Studies on the Carbon Monoxide Dehydrogenase Enzyme Complex present in Acetate-grown Methanosarcina thermophila strain TM-1

by

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ABSTRACT

The carbon monoxide dehydrogenase complex was purified from acetate-grown Methanosarcina thermophila. This complex made up greater than 10% of the cellular protein and the native enzyme formed aggregates with a Mr of approximately 1,000,000. The enzyme contained five subunits of different molecular weight suggesting a multifunctional enzyme complex. Nickel, iron, cobalt, zinc, inorganic sulfide, and a corrinoid were present in the complex. The electron paramagnetic resonance spectrum of CO-reduced enzyme at 113K contained g values of 2.073, 2.049, and 2.028. Isotopic substitution with ^{61}Ni, ^{57}Fe, or ^{13}CO resulted in broadening of the spectrum consistnet with a Ni-Fe-C spin-coupled complex. Acetyl-CoA caused a perturbation of the signal that was not caused by acetyl-phosphate or mercaptoethanol indicating acetyl-CoA is a physiological substrate.

Cell extracts from acetate-grown M. thermophila contained CO-oxidizing:H_2-evolving activity 16-fold greater than extracts of methanol-grown cells. CO-oxidizing:H_2-evolving activity was reconstituted upon combination of: (i) CO dehydrogenase complex, (ii) a ferredoxin, and (iii) purified membranes with associated hydrogenase and b-type cytochrome.

The ferredoxin was a direct electron acceptor for the CO dehydrogenase complex. The molecular weight of the isolated protein was 16,400, and the apparent minimum molecular weight was 4,900. The ferredoxin contained 2.8 ± 0.56 Fe atoms and 1.98 ± 0.12 acid-labile sulfide. UV-visible absorption maxima were 395 and 295 nm with a A_{395}/A_{295} ratio range of 0.80 to 0.88. The N-terminal amino acid sequence revealed a 4-cysteine cluster, similar to other Fe:S centers that coordinate a Fe:S center.

A CH_3-B_{12}:HS-CoM methyltransferase activity was characterized in extracts of acetate- and methanol-grown cells. The activity from extracts of acetate-grown M. thermophila was stable at 70°C for 30 minutes. The activity in cell extracts of acetate- and methnol-grown cells was fractionated with ammonium sulfate treatment and FPLC phenyl superose chromatography. Two peaks of methyltransferase activity were observed in each cell extract sample following phenyl superose fractionation.

FORWARD

This dissertation contains ten sections. An introduction (Section I) and Literature Review (Section II) serve as an introduction to the research problem. Section III through VII are written as manuscripts for publication. Section VIII summarizes the work. Literature cited in Sections I, II and VIII is included in Section IX; all other literature cited is listed at the end of the respective sections.

The following are the manuscripts that have been published:

Section III: Terlesky, K. C., M. J. K. Nelson, and J. G. Ferry. 1986. Isolation of an Enzyme Complex with Carbon Monoxide Dehydrogenase Activity Containing Corrinoid and Nickel from Acetate-grown Methanosarcina thermophila. Journal of Bacteriology 168: 1053-1058.

Section IV. Terlesky, K. C., M. J. Barber, D. A. Aceti, and J. G. Ferry. 1987. EPR Properties of the Ni-Fe-C Center in an Enzyme-Complex with Carbon Monoxide Dehydrogenase Activity from acetate-grown Methanosarcina thermophila: Evidence that Acetyl-CoA is a Physiological Substrate. Journal of Biological Chemistry 262: 15392-15395.

Section V. Terlesky, K. C., and J. G. Ferry. 1988. Purification and Characterization of a Ferredoxin from Acetate-grown Methanosarcina thermophila. Journal of Biological Chemistry 263: 4075-4079.

Section VI. Terlesky, K. C., and J. G. Ferry. 1988. Ferredoxin Requirement for Electron Transport from the Carbon Monoxide Dehydrogenase Complex to a Membrane-bound Hydrogenase in acetate-grown Methanosarcina thermophila. Journal of Biological Chemistry 263:

4080-4082.

The following manuscript is in preparation for publication:

Section VII. Terlesky, K. C., D. A. Abbanat, and J. G. Ferry. CH_3-B_{12}-HBI:HSCoM methyltransferase activity from Methanosarcina thermophila.

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INTRODUCTION

In 1776 the physicist, Allessandro Volta, noted the presence of "combustible air" originating from lakes and ponds rich in decaying vegetation. The release of large amounts of methane from these sediments is indicative of the importance of methane in the final decomposition of organic matter in the global carbon cycle. However, it was not until 171 years later that two anaerobic, methane-producing bacteria were isolated in pure culture. Since that time, the importance of the methane-producing bacteria in agricultural and industrial applications has been realized and research in the area has exploded. The most studied methanogenic habitats are the rumen and sewage waste digestors. Most of the methane in the rumen is derived from carbon dioxide but in sewage waste and freshwater ecosystems most of the methane is derived from the methyl group of acetate. Methods were developed recently to obtain large amounts of the slow-growing acetotrophic methanogens (62), allowing detailed studies on the physiology and energy conserving mechanisms in these organism.

Methanosarcina thermophila strain TM-1 (79) is a thermophilic methanogen isolated from sewage sludge that can use acetate, methanol or trimethylamine for growth and methanogenesis (80). Our laboratory chose M. thermophila to study the pathways of carbon and electron flow during acetotrophic methanogenesis because it is unable to use H_2/CO_2 for methanogenesis, and it has a higher growth rate on acetate than the mesophilic organisms. The pathway used for acetate conversion to methane by these organism is the recently described acetyl-CoA pathway and the purpose of this study was to examine the role of the central enzyme, carbon monoxide dehydrogenase (CO dehydrogenase), in this pathway.

SECTION II. LITERATURE REVIEW

Microbiology of Methanogenesis

In sediments of lakes and streams where dead vegetative matter is decomposed, a variety of organisms are required to break down the organic matter and return the carbon dioxide fixed by plants to the atmosphere. Complex organic substrates are digested to monomers, and the monomers are fermented by anaerobic bacteria producing formate, acetate, carbon dioxide and hydrogen. These are the major substrates for methanogenic bacteria which complete the microbial food chain. Thus methane is the final product in the decomposition of organic matter, and anaerobic methane-producing bacteria are the major biogenic source of methane. Methanogenic food chains, found in anaerobic sediments where sulfate and nitrate are absent, include forest soils, freshwater and marine aquatic habitats, rumens, the intestines of man, animals, and insects. In most habitats, about 70% of the total methane produced is derived from the methyl group of acetate.

Methanogens are morphologically diverse and distinguished from eubacteria by the presence of branched ether-linked lipids and unique coenzymes and the absence of peptidoglycan in their cell walls. Comparison of the 16s rRNA sequences divides the group into 3 orders, 4 families and 7 genera (17). M. thermophila belongs to the Methanomicrobiales order and Methanosarcinaceae family. Organisms in the family Methanosarcinaceae are the most physiologically diverse methanogens and are capable of using more than one substrate for methanogenesis. They have a protein cell wall with a heteropolysaccharide outer layer when grown in low salt medium, but lose the heteropolysaccharide layer when grown in high salt medium (22, 61).

Methanogenesis from H_2CO_2

The pathway of carbon dioxide conversion to methane proceeds by sequential reduction of the carbon dioxide on enzyme-bound one-carbon carriers (56), and release of methane using electrons supplied by formate or hydrogen (Equation 1).

$$4H_2 + CO_2 \text{ -------> } CH_4 + 2H_2O \quad \text{Eq. 1}$$

Coenzyme M (CoM), $HSCH_2CH_2SO_3^-$, was the first one carbon carrier shown to be required for methanogenesis in dialyzed cell extracts of Methanobacterium bryantii (39), and the methylated form, CH_3-S-CoM, was demonstrated as an intermediate in methanogenesis (39, 66). It is now known that methyl-coenzyme M is the final methylated intermediate in methanogenesis from all substrates. The discovery of coenzyme M was just the first of several coenzymes and cofactors unique to the methanogens (74).

The pathway of CO_2 reduction to methane is depicted in Figure 1, and two other one carbon-carriers, methanofuran (72) and methanopterin (36), are necessary for the methylation of CoM. Following activation and reduction of the CO_2 to formyl methanofuran, the formyl group is transferred to methanopterin and reduced to the methyl level. The methyl group is then transferred to CoM, which is reductively demethylated to methane by the methyl-CoM methylreductase system.

Studies on the methylreductase system of Methanobacterium thermoautotrophicum (21) have revealed more coenzymes and cofactors unique to methanogens. The system has been resolved into four protein components including hydrogenase and methyl-CoM-methylreductase. An inhibitor of the methylreductase reaction that has aided studies on the reaction is the coenzyme M analog, 2-bromoethanesulfonic acid. The site

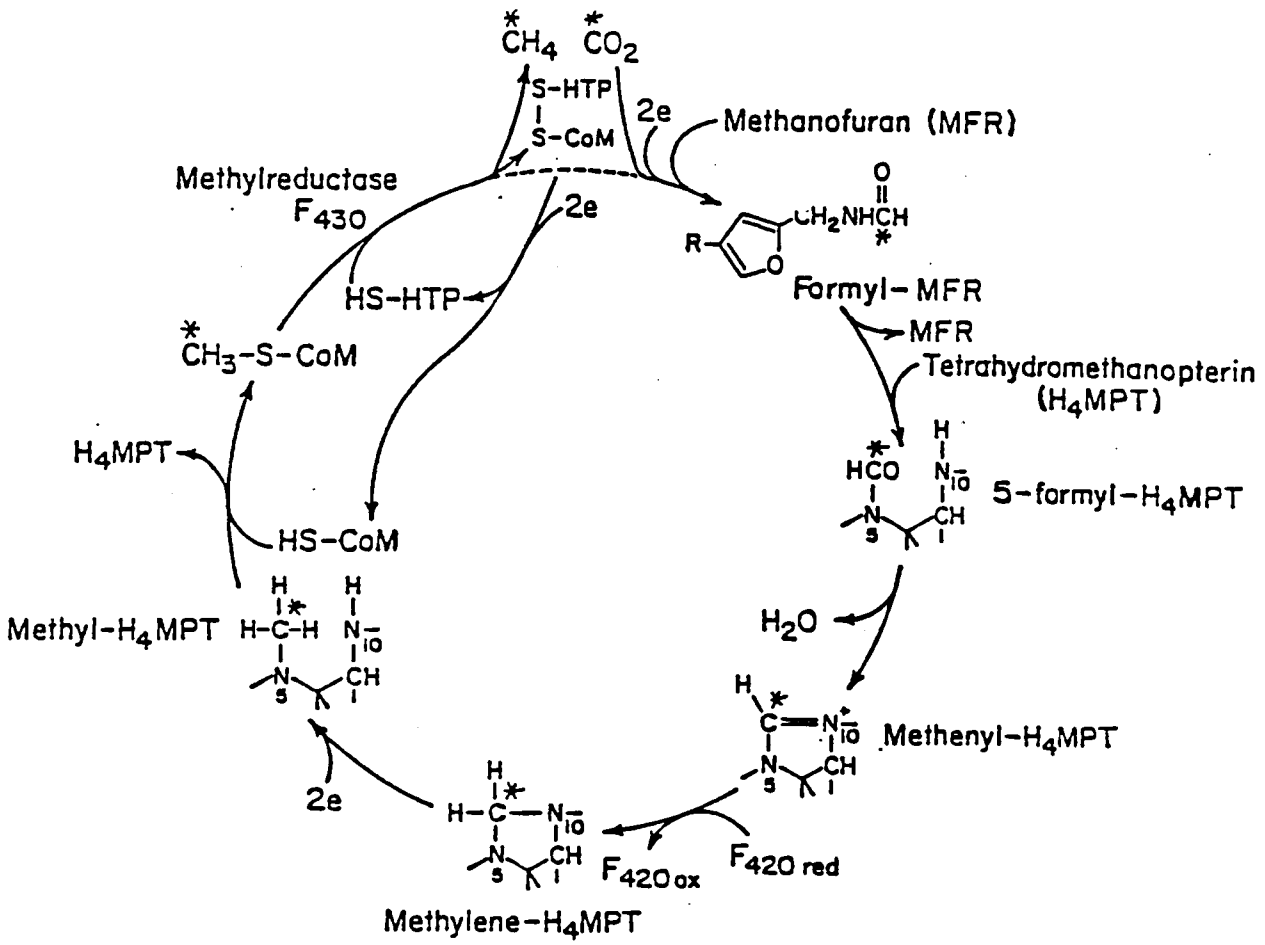

Figure 1. The proposed pathway of CO_2 reduction to methane. The CO_2 is sequentially reduced and transferred bound to the coenzymes, methanofuran (MFR), methanopterin (MPt), and coenzyme M. HS-HTP, component B; F_{430}, factor F_{430}; F_{420}, coenzyme F_{420}. (Adapted from Rouviere et al, 1988 (56))

of methyl-group reduction is the methylreductase enzyme (15), and this has been shown to contain a nickel tetrapyrrole cofactor, F_{430} (14), whose function is not known. Coenzyme M is also associated with this protein fraction. ATP, FAD and a methanogenic electron carrier, F_{420}, are required for the hydrogen-dependent reduction of methyl-CoM to methane. Though hydrogen is the source of electrons for the methylreductase reaction, the direct electron donor to the methylreductase enzyme is component B, 7-mercaptoheptanoylthreonine phosphate (43, 44). Following transfer of the electrons from component B to methyl-CoM, CoM and Component B form a heterodisulfide (4, 5). Reduced component B and HSCoM are regenerated using electrons indirectly supplied by H_2 and the F_{420}-reducing hydrogenase; the intermediate electron carriers to the disulfide are not known.

