograph equipped with a Porapak QS column, nitrogen carrier gas (15 ml/min), and flame ionization detector.

Hydrogenase and formate dehydrogenase assays were performed as previously described (21, 22) with methyl viologen as the electron acceptor. Assays were also performed with coenzyme F_{420} (F_{420}), FAD, FMN, NAD^+, or $NADP^+$ (45 μM) substituted for methyl viologen. These assays were done the same way as the methyl viologen assays except that the reduction of the electron acceptor was monitored spectrophotometrically at the appropriate wavelength for the electron acceptor used. The following extinction coefficients (mM^{-1} cm^{-1}) were used: F_{420}, E_{420} = 42.5; FAD, E_{450} = 11.3; FMN, E_{450} = 12.2; NAD^+, E_{340} = 6.2; and $NADP^+$, E_{340} = 6.2. In all cases one unit of activity equals the amount of enzyme required to reduce 1 μmol/min of the electron acceptor used. Control assays were performed without electron donors (N_2 atmosphere), and reaction rates were corrected for endogenous reduction of the electron acceptor by the cell extract. CO dehydrogenase assays were performed the same way as the hydrogenase assays but under an atmosphere of 100% CO.

Protein determination. Protein was assayed by the method of Bradford (2) with bovine serum albumin as the standard.

Chemicals. F_{420} was purified from extracts of *M. formicicum* as previously described (22). CH_3-S-CoM was synthesized as previously described (20) and isolated as the ammonium salt. All other chemicals were obtained commercially.

RESULTS

Electron donors for CH_3-S-CoM methylreductase. Both *Methanosarcina* strain TM-1 and *M. acetivorans* were grown on acetate, and extracts of the cells were tested for CH_3-S-CoM methylreductase activity with CO, H_2, or formate as the potential electron donor. *M. formicicum*, a methanogen incapable of utilizing acetate for methanogenesis, was grown on H_2-CO_2, and extracts of this organism were tested in a similar manner for comparison with the two acetotrophs.

A CO-dependent CH_3-S-CoM methylreductase (CO-methylreductase) activity was present in extracts of acetate-grown strain TM-1 (Fig. 1). After a brief lag, the rate of methane formation was constant and was dependent upon the presence of both CH_3-S-CoM and CO. The specific activity was similar (89%) to that of the H_2-methylreductase activity in extracts of H_2-CO_2-grown *M. formicicum* (Table 1). Acetate-grown strain TM-1 also contained H_2-methylreductase activity (Fig. 1 and Table 1) that was equal to the CO-methylreductase activity. Assays continued for more than 2 h produced stoichiometric amounts of methane (90 to 100% of the theoretical amount) from CH_3-S-CoM with either CO or H_2 as the electron donor. Formate was not utilized as an electron donor in the CH_3-S-CoM methylreductase system of strain TM-1.

Significant CO-methylreductase activity was also present in extracts of acetate-grown *M. acetivorans*, although the amount was lower than that in strain TM-1 (Fig. 2 and Table 1). Unlike strain TM-1, the H_2-methylreductase activity in this organism was only 20% that of the CO-methylreductase activity. Little methane was formed from CH_3-S-CoM when 20 mM formate was the electron donor (Fig. 2 and Table 1), and no significant methane formation occurred when 2 mM formate was used (Fig. 2). Thus, free formate did not support significant CH_3-S-CoM methylreductase activity in either acetotroph.

M. formicicum utilizes H_2 and formate as electron donors

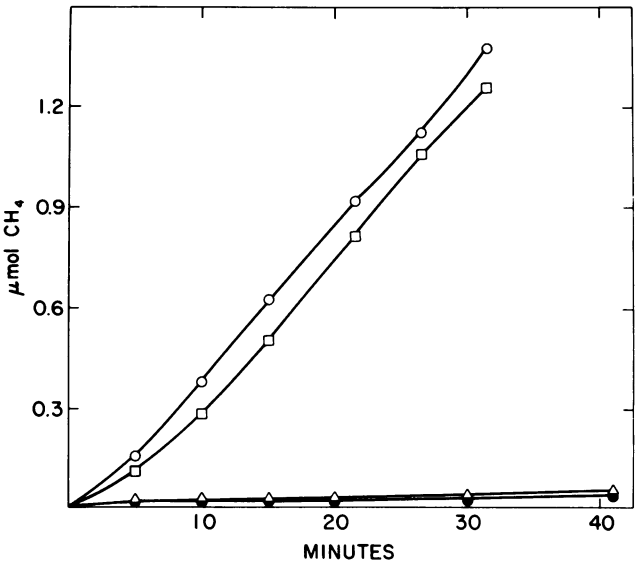

FIG. 1. Time course of methane formation by the CH_3-S-CoM methylreductase system in acetate-grown *Methanosarcina* strain TM-1 with CO, H_2, or formate as the electron donor. Each standard assay contained 100 μl (1.5 mg of protein) of strain TM-1 cell extract with the following electron donors: CO, ○; H_2, □; sodium formate (20 mM), △; no electron donor or either CO or H_2 in the absence of CH_3-S-CoM, ●.

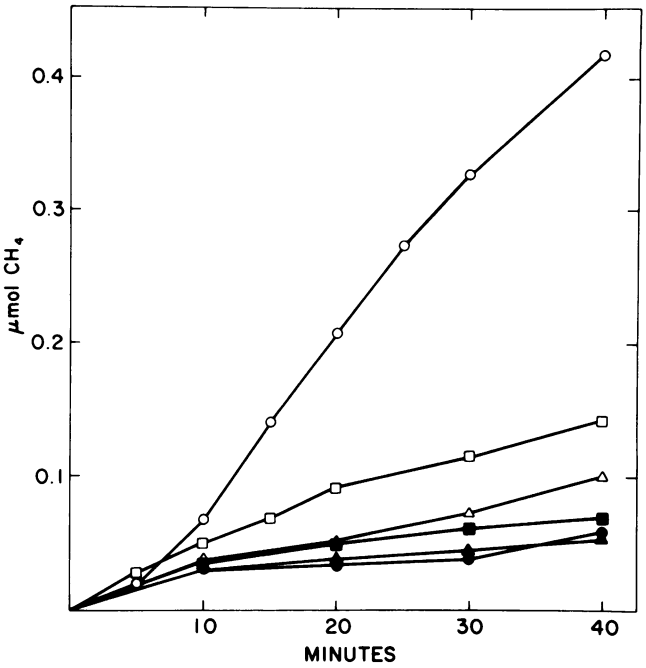

FIG. 2. Time course of methane formation by the CH_3-S-CoM methylreductase system in acetate-grown *M. acetivorans* with CO, H_2, or formate as the electron donor. Each standard assay contained 100 μl (3.2 mg of protein) of *M. acetivorans* cell extract with the following electron donors: CO, ○; H_2, □; sodium formate (20 mM), △; no electron donor, sodium formate (2 mM), or H_2 in the absence of CH_3-S-CoM, ●; CO in the absence of CH_3-S-CoM, ■; sodium formate (20 mM) in the absence of CH_3-S-CoM, ▲.

for the reduction of CO_2 to methane. Both H_2 and formate supported substantial CH_3-S-CoM methylreductase activity in extracts of this organism, but CO-methylreductase was not detected (Table 1). Thus, both acetate-grown methanogens contained CO-methylreductase systems, but the non-acetate utilizer contained no detectable CO-methylreductase.

Methanosarcina strain TM-1 was cultured on methanol as an alternate substrate and tested for H_2-methylreductase and CO-methylreductase (Table 1). Methanol-grown and acetate-grown strain TM-1 contained similar H_2-methylreductase activities. However, methanol-grown strain TM-1 contained only 5% of the CO-methylreductase activity in acetate-grown cells. Thus, the ability to use CO as an electron donor for the CH_3-S-CoM methylreductase system is correlated with growth on acetate.

Properties of the CO-methylreductase of *Methanosarcina* strain TM-1. CO-methylreductase activity occurred over a broad pH range, with optimal activity between pH 5.2 and 6.0 (Fig. 3A). Activities were similar at pH 6.5 in either buffer tested. The activity increased with temperature up to 60°C (Fig. 3B). The CO-methylreductase of strain TM-1 was stimulated by the addition of $MgCl_2$ and ATP, although substantial activity was obtained without the addition of either component. Activity was stimulated 182% by the addition of 4 mM ATP in the presence of 22 mM $MgCl_2$. In the presence of 2 mM ATP, maximum activity was obtained in the presence of 12 mM $MgCl_2$.