It has not been established if corrinoids are intermediates in methanogenesis from H_2/CO_2, though there have been reports suggesting a role for corrinoids in the methyl-group or electron transfer reactions. Corrinoid-containing membrane proteins were found in methanogenic bacteria grown on different substrates, and they were concluded to play a role in the energy conservation of the central reaction of methanogenesis, the methylreductase system (10, 60). Also, purified methyl-CoM methyl reductase was found to catalyze methane production from methyl-CoM using electrons supplied by B_{12} (1). Further research is necessary to delineate a role for corrinoids in methanogenesis from H_2/CO_2.

Methanogenesis from methanol

Members of the Methanosarcinaceae family are the only methanogens capable of utilizing methanol as a growth substrate. When both acetate and methanol are present in the growth medium, a biphasic response is observed in which methanol is utilized before acetate for methanogenesis. (29, 49, 78). A similar biphasic growth response is observed when M. barkeri is grown in the presence of trimethylamine and acetate (3). In both cases, the acetate is either assimilated for cell carbon, or the methyl group is oxidized to CO_2 supplying electrons for the reductive demethylation of methyl-coenzyme M until the methanol or trimethylamine is depleted (3).

Coenzyme M is also the terminal methyl carrier in methanogenesis from methanol. The pathway of methanol to methane is shown in Figure 2. In contrast to methanogenesis from H_2/CO_2, corrinoids are known intermediates in methanogenesis from methanol. They were initially implicated as methyl carriers in methanogenesis due to their high concentrations in methanogens and rumen fluid (63). The corrinoids found in methanogens are different from their eubacterial and eukaryotic counterparts by the structure of the α-ligand. Methanogens contain a 5-hydroxybenzimidazole base (HBI) on the α-ligand rather than a 5,6 dimethylbenzimidazole (50).

There are two enzymes required for the methylation of CoM by methanol (Figure 2) (69). The first is methanol:B12-HBI methyltransferase (MT1), an oxygen sensitive, corrinoid-containing enzyme that catalyzes the transfer of the methyl group from methanol to the enzyme-bound corrinoid (70). The second protein required is CH_3-B_{12}-HBI:HS-CoM methyltransferase (MT2) that catalyzes the transfer of the methyl group from the methylated cobamide of MT1 to HS-CoM (69).

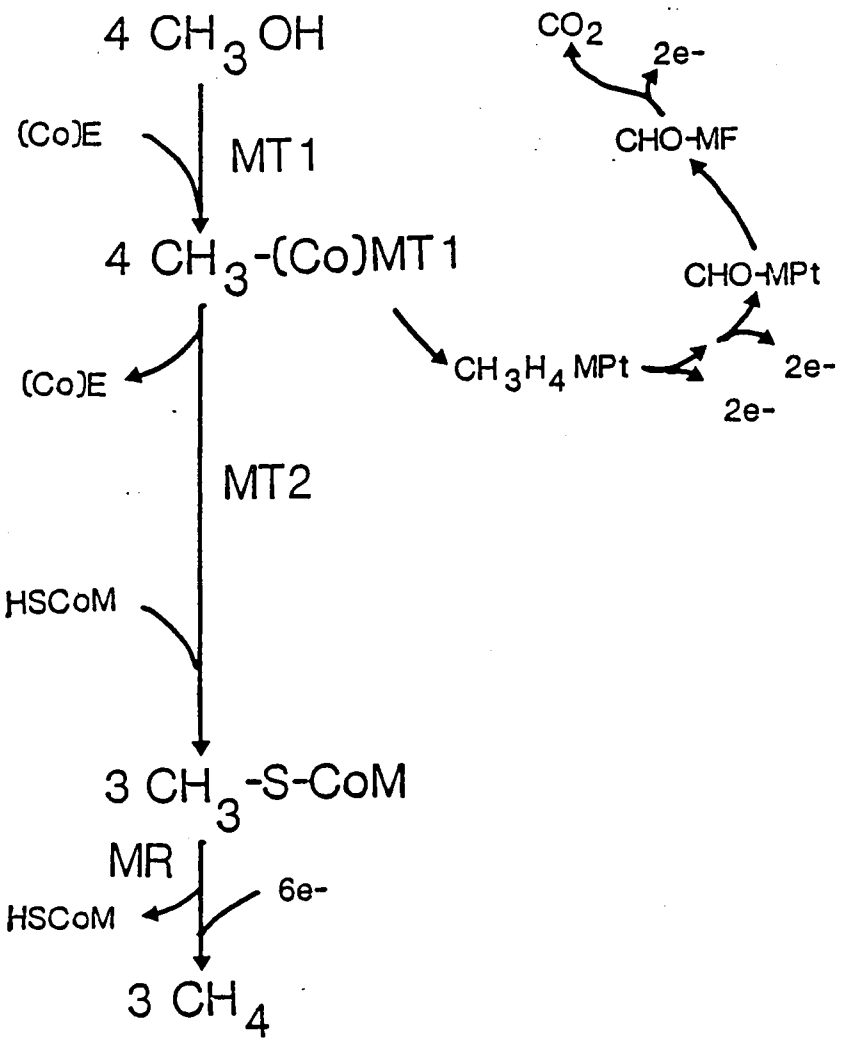

Figure 2. The pathway of methanol conversion to methane. The methyl group of methanol is transferred by the MT1 and MT2 to coenzyme M forming CH_3-S-CoM which is reductively demethylated by the methyl reductase system to methane. The electrons required for this reductive step are obtained by the oxidation of one methanol to CO_2. MT1. methanol:B_{12}-HBI methyltransferase; MT2, CH_3-B_{12}-HBI methyltransferase; MR, CH_3-S-CoM, methylreductase; [Co]E corrinoid-containing moiety of MT1; H_4MPt, tetrahydromethanopterin; MF, methanofuran; HSCoM, coenzyme M.

These enzymes have been characterized from methanol-grown Methanosarcina barkeri, though the second methyltransferase was only partially purified (69). The final step is the reductive demethylation of methyl-CoM by the methyl reductase. The electrons for the reductive demethylation of CH_3-S-CoM are derived from the oxidation of one methanol to CO_2 via reversal of the CO_2 reduction pathway for every three molecules of methanol converted to methane.

The Acetyl-CoA Pathway for Autotrophic Growth

A. Acetogenic clostridia

The acetyl-CoA pathway (Wood Pathway) for autotrophic CO_2 fixation was the third CO_2 fixation pathway to be described following the Calvin cycle (reductive pentose cycle) and the reductive tricarboxylic acid cycle (TCA). Key enzymes of the Calvin cycle are phosphoribulokinase and ribulose-1,5,-diphosphate carboxylase. The reductive TCA cycle fixes CO_2 using reverse reactions of the TCA. Carbon monoxide dehydrogenase is a key enzyme of the acetyl-CoA pathway.

The acetyl-CoA pathway was first discovered in the homoacetogenic clostridia which ferment substrates such as hexoses, pentoses, and polyols producing acetate as the main or only product. During fermentation of glucose by Clostridium thermoaceticum, three moles of acetate are formed from one mole of glucose. Two of the acetate are derived from two pyruvate, and the last is synthesized autotrophically. In experiments using $^{13}CO_2$, it was shown that both the methyl- and carbonyl-groups of the third acetate were derived entirely from CO_2 (59). Most acetogens can also grow autotrophically with H_2/CO_2, synthesizing acetate from the CO_2. This pathway has several unique

enzymes, and the enzyme hypothesized to catalyze the condensation of the methyl and carbonyl groups to acetyl-CoA is the carbon monoxide dehydrogenase (CO dehydrogenase) (54).

Outline of the pathway

The carbon flow in the acetyl-CoA pathway was first described for C. thermoaceticum. An outline of the pathway is depicted in Figure 3. During chemoautotrophic growth, hydrogen is the required electron donor. The first CO_2 is reduced to the formyl level by the formate dehydrogenase enzyme followed by reduction to the methyl level on enzyme-bound tetrahydrofolate intermediates via the tetrahydrofolate pathway. A methyltransferase enzyme then transfers the methyl group from 5-methyl-tetrahydrofolate to the corrinoid protein producing enzyme-bound CH_3-B_{12}. The methyl group, is then transferred from the CH_3-B_{12} corrinoid protein to the CO dehydrogenase. The second CO_2 is reduced to an enzyme-bound carbonyl group by the CO dehydrogenase enzyme. Following subsequent addition of coenzyme A, the three moieties condense and acetyl-CoA is released.

Enzymology of the Acetyl-CoA Pathway

CO dehydrogenases have been described in several anaerobic bacteria including the acetogenic clostridia (51, 53), methanogens (19, 31, 68) sulfate reducing bacteria (57, 58) and purple, non-sulfur photosynthetic bacteria (6); all of the enzymes are oxygen-sensitive, Ni-containing enzymes. The enzymes catalyze the oxidation of carbon monoxide to carbon dioxide with the reduction of exogenous electron acceptors such as

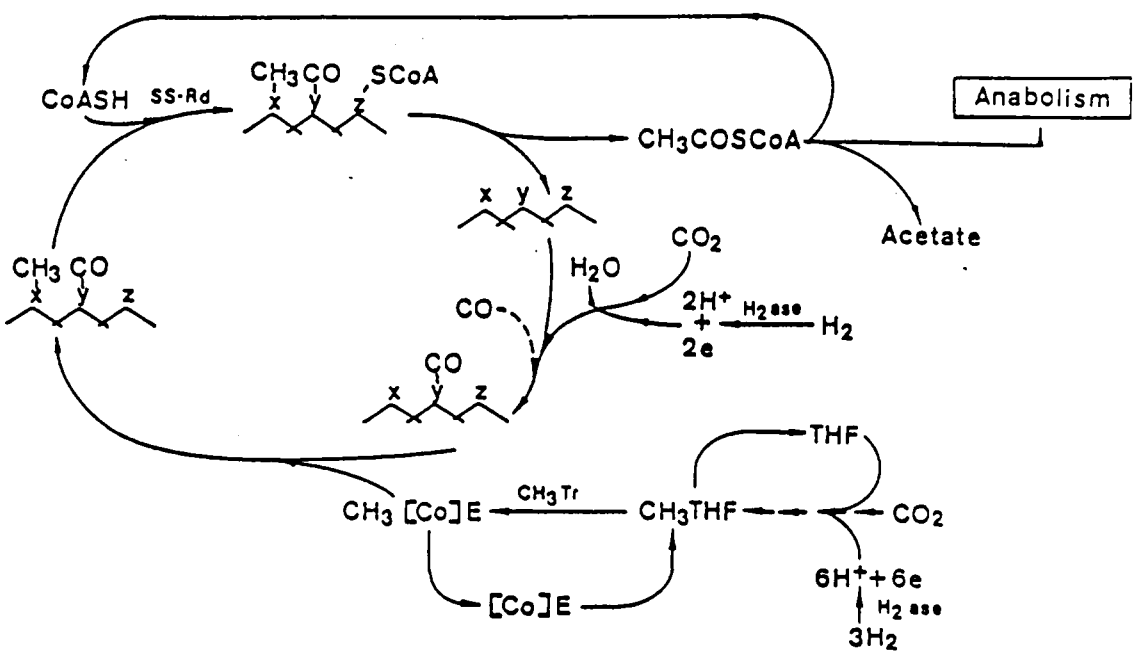

Figure 3. The acetyl-CoA pathway for autotrophic growth by acetogenic bacteria. THF, tetrahydrofolate; CH_3Tr, methyltransferase; [Co]E, corrinoid enzyme; , CO dehydrogenase with three binding sites (x, y, and z); SS-Rd, CO dehydrogenase disulfide reductase; H_2ase, hydrogenase. (Adapted from Pezacka and Wood, 1988. (47))

viologen dyes or ferredoxin. Though some aerobic bacteria such as Pseudomonas carboxydovorans, also catalyze the oxidation of CO to CO_2, the aerobic CO oxidases contain molybdenum rather than nickel (40).

The CO dehydrogenases from C. thermoaceticum and Acetobacterium woodii have a subunit composition of α3ß3 with subunit molecular weights of approximately 80 and 70 kD (51, 53). The hexameric native enzyme contains 6 Ni, 3 Zn, Mg and several iron-sulfur centers. The oxidation of CO to CO_2 by the enzyme is only part of the enzyme's proposed in vivo function which is to catalyze the condensation reaction in acetyl-CoA synthesis (Figure 4). A second activity of CO dehydrogenases, more indicative of their physiological function, is the catalysis of an exchange reaction between ^{14}CO and the carbonyl group of acetyl-CoA (54). This carbonylation/decarbonylation reaction is unique to this enzyme and the CO_2 fixation pathway.

C. thermoaceticum catalyzes an exchange reaction between [^{3}H]-CoA and acetyl-CoA (46), and the condensation reaction of the carbonyl, methyl, and CoA groups forming acetyl-CoA. This can be experimentally confirmed by the ability of the CO dehydrogenase to catalyze the formation of acetate in vitro from CH_3-corrinoid enzyme, CO and coenzyme A (45).