Oxidoreductase activities. Extracts of strain TM-1 and *M. acetivorans* were assayed for CO dehydrogenase, hydrogenase, and formate dehydrogenase with the nonspecific electron acceptor methyl viologen (Table 2). When cultured on acetate, both strain TM-1 and *M. acetivorans* contained high amounts of CO dehydrogenase activity; the rates were 96- and 40-fold greater, respectively, than that of CO dehydrogenase activity in extracts of H_2-CO_2-grown *M. formicicum*. As expected (21), *M. formicicum* contained substantial hy-

TABLE 1. CH_3-S-CoM methylreductase activities in *Methanosarcina* strain TM-1, *M. acetivorans*, and *M. formicicum* with H_2, CO, or formate as the electron donor[a]

Organism	Growth substrate	Sp act (nmol of CH_4/min per mg) with electron donor:		
		H_2	CO	Formate
Methanosarcina strain TM-1	Acetate	29.9 ± 1.5	30.2 ± 2.4	ND[b]
	Methanol	20.1 ± 0.4	1.4 ± 0.2	
M. acetivorans	Acetate	0.70 ± 0.10	3.6 ± 0.20	0.27 ± 0.12
M. formicicum	H_2-CO_2	33.9 ± 4.7	ND	38.4 ± 6.2

[a] Standard methylreductase assays were performed in an atmosphere of H_2 or CO or with 20 mM sodium formate in an atmosphere of N_2. Assays were initiated by the addition of 100 μl of cell extract of strain TM-1 (1.5 to 1.7 mg of protein), *M. acetivorans* (3.2 mg of protein), or *M. formicicum* (1.5 mg of protein). Each assay was repeated a minimum of three times.

[b] ND, Nondetectable. Minimum detectable level, 0.09 nmol/min per mg.

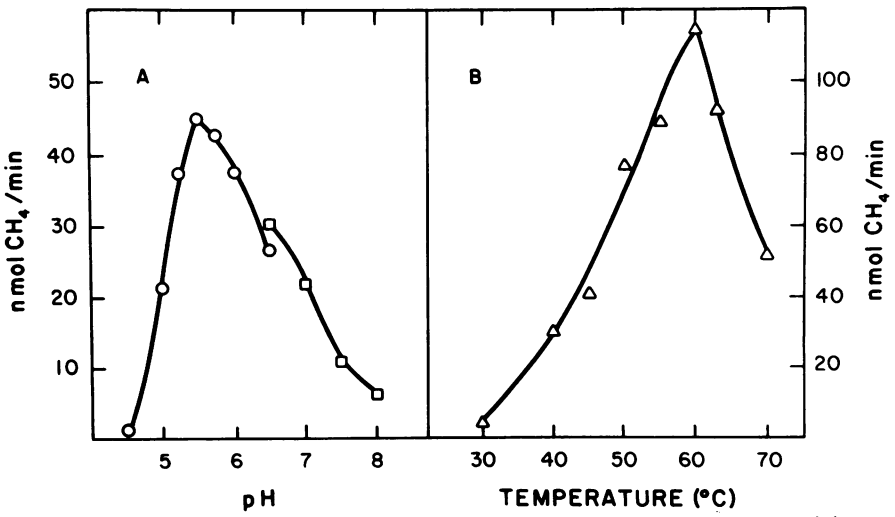

FIG. 3. Temperature and pH profiles of the CO-methylreductase of *Methanosarcina* strain TM-1. (A) Activity versus pH with 100 mM 2-(*N*-morpholino)ethanesulfonate (○) or *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (□) buffer in the assay mixture, adjusted to the indicated pH with KOH. (B) Activity versus temperature. All other conditions were as indicated for the standard assay. Each assay contained 100 μl (1.5 mg of protein) of acetate-grown strain TM-1 cell extract.

drogenase and formate dehydrogenase activities, although the CO dehydrogenase activity was low. Hydrogenase was also present in acetate-grown strain TM-1, which had H_2-methylreductase activity. *M. acetivorans*, which had low H_2-methylreductase activity (Fig. 2 and Table 1), contained little hydrogenase. No formate dehydrogenase was detected in either acetate-grown acetotroph when assayed with methyl viologen (Table 2) or FAD, FMN, or F_{420} (data not shown).

The CO dehydrogenase activity in acetate-grown strain TM-1 increased linearly with the CO concentration up to 1 mM when assayed with methyl viologen. These results indicate that the apparent K_m for CO was >1 mM with the assay conditions used.

The CO dehydrogenase and hydrogenase activities in methanol-grown strain TM-1 were both ca. 20% of the respective activities in acetate-grown cells (Table 2). Although this represents a significant decrease for both enzymes, substantial amounts of activity were still present.

The hydrogenase and CO dehydrogenase activities in extracts of both acetate-grown organisms were also assayed with several physiological electron acceptors. NAD^+ was not reduced with either CO or H_2 in either acetotroph. $NADP^+$ was slowly reduced with either H_2 or CO at the same rate (0.01 μmol/min per mg) in strain TM-1. Only CO donated electrons for $NADP^+$ reduction (0.003 μmol/min per mg) in *M. acetivorans*. Both FAD and FMN were rapidly reduced with either CO or H_2 in strain TM-1 (Table 3). F_{420} was also rapidly reduced with H_2 strain TM-1, but the reduction of F_{420} with CO was much slower. Thus, although flavins or F_{420} were reduced by H_2, only flavins were readily reduced by CO. Similar results for the reduction of F_{420} and flavins in the presence of CO were obtained in *M. acetivorans* (Table 3). The reduction of F_{420}, FAD, or FMN with H_2 was not detected in extracts of *M. acetivorans*.

DISCUSSION

In this study, we investigated potential electron donors for the CH_3-S-CoM methylreductase systems in acetate-grown *Methanosarcina* strain TM-1 and *M. acetivorans*. A novel feature of the CH_3-S-CoM methylreductase systems in these organisms is the ability to use CO as the electron donor when grown on acetate. Based on our results and the recent finding that CH_3-S-CoM is an intermediate in the conversion of acetate to methane (15), a pathway for methanogenesis from acetate is postulated in Fig. 4. In this pathway, the methyl group of acetate is transferred to HS-CoM, forming CH_3-S-CoM as the terminal methyl carrier. Other methyl carriers may be involved before HS-CoM. The carboxyl

TABLE 2. Methyl viologen-linked hydrogenase, CO dehydrogenase, and formate dehydrogenase in extracts of *Methanosarcina* strain TM-1, *M. acetivorans*, and *M. formicicum*

Organism	Growth substrate	Sp. act[a] with electron donor:		
		H_2	CO	Formate
Methanosarcina strain TM-1	Acetate	8.4 ± 1.0	9.1 ± 1.9	ND[b]
	Methanol	1.9 ± 0.2	1.7 ± 0.05	
M. acetivorans	Acetate	0.070 ± 0.004	3.8 ± 0.5	ND
M. formicicum	H_2–CO_2	129 ± 25	0.095 ± 0.007	8.2 ± 0.3

[a] Expressed as micromoles of methyl viologen reduced per minute per milligram. Assays were performed as described in the text. Aliquots of extracts containing 15 to 650 μg of protein were assayed. Each assay was repeated a minimum of three times. Endogenous activities with no added electron donor were <0.005, <0.002, and <0.06 μmol/min per mg for assays of strain TM-1, *M. acetivorans*, and *M. formicicum*, respectively.

[b] ND, Nondetectable. Minimum detectable level, 5×10^{-4} μmol/min per mg.

TABLE 3. F_{420}-, FAD-, and FMN-linked hydrogenase and CO dehydrogenase in extracts of acetate-grown *Methanosarcina* strain TM-1 and *M. acetivorans*

Organism	Electron donor	Sp act[a] with electron acceptor:		
		F_{420}	FAD	FMN
Methanosarcina strain TM-1	H_2	0.15 ± 0.03	0.41 ± 0.03	0.50 ± 0.04
	CO	0.01[b]	2.1 ± 0.2	1.9 ± 0.7
M. acetivorans	H_2	ND[c]	ND	ND
	CO	0.03[b]	0.77 ± 0.13	0.57 ± 0.002

[a] Expressed as micromoles of electron acceptor reduced per minute per milligram. Assays were performed as described in the text. Aliquots of extracts containing 15 to 650 μg of protein were assayed. Each assay was repeated a minimum of three times. Endogenous activities with no added electron donor were <0.0002 and <0.002 μmol/min per mg for F_{420} and flavin assays, respectively.