Perturbations of a nickel electron paramagnetic resonance (EPR) signal by carbon monoxide, are consistent with binding of the carbonyl group at a nickel-iron site (55) in the C. thermoaceticum enzyme (52). The nickel center of this enzyme is different from other nickel enzymes including urease, hydrogenase and methyl-CoM methylreductase. The nickel of the CO dehydrogenase is not believed to be part of a

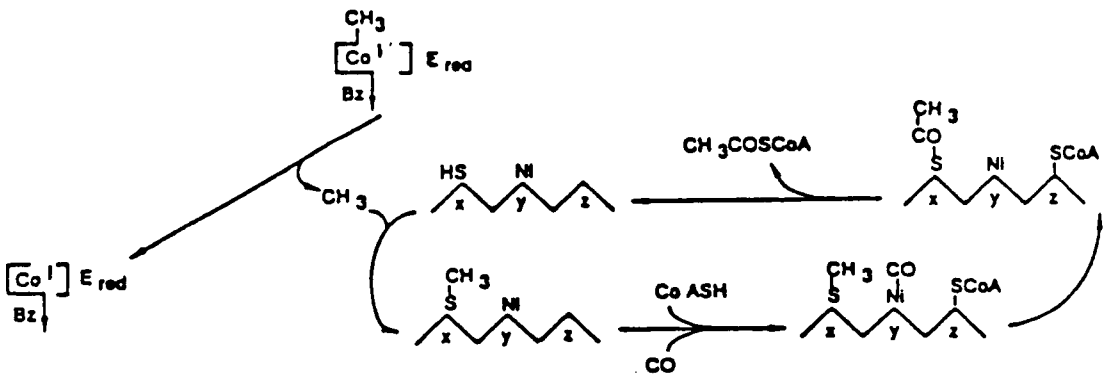

Figure 4. The proposed mechanism for acetyl-CoA synthesis by CO dehydrogenase. [Co]E, corrinoid enzyme; ΛΛΛ , CO dehydrogenase with three binding sites (x, y, and z). (Adapted from Pezacka and Wood, 1988) (47)

cofactor, but EPR (52, 55) and extended X-ray analysis of fine structure (EXAFS) (9) spectroscopic studies of the nickel environment revealed a Fe-Ni-C spin-coupled center in which Ni-(N,O) and Ni-S interactions are present (9). Since binding of CoA to the CO dehydrogenase caused perturbation of the Ni EPR signal, it was proposed that the CoA binding site is near the carbonyl-nickel site. The CoA is believed to bind to a thiol group that must first be reductively activated by the CO dehydrogenase disulfide reductase (46). The disulfide reductase is a 225 kD tetramer containing 8 calcium and 4 zinc atoms. It catalyzes the reduction of disulfides such as CoAS-SCoA, CoAS-glutathione and cystine. Following treatment of the CO dehydrogenase with disulfide reductase, the detectable SH groups of CO dehydrogenase increased from 22 to 33, and there was a 3.5 fold increase in the amount of acetyl-CoA synthesized (46).

There was no perturbation of the Ni EPR signal by the methyl carbon of acetyl-CoA which suggested it does not bind near the Ni center. Recently it was found that the methyl group binding site is a cysteine residue of the smaller subunit of the CO dehydrogenase (47).

The corrinoid protein that donates the methyl carbon to the CO dehydrogenase has two subunits of 34 and 55 kd and contains 0.69 mol corrinoid per mol native enzyme (25). This corrinoid protein is different from other B_{12}-containing proteins because it also contains a redox-active iron-sulfur center. The Co^{2+} and [4Fe-4S] center in the as-isolated protein must first be reduced with CO and CO dehydrogenase prior to accepting the methyl group from 5-methyl-tetrahydrofolate (25).

A methyltransferase enzyme catalyzes the transfer of the methyl group from 5-methyl-tetrahydrofolate to the corrinoid enzyme. This enzymatic activity has been

described in C. thermoautotrophicum and C. thermoaceticum (25), though no extensive characterization of the purified enzyme has been reported.

B. The Sulfate-Reducing bacteria

Certain sulfate-reducing bacteria are considered homoacetogens because they are able to grow on CO_2 as a sole carbon source and acetate is the assimilated product. There is evidence for both the reductive TCA cycle and the acetyl-CoA pathway within this group of organisms. In Desulfobacter hydrogenophilus, it appears the reductive TCA cycle is operative, but in Desulfobacterium autotrophicum, Desulfotomaculum acetoxidans, and Desulfovibrio baarsii there is evidence for the acetyl-CoA pathway (57). D. acetooxidans cells catalyzed an exchange between the carbonyl group of acetate and free carbon monoxide (57), characteristic of CO dehydrogenase enzymes.

C. Cell carbon synthesis in methanogens

During methanogenesis from methanol or H_2/CO_2, the synthesis of acetate for cell carbon occurs by the acetyl-CoA pathway in which the condensation reaction is catalyzed by a CO dehydrogenase enzyme. During growth of Methanobacterium thermoautotrophicum on H_2/CO_2, acetyl-CoA is the first detectable CO_2 fixation product (18). Several lines of evidence support the involvement of a CO dehydrogenase in cell carbon synthesis. First, in M. thermoautotrophicum, the carboxyl group of acetate is derived from a cyanide-sensitive enzyme that reduces CO_2 to a CO-bound intermediate (65), and ^{14}CO is incorporated into the carboxyl group of acetyl-CoA (65). Recently, a nickel-containing CO dehydrogenase was purified from Methanococcus vannielii (11).

Alkyl halides were found to inhibit growth but not methanogenesis; this effect was abolished by exposure to light (24). When concentrations of methyl iodide were used that were below the inhibitory concentration, $^{14}CH_3I$ was incorporated into the methyl group of acetate (24). Such observations are consistent with the involvement of corrinoids in cell carbon synthesis in these organisms. A proposed mechanism for acetyl-CoA synthesis in H_2/CO_2-grown and methanol-grown cells is shown in Figure 5 and Figure 6. In contrast to the tetrahydrofolate pathway used in the acetogenic clostridia, the methanogens utilize the methanopterin and methanofuran intermediates to oxidize one methyl group to CO_2. The branch point for cell carbon synthesis is proposed to be at the methyl-H_4MPT level since it is a precursor of the methyl group of acetate in Methanobacterium, and CH_3-S-CoM is not a precursor of acetate (34). Following synthesis of acetyl-CoA, the reverse reactions of the TCA cycle are used to generate the necessary precursors for required cell components.

Methanogenesis from Acetate

A. Whole cell studies

The proposed pathway of acetate conversion to methane is depicted in Figure 7. The initial work on the pathway of methanogenesis from acetate studied carbon conversions in cell cultures. M. barkeri cell suspensions catalyzed methane formation from acetate, and ^{14}C-acetate, and ^{3}H-acetate tracer studies revealed that the methyl group of acetate was transferred intact to methane and the carbonyl group to CO_2 (48, 64, 73). This provided the first evidence that substrates other than CO_2 were precursors for methane. The possible involvement of a CO dehydrogenase enzyme and

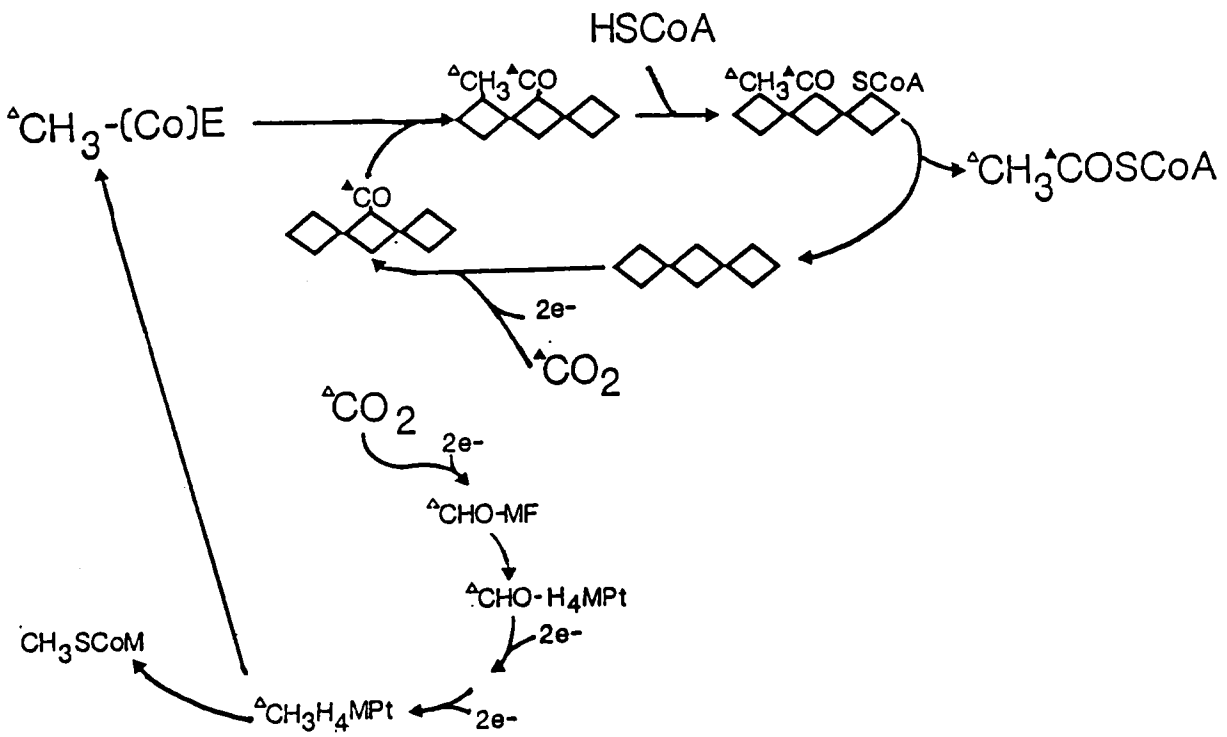

Figure 5. A proposed pathway for cell carbon synthesis during methanogenesis from CO_2. [Co]E, corrinoid enzyme; MF, methanofuran; MPt, methanopterin;◇◇◇, CO dehydrogenase with three binding sites.

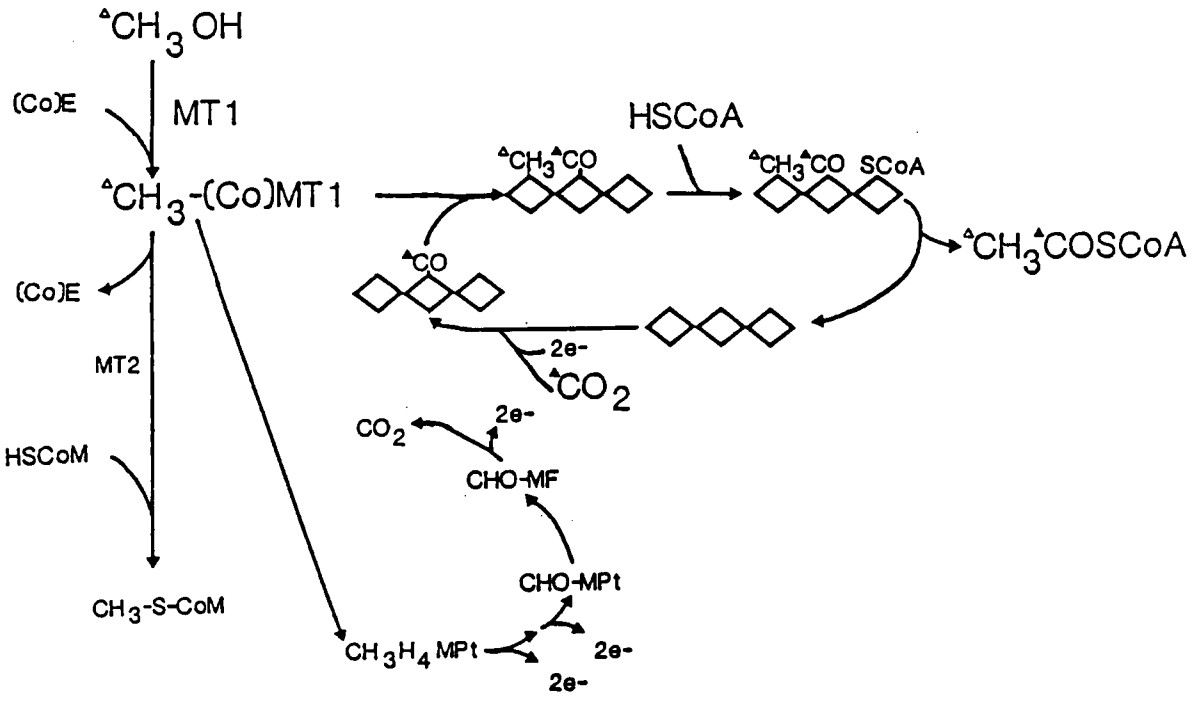

Figure 6. A proposed pathway for cell carbon synthesis during methanogenesis from methanol. MT1, methanol:B_{12}-HBI methyltransferase; MT2, CH_3-B_{12}-HBI methyltransferase; [Co]E, the corrinoid-containing moiety of MT1; MF, methanofuran; MPt, methanopterin; ◇◇◇, CO dehydrogenase with three binding sites.

the acetyl-CoA pathway in aceticlastic methanogenesis was suggested when cell suspensions of M. barkeri catalyzed an isotopic exchange reaction between the carbonyl group of acetate and $^{14}CO_2$ (12). M. barkeri cells also catalyzed the synthesis of acetate from methyl iodide, CO_2 and H_2 (35). Consistent with other methanogenic pathways, CH_3-S-CoM was demonstrated as an intermediate in aceticlastic methanogenesis in whole cells of M. thermophila (38) and M. barkeri (28). To further examine the intermediates and enzymes involved in aceticlastic methanogenesis, it was necessary to reconstitute methane production from acetate in broken cells.