[b] Maximum activity out of eight assays.

[c] ND, Nondetectable. Minimum detectable level, 4×10^{-4} μmol/min per mg.

group of acetate is converted to an unidentified, bound C_1 intermediate before oxidation to CO_2. High concentrations of exogenous CO may also form this bound C_1 intermediate. The oxidation of the C_1 intermediate by a protein with CO dehydrogenase activity provides the reducing equivalents necessary for the reduction of CH_3-S-CoM to CH_4. The reducing equivalents are transferred from the CO dehydrogenase to the CH_3-S-CoM methylreductase by a presently unidentified electron carrier(s).

The low affinity for CO of the CO dehydrogenase in strain TM-1 in the present study and similar findings for the CO dehydrogenase in acetate-grown *M. barkeri* (13) suggest that free CO is not an intermediate in the acetate dissimilation pathway. However, high concentrations of free CO may equilibrate with a component of the pathway, forming a physiological C_1 electron donor for the methylreductase (Fig. 4). Furthermore, evidence has been presented that a bound C_1 intermediate formed from CO is incorporated into the carboxyl group of acetate during its biosynthesis in the H_2-CO_2 utilizer *M. thermoautotrophicum* (25). A protein with CO dehydrogenase activity is also required for acetate formation by *C. thermoaceticum* from physiological C_1 donors (18).

The following results were consistent with an involvement of components of the CO-methylreductase system in the pathway of methanogenesis from acetate. (i) High levels of CO dehydrogenase activity were present in both acetate-grown organisms. *M. formicicum*, grown on H_2-CO_2, contained only 1% of the CO dehydrogenase activity present in acetate-grown strain TM-1. Activities reported for other nonacetate utilizers (3, 9) were from 2 to 4% of those in strain TM-1. (ii) High levels of CO-methylreductase activity were also present in both acetate-grown organisms, suggesting its involvement in a major metabolic pathway. The CO-methylreductase activities in these organisms were comparable to the H_2-methylreductase activities reported for H_2-CO_2-utilizing methanogenic organisms (1, 5, 7). (iii) A CO-methylreductase system was not present in the nonacetate utilizer *M. formicicum*. (iv) When strain TM-1 was cultured on methanol as an alternate energy source, the CO-methylreductase activity was 5% of that in acetate-grown cells. It was previously reported that cell suspensions of methanol-grown strain TM-1 produce CH_4 from acetate at 3% of the rate in acetate-grown cells (S. H. Zinder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I88, p. 109).

The possibility exists that in the pathway of methanol utilization for methanogenesis, an oxidized C_1 intermediate identical to that formed during the oxidation of the C-1 of acetate is formed from methanol (presumably at the step just before the final two-electron oxidation to CO_2) and subsequently consumed in a manner identical to that in the acetate utilization pathway. If this were the case, methanol-grown cells should also contain substantial CO-methylreductase activity. The results of the present study suggest that this is not the case and that the CO-methylreductase system may be uniquely associated with the conversion of acetate to methane.

Although the CO-methylreductase specific activity in methanol-grown strain TM-1 decreased to 5% of that in acetate-grown cells, the CO dehydrogenase specific activity decreased to only 19% of that in acetate-grown cells. Thus, the decreased CO dehydrogenase activity in methanol-grown cells apparently cannot account for the much larger decrease in the CO-methylreductase activity. Possible explanations for these results which are consistent with both CO dehydrogenase and CO-methylreductase involvement in methanogenesis from acetate are the following. (i) The CO dehydrogenase was constitutive, but another inducible component(s) of the CO-methylreductase system limited the CO-methylreductase activity in methanol-grown cells. (ii) More than one CO dehydrogenase was present in strain TM-1, but only one coupled to the CH_3-S-CoM methylreductase system, and it was the absence of the other enzyme that was responsible for the decreased CO dehydrogenase activity and loss of CO-methylreductase activity in methanol-grown cells.