B. Cell extract studies

Research on the conversion of acetate to methane and carbon dioxide in methanogens has been hampered by the difficulty in growing the cells and the resulting low cell yields of the organisms grown on acetate. A method was developed in 1984 by Sowers et al. for growing M. thermophila on acetate in a pH auxostat (62). Sufficient cell yields are obtained in this manner for enzyme purification.

Recently, several investigators have obtained methanogenesis from acetate in cell extracts of Methanosarcina spp. (2, 30, 71). Acetyl-CoA was determined to be an intermediate since the rates of methanogenesis from acetate and ATP or acetyl-phosphate in M. barkeri extracts increased upon the addition of coenzyme-A (16, 20). The involvement of CH_3-S-CoM as an intermediate methyl carrier was implicated in whole cells and confirmed in extracts (28); the methylreductase enzyme was also implicated due to inhibition of methanogenesis by bromoethanesulfonic acid (28). van der Wijngaard et al demonstrated methanogenesis from cofactor-free extracts that was

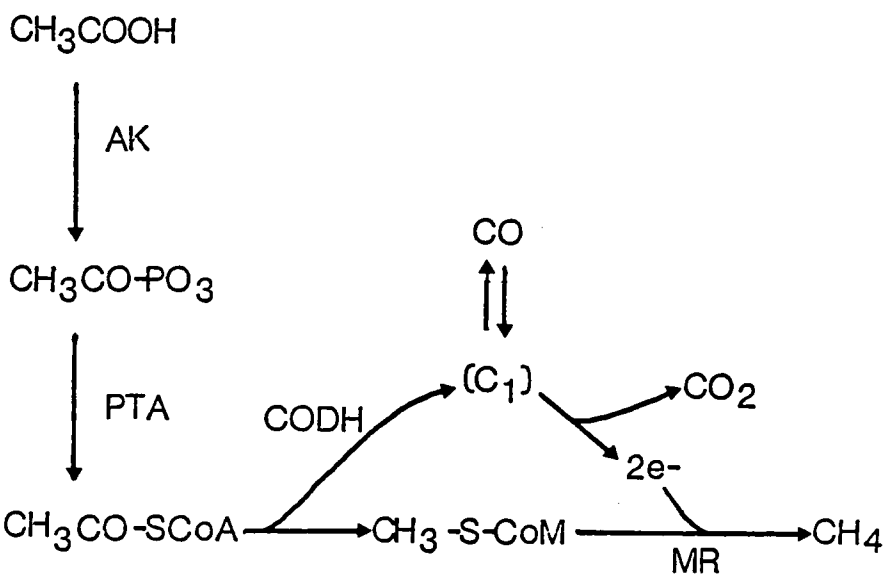

Figure 7. A proposed pathway of carbon and electron flow during methanogenesis from acetate. AK, acetate kinase; PTA, phosphotransacetylase; CODH, carbon monoxide dehydrogenase; MR, methyl coenzyme M methylreductase.

dependent on ATP, CoA, HS-CoM and component B (71).

Corrinoid compounds have been shown to be methyl-carriers during methanogenesis from methanol and are believed to have a parallel role in acetate conversion to methane. Propyl iodide inhibited methanogenesis in extracts of M. barkeri which was relieved by exposure to light due to photolysis of the Co-propyl bond (13). In another study, CH_3-B_{12}-HBI was isolated as an enzyme-bound intermediate during the conversion of acetate or acetyl-phosphate and ATP to methane in cofactor-free M. barkeri extracts (71).

C. Enzymology of the pathway

The initial steps in the formation of methane and CO_2 from acetate are the activation of acetate. Nearly simultaneous to the cell extract studies implicating acetyl-CoA as an intermediate in the pathway, activities of certain acetate-activating enzymes were reported. In Methanothrix soengenii, acetate thiokinase was found (27). However, in M. barkeri and M. thermophila acetate kinase and phosphotransacetylase activities were found and no thiokinase activity was detected (67, 35). In each enzyme systems the activated form of acetate is acetyl-CoA; the acetate kinase enzyme however first activates the acetate to acetyl-phosphate followed by derivatization to acetyl-CoA by the phosphotransacetylase.

The central enzyme in the pathway believed to cleave the carbon-carbon bond of acetyl-CoA is CO dehydrogenase, which was initially implicated due to the CO-carbonyl group exchange reaction observed in M. barkeri cell suspensions (12). Furthermore, CO dehydrogenase activity and protein levels were amplified in Methanosarcina species

cultured on acetate compared to cells grown on methanol (29). Following cleavage of acetyl-CoA by the CO dehydrogenase, the methyl group is transferred to unkown methyl-intermediates to coenzyme M.

The final step in the carbon clow pathway is the formation of CH_3-S-CoM, and it's subsequent conversion to methane by the CH_3-S-CoM methylreductase enzyme. The electrons required for this reaction are derived from the oxidation of the carbonyl group bound to the CO dehydrogenase. The electrons derived from this oxidation probably pass through various electron transfer proteins prior to the methylreductase enzyme. The exact steps involved are unknown.

D. Electron transfer in aceticlastic methanogenesis

Researchers hypothesize that the energy-conserving step in methanogenesis from all substrate is the methylreductase reaction. Though the origin of the electrons required for this reaction is different in methanogenesis from different substrates, their route to methyl CoM is believed to be important for ATP synthesis. In acetate-grown methanogens these electrons are derived from the oxidation of the carbonyl group to CO_2.

The pathway of electron transfer following oxidation of the carbonyl group is of interest because the free energy change for acetate conversion to methane is only slightly greater than the free energy required for the synthesis of one ATP. This low free energy change is suggestive of a chemiosmotic mechanism of ATP synthesis rather than substrate-level phosphorylation. Figure 8 is a proposed model of carbon and electron flow demonstrating a possible mechanism for ATP synthesis in which electrons

are channelled through membrane carriers with the concomitant translocation of protons across the membrane generating a proton gradient. The importance of the carbonyl group oxidation in acetotrophic methanogenesis was studied in whole cells of M. barkeri. Whole cell suspensions of acetate-grown M. barkeri catalyzed the conversion of CO and H_2O to CO_2 and H_2 coupled to the phosphorylation of ADP via a chemiosmotic mechanism (7). They were also capable of catalyzing the reverse reaction forming CO from CO_2 when the cells were metabolizing methanol (8). The reaction was inhibited when the protonophore 3,4,3'4'-tetrachlorosalicylanilide (TCS) was included. However, an ATPase inhibitor did not inhibit the reaction indicating a proton motive force was responsible for the endergonic formation of CO from CO_2 (8). Nelson et al demonstrated that the electrons derived from the carbonyl group oxidation can be used for the reduction of methyl-coenzyme M to methane (42). Elucidation of the electron transfer steps should provide insight to the energy conserving mechanisms in these organisms.

Several electron transfer proteins and enzymes have been described in methanogens including ferredoxins, cytochromes and hydrogenases. Ferredoxins are low-potential electron carriers, and two ferredoxins have been purified from methanol-grown M. barkeri (23, 41), though their exact physiological function is not known. One of these ferredoxins has been reported to couple H_2 evolution to the pyruvate dehydrogenase reaction (23).

Cytochromes have been found in membranes of several Methanosarcina species growing on methanol and acetate (32, 33). Two membrane-bound b-type cytochromes and one c-type cytochrome was detected in methanol-grown cells. An additional b-type

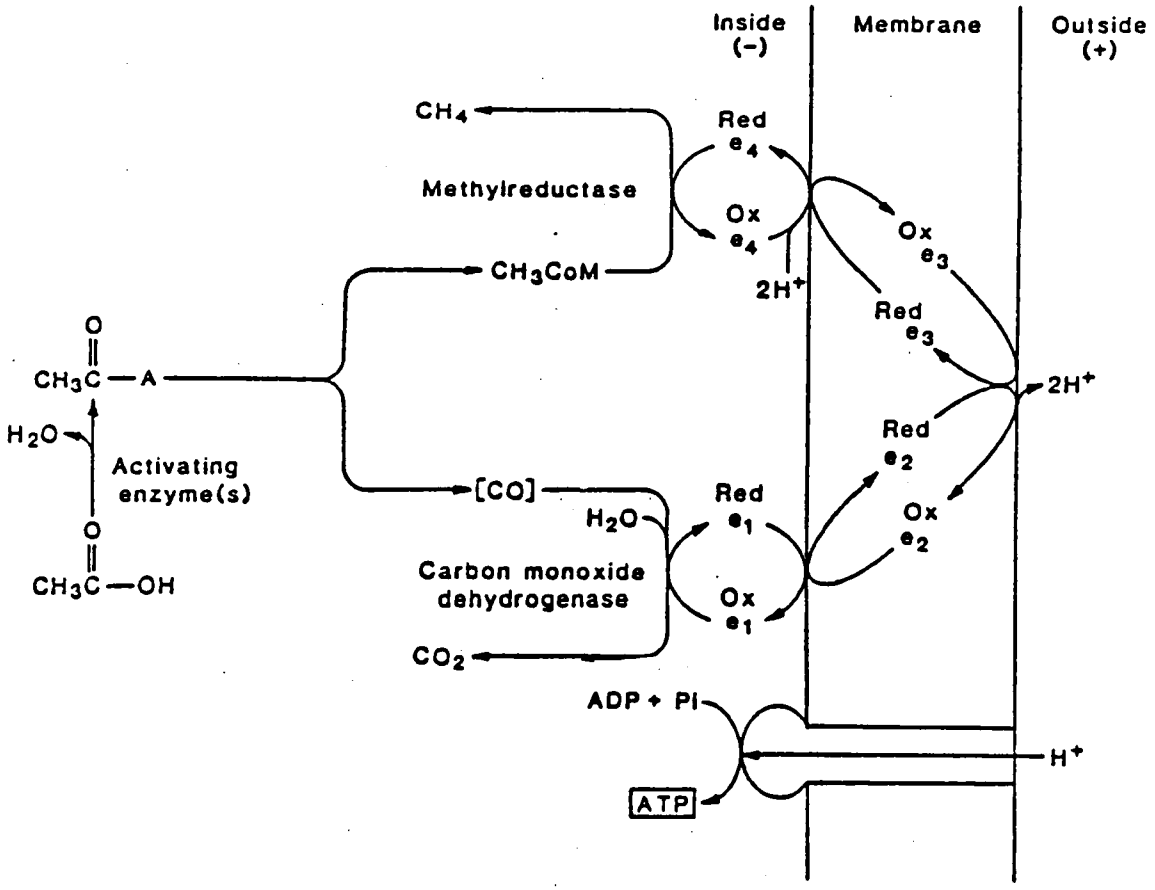

Figure 8. A proposed model for carbon and electron flow for energy conservation and ATP synthesis in acetotrophic methanogenesis. (Adapted from Zeikus et al (77)).

cytochrome was noted in acetate-grown cells (33). A b-type cytochrome, described in membranes of M. barkeri, was reduced by a membrane-bound hydrogenase and molecular hydrogen (26), and reoxidized by coenzyme M releasing an oxidized CoM heterodisulfide. Thus cytochromes and hydrogenases may have a role in electron transfer reactions during methanogenesis from methanol or acetate.

Though hydrogenase enzymes have not been purified from acetate-grown methanogens, Lovley et al. noted that two Methanosarcina species were found to maintain a steady state hydrogen concentration during growth (37). Furthermore, in whole cell studies, hydrogen was found to act as an electron donor for acetate-dependent methane production (30). Thus hydrogen metabolism may be physiologically significant not only in H_2/CO_2 grown methanogens but also in methanogens metabolizing methyl-group substrates.

SECTION III. ISOLATION OF AN ENZYME COMPLEX WITH CARBON MONOXIDE DEHYDROGENASE ACTIVITY CONTAINING CORRINOID AND NICKEL FROM ACETATE-GROWN Methanosarcina thermophila

SUMMARY

Fast protein liquid chromatography of cell extract from methanol- or acetate-grown Methanosarcina thermophila resolved two peaks of CO dehydrogenase activity. The activity of one of the CO dehydrogenases was 6-fold greater in acetate-grown compared to methanol-grown cells. This CO dehydrogenase was purified to apparent homogeneity (70 μmoles methyl viologen reduced/min/mg protein), and comprised greater than 10% of the cellular protein of acetate-grown cells. The native enzyme (M_r = 250K) formed aggregates of approximately M_r = 1,000K. The enzyme contained five subunits of M_r = 89K, 71K, 60K, 58K and 19K suggesting a multifunctional enzyme complex. Nickel, iron, cobalt, zinc, inorganic sulfide and a corrinoid were present in the complex. The UV-visible spectrum suggested the presence of iron-sulfur centers. The electron paramagnetic resonance spectrum contained g values of 2.073, 2.049, and 2.028; these features were broadened in enzyme purified from cells grown in the presence of medium enriched with ^{61}Ni indicating the involvement of this metal in the spectrum. The pattern of potassium cyanide inhibition indicated that cyanide binds at or near the CO binding site. The properties of the enzyme imply an involvement in the dissimilation of acetate to methane; possibly cleavage of acetate or an activated form.

INTRODUCTION

The pathway of methanogenesis from acetate in Methanosarcina thermophila involves transfer of the intact methyl group of acetate to coenzyme M (HSCoM) (11) followed by reductive demethylation catalyzed by methyl coenzyme M methylreductase (13). The mechanism of methyl transfer to HSCoM is unknown. It is postulated that cleavage of the carbon-carbon bond of activated acetate is catalyzed by an enzyme with CO dehydrogenase activity, with transfer of the carbonyl carbon to a bound one-carbon intermediate (9,13). The CO dehydrogenase activity of M. thermophila is several-fold greater in acetate-grown cells compared to cells grown with alternate substrates, and acetate-grown cells contain CO-dependent methyl coenzyme M methylreductase activity (13). Recent evidence for transfer of the methyl group of activated acetate to a corrinoid protein stems from the propyl iodide inhibition of methanogenesis from acetate in Methanosarcina barkeri (5).

The mechanism proposed for cleavage of activated acetate by M. thermophila (13) is similar to a reversal of the pathways for the synthesis of acetyl-CoA in strict anaerobes. A CO dehydrogenase and a corrinoid protein are involved in acetyl-CoA synthesis for cell carbon of methanogenic bacteria (6). A purified CO dehydrogenase from the homoacetogenic clostridia catalyzes the synthesis of acetyl-CoA from CoA, methyl corrinoid, and an enzyme-bound one-carbon intermediate (17). The clostridial enzyme forms a unique paramagnetic nickel(III)-iron-carbon species when incubated with CO (18).

Here we describe the purification of the CO dehydrogenase complex synthesized in acetate-grown M. thermophila which displays properties that implicates an involvement

in acetate dissimilation to methane.

MATERIALS AND METHODS

Organism and culture conditions. Methanosarcina thermophila strain TM-1 (26) was cultured on acetate in a 10-liter pH auxostat as described previously (22). The basal medium contained (in grams per liter, final concentration): NH_4Cl, 1.44; K_2HPO_4, 1.13; KH_2PO_4, 1.13; NaCl, 0.45; $MgSO_4 \cdot 2H_2O$, 0.09; $CaCl_2 \cdot 2H_20$, 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, 0.001. Trace elements and vitamin solutions (24) were each added at a final concentration of 1% (vol/vol); $NiCl \cdot 6H_2O$ was added to a final concentration of 0.5 g/liter. 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. When cells were cultured in the presence of ^{61}Ni, $NiCl_2 \cdot 6H_2O$ was omitted, yeast extract was decreased to 0.1 g/liter, and ^{61}Ni (dissolved in nitric acid) was added to a final concentration of 0.5 mM. 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 enzyme assays were as previously described (19). 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 free of trace amounts of O_2 by passage through reduced BASF catalyst R3-11

(Chemical Dynamics, South Plainfield, NJ).

Preparation of cell extract. Cell extracts were prepared anaerobically under a H_2 atmosphere as previously described (13). Breakage buffer consisted of 50 mM potassium N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate buffer (TES) (pH7.0) containing 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5% (vol/vol) glycerol, and 0.015 mg/ml of DNase I (Sigma, St. Louis, MO).

Enzyme Purification. All steps were performed at 23°C in a Coy anaerobic chamber (Coy Manufacturing Co., Ann Arbor, MI) unless otherwise noted. Buffer A contained 50 mM TES (pH 6.8), 10% (vol/vol) ethylene glycol, and 10 mM $MgCl_2$. Buffer B and buffer C were identical to buffer A except 1.0 M KCl and 0.15 M KCl were included.

Saturated ammonium sulfate solution in 50 mM TES (pH 6.8) and 10 mM $MgCl_2$ was added to 10 ml of cell extract to a final concentration of 0.35 saturation. This mixture was incubated for 30 min at 4°C and then centrifuged at 41,000 x g for 20 min in a DuPont Sorvall RC-5B centrifuge at 4°C. The brown supernatant solution containing CO dehydrogenase activity was dialyzed at 4°C against 1.5 l of buffer A without ethylene glycol. The remaining steps in the purification utilized a high resolution fast protein liquid chromatography (FPLC) system (Pharmacia, Piscataway, NJ) equipped with a model GP-250 gradient programmer. A sample (10 ml) of the dialyzed enzyme solution was injected onto a Mono-Q HR 10/10 ion exchange column (Pharmacia) previously equilibrated with Buffer A. A linear gradient from 0.0 to 0.5M KCl was applied at a flow rate of 2.0 ml/min. Two peaks of CO dehydrogenase activity eluted. The second, larger peak was collected and injected again onto the Mono-Q HR 10/10 column equilibrated with buffer A. The enzyme was concentrated 10-fold by

batch elution with 0.4 M KCl. Aliquots (0.5 ml) of the concentrated protein solution were injected on a Superose-6 (Pharmacia) gel filtration column previously equilibrated with Buffer C. The column was developed at a flow rate of 0.4 ml/min. Purified CO dehydrogenase was collected and stored in liquid N_2 until use.

Enzyme Assays. All assays were performed at 23°C. The standard assay for CO dehydrogenase was performed by following the CO-dependent reduction of methyl viologen (MV) (ϵ_{603} = 11.3 $mM^{-1}cm^{-1}$) (19) with a Perkin-Elmer Lambda 1 spectrophotometer. The standard reaction mixture contained: 50 mM Tris-HCl (pH 8.4), 20 mM MV and 20 mM 2-mercaptoethanol. Reaction mixture (0.5 ml) was equilibrated with CO for the test assay and N_2 for control assays. The mixture was added to serum stoppered cuvettes (1 ml) filled with either CO or N_2. The reaction was initiated by the addition of enzyme. FAD (20 μM), FMN (20 μM) and F_{420} (49.5 μM) were also tested as electron acceptors. In the case of F_{420}, the enzyme was preincubated under N_2 with either 30 μM FAD or 0.3 M KCl or both FAD and KCl. The extinction coefficients at pH 7.5 were as follows: FAD, ϵ_{450} = 11.3 mM^{-1} cm^{-1}; FMN, ϵ_{450} = 12.2 $mM^{-1}cm^{-1}$; and F_{420}, ϵ_{420} = 42.5 $mM^{-1}cm^{-1}$. One unit of enzyme activity was the amount that reduced 1 μmol of acceptor per min.

Analytical. Protein was determined with protein dye reagent (Bio-Rad, Richmond, CA) by the method of Bradford (3) with bovine serum albumin (Sigma) as the standard. Metals were analyzed by plasma emission spectroscopy at the Institute of Ecology, University of Georgia as described elsewhere (15). Inorganic sulfide was determined by the micro method of Beinert (2) in 0.5 ml culture tubes fitted with serum stoppers (7 x 15 mm).

Gel electrophoresis. Native polyacrylamide gel electrophoresis was performed using the Laemmli buffer system without sodium dodecyl sulfate (SDS) (10). CO dehydrogenase activity was visualized on native gels prior to staining for protein. The gel was placed in a glass dish (20.5 x 20.5 x 5 cm) modified with silicone rubber molded on to the edges to provide a gastight seal with a glass cover. The atmosphere in the dish was flushed with O_2-free N_2 at 150 ml/min for 20 min using entrance and exit syringe needles inserted through the rubber mold. The N_2 atmosphere was then replaced with CO. The reaction mixture used in the standard CO dehydrogenase assay was flooded onto the gel and incubated at room temperature. Protein bands that contained CO dehydrogenase activity were visualized as a dark blue band of reduced MV against a colorless background. The gels were stained for protein using Coomassie blue R-250. An Isophore (Isolabs Inc., Akron, OH) 4-30% linear gradient gel was used to estimate native molecular weights (12). The Tris-boric acid buffer system (pH 8.3) contained per liter: 10.75 g Tris-HCl, 5.0 g borate and
0.93 g sodium EDTA. The molecular weight standards (Sigma) were: bovine thyroglobulin, 700,000; horse serum ferritin, 450,000; bovine catalase, 240,000; rabbit muscle aldolase, 160,000; bovine serum albumin, 67,000; ovalbumin, 45,000. The molecular weight standards used in SDS gel electrophoresis were phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

Spectroscopy. Electron paramagnetic resonance (EPR) experiments were performed with a Varian E109 Century Series spectrometer. The temperature was kept constant with a Varian variable temperature accessory. A copper-EDTA standard was used for

integration of the signals. UV-visible spectra of the CO dehydrogenase and corrinoid were obtained with a Cary model 219 and a Perkin-Elmer model 552 spectrophotometer.

Chemicals. F_{420} was purified from extracts of Methanobacterium formicicum as previously described (19). The following were purchased from Sigma: ammonium sulfate (grade III), MV, 2-mercaptoethanol (type I), FMN and FAD. ^{61}Ni (87% isotopic purity) was obtained from the Oak Ridge National Laboratory. Electrophoresis grade Tris, glycine, and SDS were obtained from BioRad, Richmond, CA. All other chemicals were of reagent grade and the highest purity obtainable from Sigma.

RESULTS

Purification. Anion exchange FPLC of cell extract from acetate-grown cells resolved two peaks of CO dehydrogenase activity (Fig. 1) The first peak (peak A-I) contained only 10% of the total activity recovered from the column, while the second peak (peak A-II) contained 90%. FPLC of extracts from methanol-grown cells yielded similar results except the second peak of CO dehydrogenase activity (peak B-II) was 6-fold less than the corresponding peak A-II from acetate-grown cells. The greater amount of total activity recovered from the acetate-grown cells was consistent with previous results (13). The greater amount of activity in peak A-II of acetate-grown cells suggested a specific involvement of this CO dehydrogenase in the pathway of acetate conversion to methane.

A representative purification of the CO dehydrogenase in peak A-II from acetate-grown cells is shown in Table 1. The CO dehydrogenase rapidly lost activity on

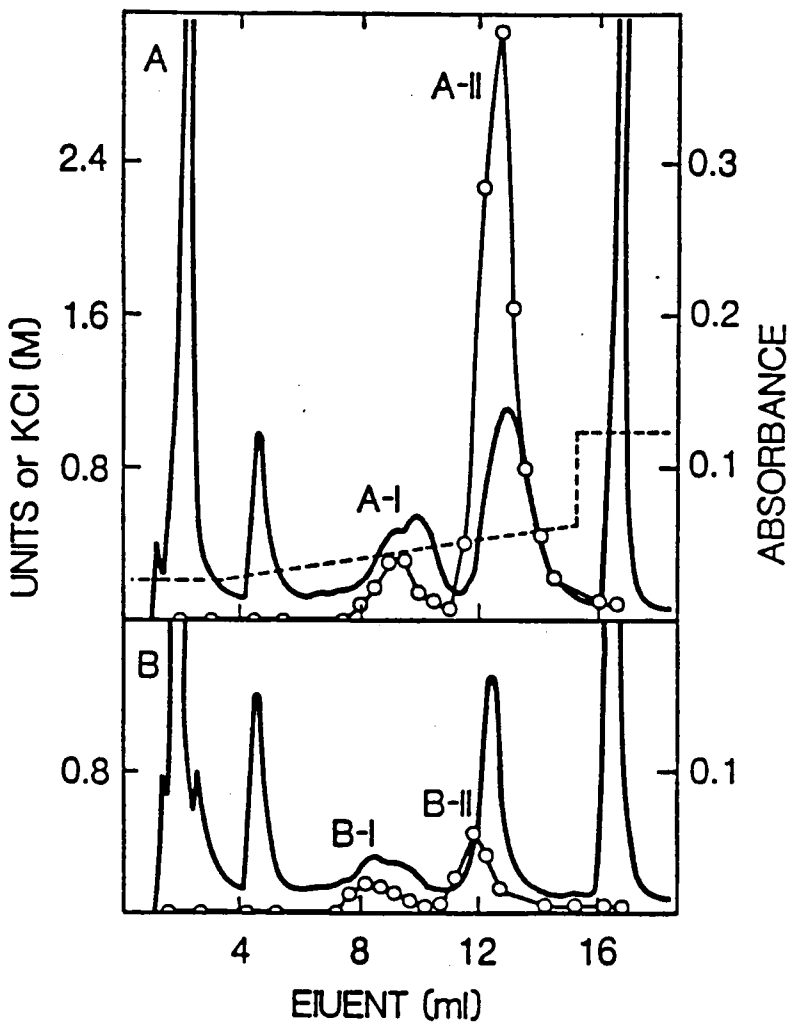

Figure 1. FPLC anion exchange chromatography of cell extracts from acetate- or methanol-grown Methanosarcina thermophila. Cell extract from acetate-grown (A) or methanol-grown (B) cells each containing 1.4 mg protein was injected onto a Pharmacia Mono-Q 5/5 anion exchange column previously equilibrated with 50 mM TES (pH 7.0) and developed with a gradient of KCl at 0.5 ml/min as indicated. The total activity in each fraction (0.5 ml) was determined by the standard assay for CO dehydrogenase. Symbols: (o) CO dehydrogenase activity; (—) A_{280}; (- - -) KCl.