Possible mechanisms for the transfer of reducing equivalents from CO to CH_3-S-CoM include the intermediate formation of formate or H_2, with subsequent utilization by a formate dehydrogenase- or hydrogenase-dependent CH_3-S-CoM methylreductase system. The absence of detectable formate dehydrogenase activity and the inability of formate to support significant methylreductase activity in either acetotroph indicate that free formate is not an intermediate

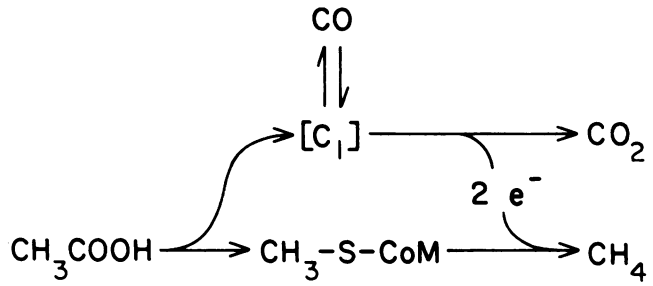

FIG. 4. Proposed pathway for the conversion of acetate to methane and CO_2. A hypothetical bound C_1 intermediate in the production of CO_2 from the C-1 of acetate or from CO is represented as $[C_1]$.

in the CO-methylreductase systems in these organisms. These results also suggest that formate is not a free intermediate in the electron flow from acetate to methane, as proposed previously (27). The presence of a H_2-methylreductase system in the acetotrophic organisms studied raises the question of whether H_2 is an intermediate in the CO-methylreductase system. If H_2 were produced from CO, followed by uptake by the H_2-methylreductase system, the H_2-methylreductase activity should be equal to or greater than the CO-methylreductase activity. The H_2-methylreductase activity in *M. acetivorans* was 20% of the CO-methylreductase activity, suggesting that the transfer of electrons from CO to CH_3-S-CoM does not involve free H_2.

At present, the physiological electron acceptor for the CO dehydrogenase coupled to the CH_3-S-CoM methylreductase system is unknown. The preliminary evidence suggests that F_{420} and pyridine nucleotides are not involved. However, both FAD and FMN were readily reduced by CO, suggesting that a flavin or flavoprotein may be linked to the CO dehydrogenase. FAD was shown to be required for the H_2-methylreductase system in *M. thermoautotrophicum* (17).

Some of the properties examined for the CO-methylreductase system in cell extracts of the thermophilic acetotroph strain TM-1 were similar to those reported for the H_2-methylreductase system in cell extracts of the thermophilic H_2-CO_2 utilizer *M. thermoautotrophicum* (7). Both systems had a similar pH optimum and temperature optimum. Like the H_2-methylreductase system in *M. thermoautotrophicum*, the CO-methylreductase system in strain TM-1 was stimulated by the addition of ATP and $MgCl_2$. However, the CO-methylreductase in strain TM-1 was active without the addition of ATP. This is in contrast to H_2-methylreductase activities reported for several other methanogens, including *M. thermoautotrophicum* (7). The ATP-independent activity of the CO-methylreductase was more consistent with the H_2-methylreductase activity in *Methanobacterium bryantii* (28), which also contained substantial activity without the addition of ATP because of the endogenous synthesis of ATP by the extracts tested. Whether the ATP-independent activity of the CO-methylreductase in the present study was the result of endogenous ATP was not examined.

The results of the present study are consistent with the hypothesis that the biochemistry of the conversion of acetate to methane involves steps analogous to a reversal of the acetate biosynthesis pathways of acetogenic anaerobes and H_2-CO_2-utilizing methanogenic bacteria. Cell extracts of acetate-grown methanogenic bacteria readily lose the ability to produce methane from acetate (unpublished data), which has precluded the in-depth study of the biochemistry of the acetate dissimilation pathway. Our results suggest that the CO-methylreductase system is involved in the terminal steps of the conversion of acetate to methane and CO_2. Studies of this system should prove useful in the understanding of the pathway of the conversion of acetate to methane. Experiments are presently under way to further characterize the CO-methylreductase system in acetotrophic methane-producing bacteria.

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