Table 1. Purification of CO dehydrogenase from acetate-grown Methanosarcina thermophila

Step	Units	Units/mg	Fold purification	Per cent recovered
Cell extract	790	10.5	1	-
35% Ammonium sulfate	732	10.9	1	93
Mono Q fractionation	687	46.0	4.4	87
Mono Q concentration	666	67.5	6.4	84
Superose S-6 gel filtration	618	70.0	6.9	78

The enzyme purified was the major CO dehydrogenase synthesized in acetate-grown cells (A-II, Figure 1)

Cell extract was 20 mg protein/ml.

exposure to air, therefore all steps were performed in the anaerobic chamber. Based on the following results, the enzyme was judged to be homogeneous. The CO dehydrogenase eluted from the high resolution FPLC Superose-6 gel filtration column in a single symmetrical protein peak with constant specific activity (+/- 2 units/mg). Native gel electrophoresis of the preparation yielded a major high-molecular-weight protein band and a minor protein band near the dye front (Fig. 2). Each of the bands contained CO dehydrogenase as shown by activity staining prior to staining for protein (data not shown). In some preparations, a minor amount of contaminating protein was observed migrating with the dye front. This did not have CO dehydrogenase activity. The native M_r of the corresponding proteins were approximately 1,000K and 250K as estimated by gradient gel electrophoresis (data notshown). SDS gel electrophoresis in the second dimension (data not shown) revealed identical subunit compositions of the two proteins which indicated that the larger protein was an aggregate of the smaller protein; the SDS gel also showed that no protein was detectable at the dye front of the native gel.

Native gel electrophoresis of cell extracts from acetate- and methanol-grown cells showed higher concentrations of the purified CO dehydrogenase in acetate-grown cells (Fig. 2). Similar patterns were obtained when the gels were stained for activity prior to protein staining (data not shown). Although the CO dehydrogenase was purified to apparent homogeneity, the specific activity increased only 7-fold (Table 1) indicating that the enzyme was a major constituent of acetate-grown cells.

Composition. Denaturing gel electrophoresis of the purified preparation revealed 5 subunits with Mr of 89K, 71K, 60K, 58K and 19K (Fig. 3). Some preparations (2 of 6)

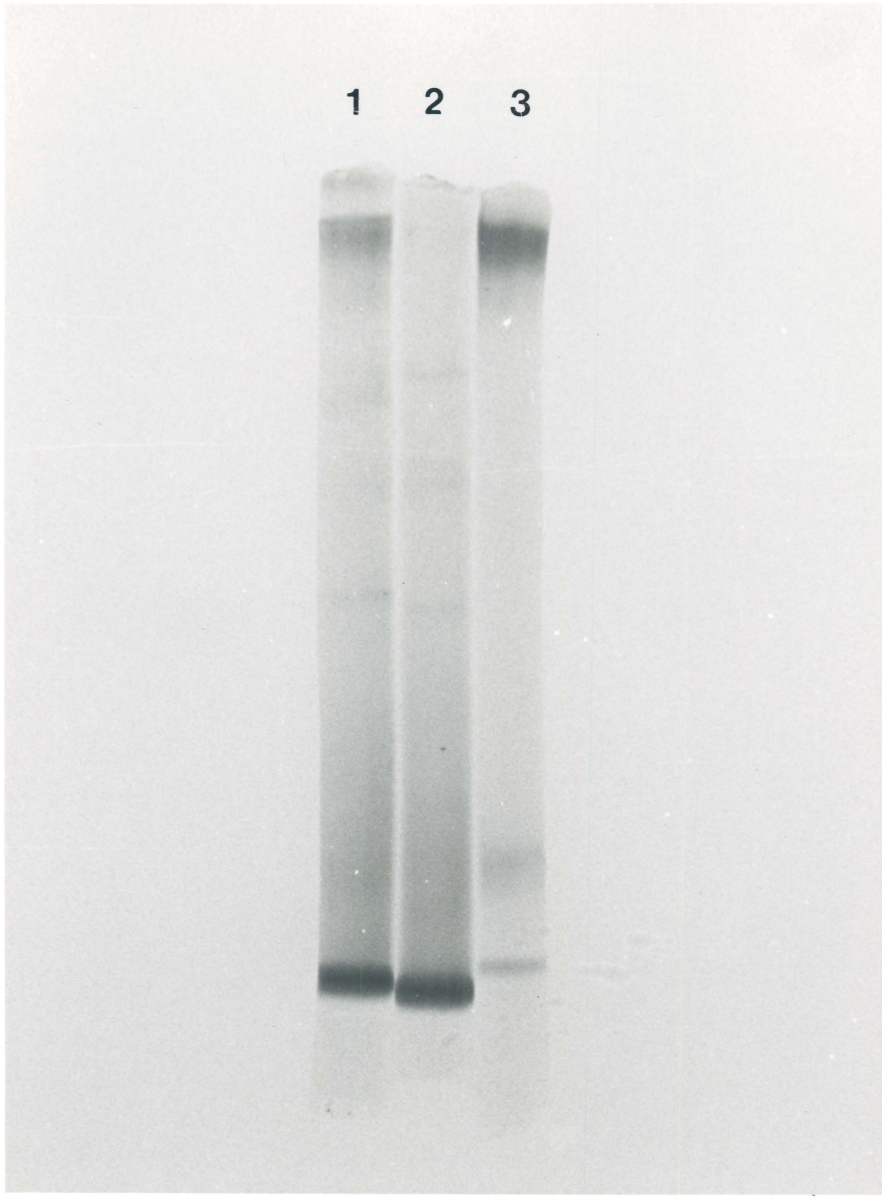

Figure 2. Native polyacrylamide tube gel electrophoresis of cell extracts and CO dehydrogenase from Methanosarcina thermophila. Gels were loaded with: 15 μg cell extract protein from acetate-grown cells, (lane 1); 15 μg cell extract protein from methanol-grown cells, (lane 2); and 5 μg of purified CO dehydrogenase, (lane 3). The gels were stained for protein with Coomassie blue R-250.

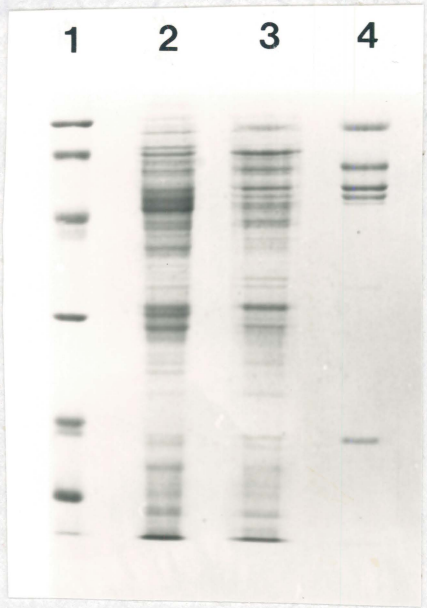

Figure 3. SDS polyacrylamide gel electrophoresis of purified CO dehydrogenase , and acetate- and methanol-grown cell extracts, from Methanosarcina thermophila. Molecular weight standards, see Materials and Methods, (lane 1); cell extract from methanol-grown cells, 50 μg protein, (lane 2); cell extract from acetate-grown cells, 50 μg protein, (lane 3); CO dehydrogenase, 5 μg (lane 4). Gels were stained for protein with Coomassie blue R-250.

yielded variable amounts of an additional protein band (M_r = 56K, Fig. 3). This additional protein never exceeded 5% of the total absorbance in densitometer scans, which suggests it was a minor contaminant or degraded protein. Densitometer scans revealed a 1.3:1.0:1.1:1.1:1.0 ratio of the subunits yielding a minimum M_r of 297K. Denaturing gel electrophoresis of cell extracts from acetate- and methanol-grown cells showed that at least the 89K, and 19K subunits of the CO dehydrogenase were absent from methanol-grown cells (Fig. 3).

Metal analysis revealed apparently 3.6 Ni, 25 Fe, 1.2 Co, and 6.1 Zn per minimum Mr of 297K when protein was determined using the Bradford assay. Inorganic sulfide analysis showed 15.2 atoms per minimum M_r.

Spectral characteristics. The UV-visible absorption spectrum of the enzyme showed a broad peak in the 380nm - 480nm region characteristic of Fe-S proteins (Fig. 4). This absorbance decreased upon incubation with CO, revealing an absorbance maximum at 395 nm characteristic of the reduced, electrophilic cobamides.

A CO-dependent, EPR spectrum with g-values of 2.073, 2.049 and 2.028 was obtained (Fig. 5). The EPR signals were broadened with enzyme isolated from cells grown in medium enriched with ^{61}Ni, showing that the signal involved nickel. Double integration of the signal showed it to represent 29% of the nickel present in the sample.

The presence of cobalt in the CO dehydrogenase suggested that the CO dehydrogenase might contain a corrinoid compound. Heating the enzyme with KCN at pH 10.5 released a compound with a visible spectrum (Fig. 6) characteristic of dicyanocobamides (14). Approximately 0.5 moles of the corrinoid were recovered per

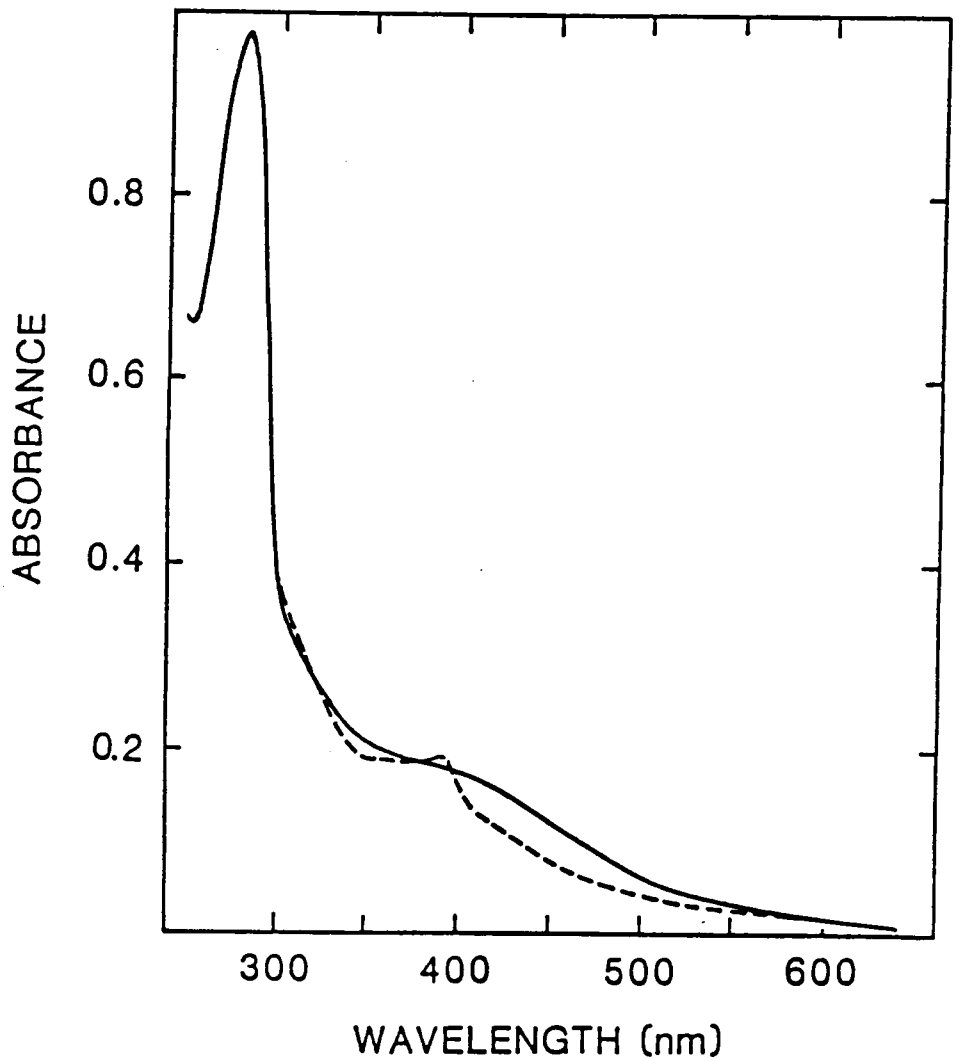

Figure 4. UV-visible absorption spectra of CO dehydrogenase from Methanosarcina thermophila. Spectrum of enzyme (0.63 mg/ml) in 0.15 M TES buffer (pH 6.8) and in a N_2 atmosphere,(__). Spectrum after the enzyme was incubated in a CO atmosphere for 15 min (- - -) .

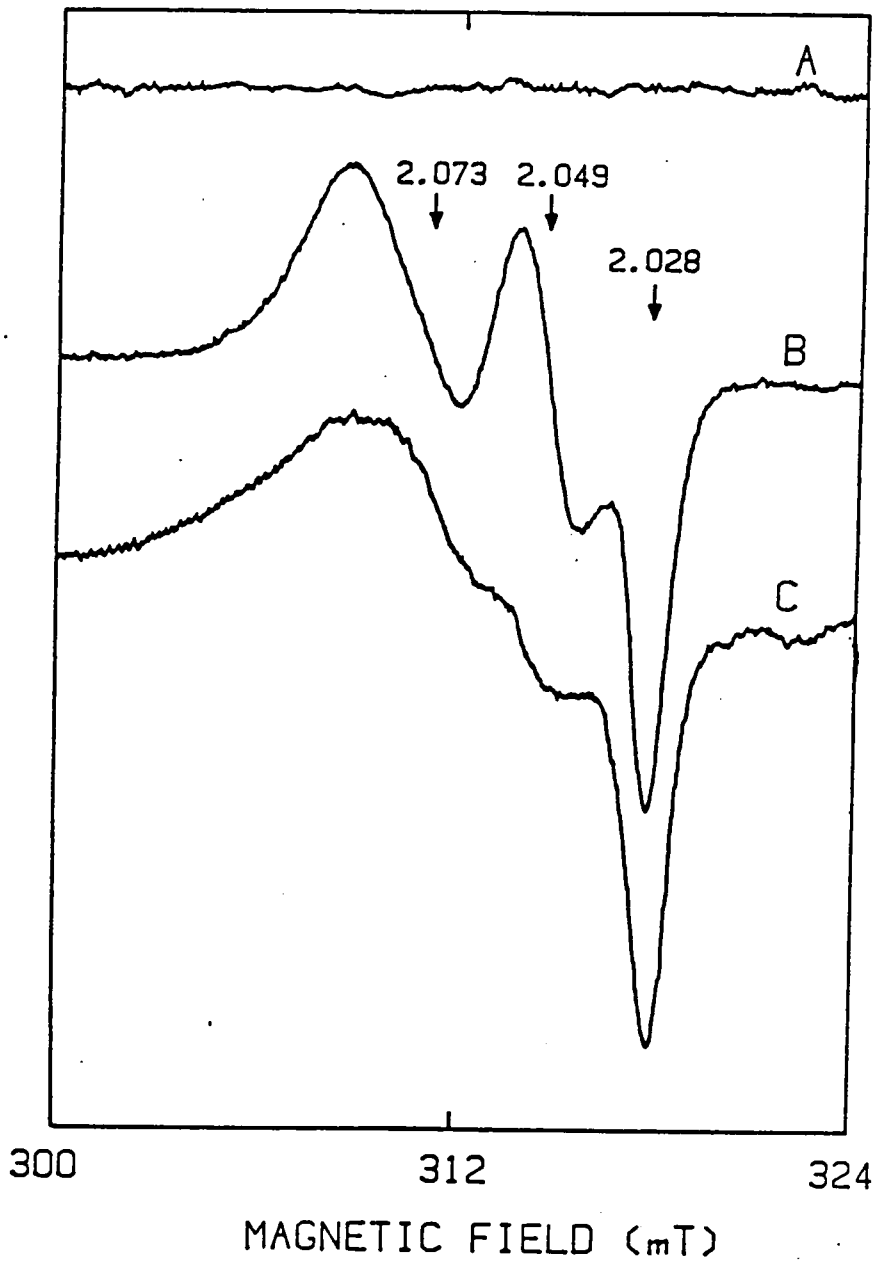

Figure 5. Electron paramagnetic resonance spectra of the CO dehydrogenase from Methanosarcina thermophila. Spectra were measured at 9 GHz and at -160°C. Other parameters were: 10 mwatt power, 3200 field set, 0.123-s time constant, 2.5 gauss modulation amplitude, 250 KHz modulation frequency, 20,000 gain, and 4-min scan time. Each enzyme preparation was in 50 mM TES pH 7.0 and 10 mM MgCl. (A) 15.5 mg/ml preincubated in an atmosphere of N_2. (B) 13.7 mg/ml preincubated in an atmosphere of CO. (C) 12.2 mg/ml of enzyme purified from cells grown in the presence of ^{61}Ni, and preincubated in an atmosphere of CO.

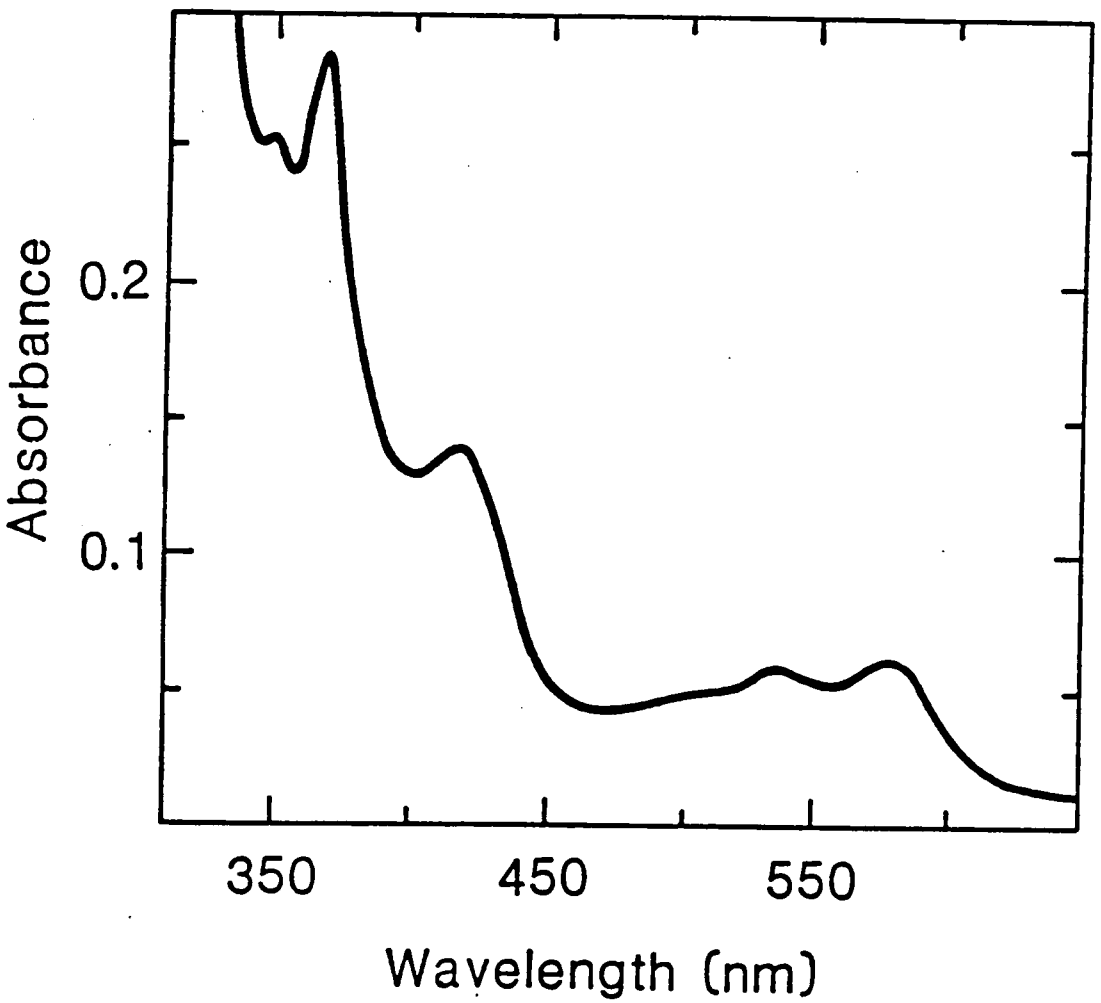

Figure 6. Visible spectrum of the corrinoid released from purified CO dehydrogenase of Methanosarcina thermophila. KCN was added to purified enzyme (4 mg in 2.0 ml 50 mM TES (pH 6.8), and 10 mM MgCl) at a final concentration of 0.01% (wt/vol), and the pH adjusted to 10.0 with concentrated KOH. The mixture was heated for 10 min at 95°C and centrifuged at 48,000 x g for 40 min.

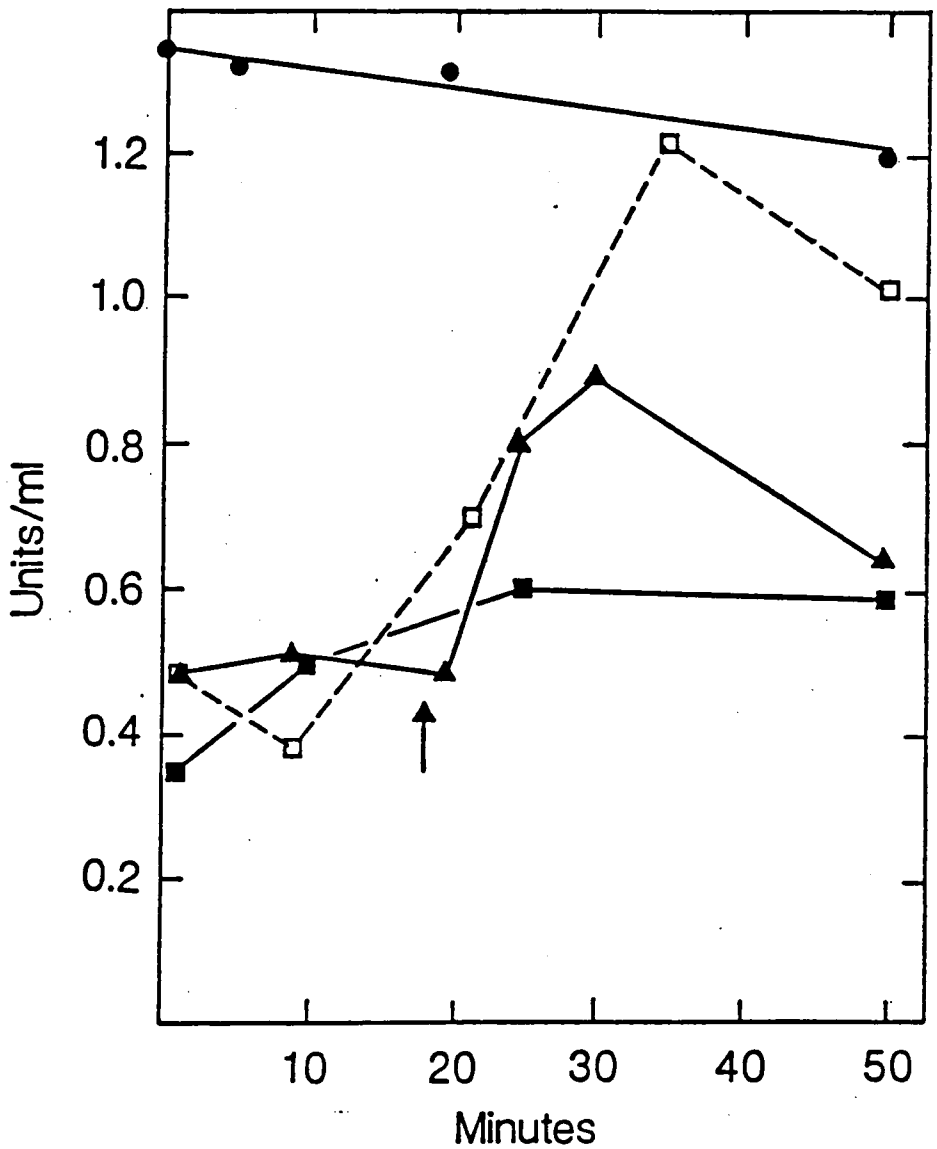

Figure 7. Inhibition of CO dehydrogenase from Methanosarcina thermophila by potassium cyanide. Solutions (0.2 ml) of enzyme (0.1 mg/ml) were contained in 2.2 ml stoppered serum vials with a N_2 or CO atmosphere as indicated. KCN was added to a final concentration of 20 μM where indicated. Samples (10 μl) were immediately assayed for activity in the standard assay except 20 uM KCN was present in the assay mixture. The control sample,(●),was not exposed to KCN in the incubation or assay mixture. Symbols: (●) N_2 atmosphere control; (■) N_2 atmosphere, KCN; (□) CO atmosphere, KCN; (▲) N_2 atmosphere replaced with CO at 17 min (arrow), KCN.

mole of enzyme assuming a minimum M_r of 297K and using ϵ_{580} = 10.2 $mM^{-1}cm^{-1}$ to estimate the amount of corrinoid (14).

Inhibition by cyanide. When the enzyme was incubated in either a N_2 or CO atmosphere and in the presence of 20 μM KCN, CO dehydrogenase activity was initially inactivated 73% (Fig. 7). However, after 10 min, a time-dependent reversal of inactivation occurred with enzyme incubated in a CO atmosphere; after 30 min, the activity was restored to 95% of the control. Replacement of the N_2 atmosphere with CO after 17 min also relieved the inactivation. These results suggest that CN^- may bind at or near the CO binding site.

Electron acceptors. The pH optimum in the standard assay with MV was 8.4 and activity was detected in the range 6 to 9. The CO dehydrogenase reduced FAD and FMN at 15% of the specific activity in the standard assay with MV, but it did not reduce F_{420}.

DISCUSSION

Several properties of the CO dehydrogenase purified from M. thermophila support a function for this enzyme in acetate conversion to methane. (i) The enzyme was highly enriched in acetate-grown compared to methanol-grown cells. (ii) The enzyme comprised greater than 10% of the cellular protein suggesting involvement in a major catabolic pathway. (iii) The EPR properties were nearly identical to the published spectra of the clostridial CO dehydrogenase (18) catalyzing the synthesis of acetyl-CoA. (iv) The enzyme contained a corrinoid, which has been implicated as a methyl carrier in the pathway of acetate conversion to methane (5). (v) The CO dehydrogenase activity

was reversibly inactivated by cyanide which also inhibits methanogenesis from acetate (21). Hypothetically, the enzyme may function to cleave an activated form of acetate transferring the methyl group to the corrinoid and the carbonyl to the nickel site forming a nickel(III)-iron-carbon center as proposed for the clostridial enzyme (18). The EPR results suggest that the environments of the nickel centers in the clostridial and methanogen enzymes are similar and may reflect similar reaction mechanisms in the synthesis and cleavage of acetyl-CoA. In addition, the pattern of cyanide inactivation of CO dehydrogenase activity was similar to the inactivation of the clostridial enzyme (16). The presence of Fe-S centers and zinc in the M. thermophila enzyme are additional features in common with the clostridial enzyme (15). Further research is necessary to prove the proposed function for the CO dehydrogenase such as demonstration of methylcobamide derived from the methyl group of acetate or an activated form of acetate. Cell extracts of M. thermophila contain high levels of acetate thiokinase activity (unpublished results) implicating acetyl-CoA as a potential activated form. Acetyl-CoA may also be the activated form in M. barkeri (5) and Methanothrix soehngenii (8).

The CO dehydrogenase contained 5 subunits suggestive of a multifunctional enzyme complex. Two of the subunits may be required for the CO dehydrogenase activity. A CO dehydrogenase has been described from M. barkeri (9) that contains two subunits with M_r of 92K and 18K, similar to the larger and smaller subunits of the M. thermophila enzyme; however, the composition of the M. barkeri enzyme is $\alpha_2\beta_2$. Another subunit in the M. thermophila CO dehydrogenase complex may be a corrinoid protein. Other subunits present may catalyze methyl transfer from the corrinoid protein

to HSCoM or function in electron transport.

The cleavage of acetyl-CoA or acetate to CH_3-S-CoM, CO_2, and two electrons may be thermodynamically unfavorable (4). An enzyme complex for the cleavage of acetyl-CoA and methylation of HSCoM may help maintain reactants and products at thermodynamically favorable concentrations. Acetate conversion to methane in extracts of M. barkeri may be associated with a complex (1). Efficient methyl transfer from methanol to HS-CoM in M. barkeri extracts also appears to require an enzyme complex (23). A cobamide-containing membrane complex has been reported in the H_2-CO_2-utilizing methanogen Methanobacterium thermoautotrophicum (20). The corrinoid protein and the CO dehydrogenase in the homoacetogenic clostridia also form a complex (7).

High resolution FPLC resolved two peaks of CO dehydrogenase activity from extracts of acetate-grown M. thermophila. The enzyme in the first peak (peak A-I) was not purified. However, SDS gel electrophoresis of peak A-I fractions revealed that none of the subunits of the purified CO dehydrogenase (peak A-II) were present (unpublished results), suggesting a different enzyme in peak A-I. Other CO dehydrogenases may be present that function to oxidize CO during growth on this substrate, or function in acetyl-CoA synthesis for cell carbon during growth on methanol or CO_2.

When Methanosarcina are grown with both methanol and acetate, growth is biphasic with methanol as the preferred substrate. A model for regulation in M. thermophila has been proposed in which methanol represses the synthesis of enzymes for acetate conversion to methane while enzymes for conversion of methanol are inducible (25). The comparison of FPLC, and native and SDS gels of extracts from

acetate- and methanol-grown cells reported here suggests that synthesis of the intact CO dehydrogenase complex may be repressed in methanol-grown cells supporting the proposed model. However the results do not rule out that three of the subunits (71K, 60K and 58K) may also be synthesized in methanol-grown cells.

ACKNOWLEDGEMENTS

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SECTION IV. EPR PROPERTIES OF THE Ni-Fe-C CENTER IN AN ENZYME COMPLEX WITH CARBON MONOXIDE DEHYDROGENASE ACTIVITY FROM ACETATE-GROWN Methanosarcina thermophila: EVIDENCE THAT ACETYL-CoA IS A PHYSIOLOGICAL SUBSTRATE

SUMMARY

The carbon monoxide dehydrogenase complex from acetate-grown Methanosarcina thermophila was further studied by EPR spectroscopy. The as-purified enzyme exhibited no paramagnetic species at 113 K; however, enzyme reduced with CO exhibited a complex EPR spectrum comprised of two paramagnetic species with g-values of g_1 = 2.089, g_2 = 2.078 and g_3 = 2.030 (signal I) and g_1 = 2.057, g_2 = 2.049 and g_3 = 2.027 (signal II). Isotopic substitution with either ^{61}Ni, ^{57}Fe or ^{13}CO resulted in broadening of the EPR spectra indicating a Ni-Fe-C spin coupled complex. Pure signal II was obtained following treatment of the CO-reduced enzyme with acetyl-CoA but not by addition of acetyl-phosphate or CoASH. Acetate-grown cells were highly enriched in acetate kinase (EC 2.7.2.1) and CoASH-dependent phosphotransacetylase (EC 2.3.1.8) activities. These results suggest acetyl-CoA is a physiological substrate for the carbon monoxide dehydrogenase complex synthesized in acetate-grown cells of M. thermophila.

INTRODUCTION

The pathway of methanogenesis from acetate in Methanosarcina thermophila involves transfer of the methyl group to HS-CoM[1] followed by reductive demethylation of the CH_3-S-CoM to methane and HS-CoM (1,2). It is hypothesized that carbon-

carbon bond breakage may be catalyzed by an enzyme complex with CO dehydrogenase activity (3). It is further postulated that the carbonyl group of acetate may bind to nickel in the complex followed by oxidation to CO_2 to supply electrons for the demethylation of CH_3-S-CoM to methane (4). The proposed mechanism is similar to a reversal of acetyl-CoA synthesis from CoASH, a methylated corrinoid compound, and CO which is catalyzed by an enzyme with CO dehydrogenase activity from Clostridium thermoaceticum (5). The clostridial enzyme forms a unique paramagnetic Ni-Fe-C species when reduced with CO (6). The CO dehydrogenase from M. thermophila is a complex of five subunits and contains Ni, Fe, Co, Zn, and a corrinoid compound (3). The CO-reduced enzyme displays a nickel EPR signal nearly identical to that of the clostridial CO dehydrogenase which suggests a similar nickel environment for both enzymes.

Based on thermodynamic considerations, it is predicted that acetate requires activation before cleavage of the carbon-carbon bond (7). Acetyl-CoA is a likely substrate for the CO dehydrogenase complex in M. thermophila since it is synthesized by an enzyme with CO dehydrogenase activity in C. thermoaceticum (5). However, methanogens also contain Component B which is postulated to be a functional analog of CoASH based on partial structural similarity (8).

In this study, we demonstrate a paramagnetic Ni-Fe-C center in the CO-reduced M. thermophila CO dehydrogenase complex and show that the EPR signal is perturbed by acetyl-CoA. This result combined with the presence of high levels of acetate kinase and CoASH-dependent phosphotransacetylase in cells suggest acetyl-CoA is a physiological substrate for the enzyme.

MATERIALS AND METHODS

Organism and culture conditions. M. thermophila (9) was cultured on acetate (10) or methanol (11) as described. Growth on acetate in the presence of ^{61}Ni was as described (3). For growth on acetate in the presence of ^{57}Fe, trace elements were prepared without $FeSO_4 \cdot 7H_2O$ and ^{57}Fe was dissolved in concentrated HCl and added to a final concentration of 27 μM Fe.

Enzyme assays. Cell extracts were prepared anaerobically as described (2) except in a N_2 atmosphere. Acetate kinase (EC 2.7.2.1) and phosphotransacetylase (EC 2.3.1.8) were assayed aerobically at 37°C unless otherwise noted. Units were μmoles of product/min. Acetate kinase was assayed in the forward direction by coupling ADP formation to the oxidation of NADH (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) with pyruvate kinase and lactate dehydrogenase. The assay mixture (0.5 ml) contained (in final concentrations): 100 mM Tricine buffer, pH 8.2; 60 mM potassium acetate; 1.5 mM ATP; 2 mM glutathione; 2 mM $MgCl_2$; 2 mM phosphoenolpyruvate; 0.4 mM NADH; 1 unit pyruvate kinase; 5 units lactic dehydrogenase. Assays were initiated with 2.5 μl of enzyme solution or cell extract. Phosphotransacetylase was routinely assayed in the forward direction by following formation of the thiol ester of acetyl-CoA (ϵ_{233} = 5.55 mM^{-1} cm^{-1}, experimentally determined) as described (12). A second method, used where noted, employed DTNB which reacts with the sulfhydryl form (non-acetylated) of CoASH or Component B releasing the nitrobenzenethiol anion [ϵ_{412} = 13.6 mM^{-1} cm^{-1} (13)]. Anaerobic assay mixtures contained (in final concentrations): 2 mM acetyl-phosphate; 20 mM potassium phosphate buffer (pH 8); 0.4 mM CoASH or 0.4 mM reduced Component B. Component B, supplied as the disulfide, was reduced with 10-fold excess

sodium borohydride to the sulfhydryl form. The enzyme reaction was stopped by boiling 100 μl for 5 min following acidification with 5 μl 1N acetic acid. The sample was brought to pH 8 anaerobically with 8 μl 1N sodium hydroxide followed by addition of 290 μl 20 mM potassium phosphate buffer (pH 8) and 100 μl DTNB/EDTA solution (2 mM DTNB and 6 mM EDTA in 20 mM potassium phosphate buffer, pH 7).

Enzyme purifications. Partial purifications of acetate kinase and phosphotransacetylase were accomplished by FPLC using a model GP-250 gradient programmer (Pharmacia, Inc., Piscataway, NJ). Extract (113 mg protein) from acetate-grown cells was chromatographed using a Mono-Q HR 10/10 anion-exchange column (Pharmacia) previously equilibrated with buffer A (50mM TES buffer, pH 6.8, with 10% [v/v] ethylene glycol and 10 mM $MgCl_2$). Ethylene glycol and $MgCl_2$ was included to stabilize activity during purification and storage. Protein was eluted with a linear gradient (110 ml) from 0.0 to 0.4 M KCl. The preparations were stored in liquid N_2.

The CO dehydrogenase complex from acetate-grown cells was purified to electrophoretic homogeneity as described (3). The enzyme was further concentrated by adsorption and elution from a Mono Q column as described (3) except ammonium chloride replaced potassium chloride in the elution buffer. The concentrated enzyme solution eluted in 0.33 M ammonium chloride. Enzyme manipulations were performed under anaerobic conditions in a Coy Anaerobic Chamber (Coy Laboratory Products, Ann Arbor, MI) or as described (3). Exchange of CO for a N_2 atmosphere above the enzyme solutions was done by flushing the head space with N_2 at a flow rate of 50 ml/min for 5 min.

EPR Spectroscopy. EPR spectra were recorded using a Varian E109 Century Series spectrometer (Varian Associates, Palo Alto, CA) operating at 9 GHz and 100 kHz modulation and equipped with a variable temperature accessory. Spectra were routinely obtained within the temperature range 77-113 K using an incident microwave power of 20 mW and a modulation amplitude of 0.4 mT. Spectra were calibrated using DPPH-Mn^{2+} as a g-value standard (14). Double integrations of experimental spectra were carried out as described by Wyard (15) using CuEDTA as standard. Computer simulations of experimental spectra were calculated using the program described by Lowe (16). Microwave power saturation measurements of EPR spectra were recorded over a 1-200 mW range of microwave power. Data of EPR signal amplitude (S) as a function of incident microwave power (P) were analyzed as described by Barber et al. (17) using the semiempirical equation:

$$S = k\sqrt{P}/[1+P/P_{½}]^{0.5b} \qquad \text{(equation 1)}$$

where b, the "inhomogeneity parameter", varies from 1.0 for inhomogeneously broadened lines to 4.0 for the homogenous case. $P_{½}$ in equation 1 is the power at half-saturation and k is a constant. Saturation curves for species containing more than one relaxing component were constructed by adding together individual saturation curves obtained for each of the components in various proportions until the best fit was obtained.

Chemicals. Acetyl-CoA, CoASH, the lithium-potassium salt of acetyl-phosphate, sodium phosphoenolpyruvate, ATP, NADH, DTNB, sodium borohydride, pyruvate kinase

(rabbit muscle), and lactic dehydrogenase (rabbit muscle) were obtained from Sigma Chemical Co., St. Louis, MO. Chemically synthesized Component B (the disulfide form) (18) was kindly provided by K. Noll. All other chemicals were of reagent grade. ^{13}CO (99% isotopic purity) was obtained from Merck, Sharp and Dohme isotopes and ^{57}Fe (93% isotopic purity) was obtained from Oak Ridge National Laboratory, Oak Ridge, TN.

RESULTS

The EPR spectrum observed at 113 K of CO dehydrogenase complex, as purified under anaerobic conditions and reduced with CO, is shown in Fig. 1A. The spectrum exhibited three features with g-values of 2.074, 2.049 and 2.028, with no other resonances detected within the g-values range of 1.85 - 4.0. In the absence of CO, no paramagnetic species were detected within this range. All CO-reduced samples of the CO dehydrogenase complex exhibited this overall line shape although the signal amplitudes of the individual features varied among enzyme preparations suggesting this spectrum was a composite of multiple species.

Examination of the EPR spectrum of the CO-reduced CO dehydrogenase complex from cells cultured in a medium enriched in ^{57}Fe (I = 1/2) (Fig. 1B) showed significant broadening of the overall spectrum, although this was most pronounced for the g = 2.028 feature. Spectral broadening was also observed for the CO-reduced enzyme isolated from cells cultured in a medium enriched in ^{61}Ni (I = 3/2) (Fig. 1C). However, in this case, broadening was most pronounced in the g = 2.074 and 2.049 features. Incubations with ^{13}CO (^{13}C I = 1/2) as reductant (Fig. 1D) also

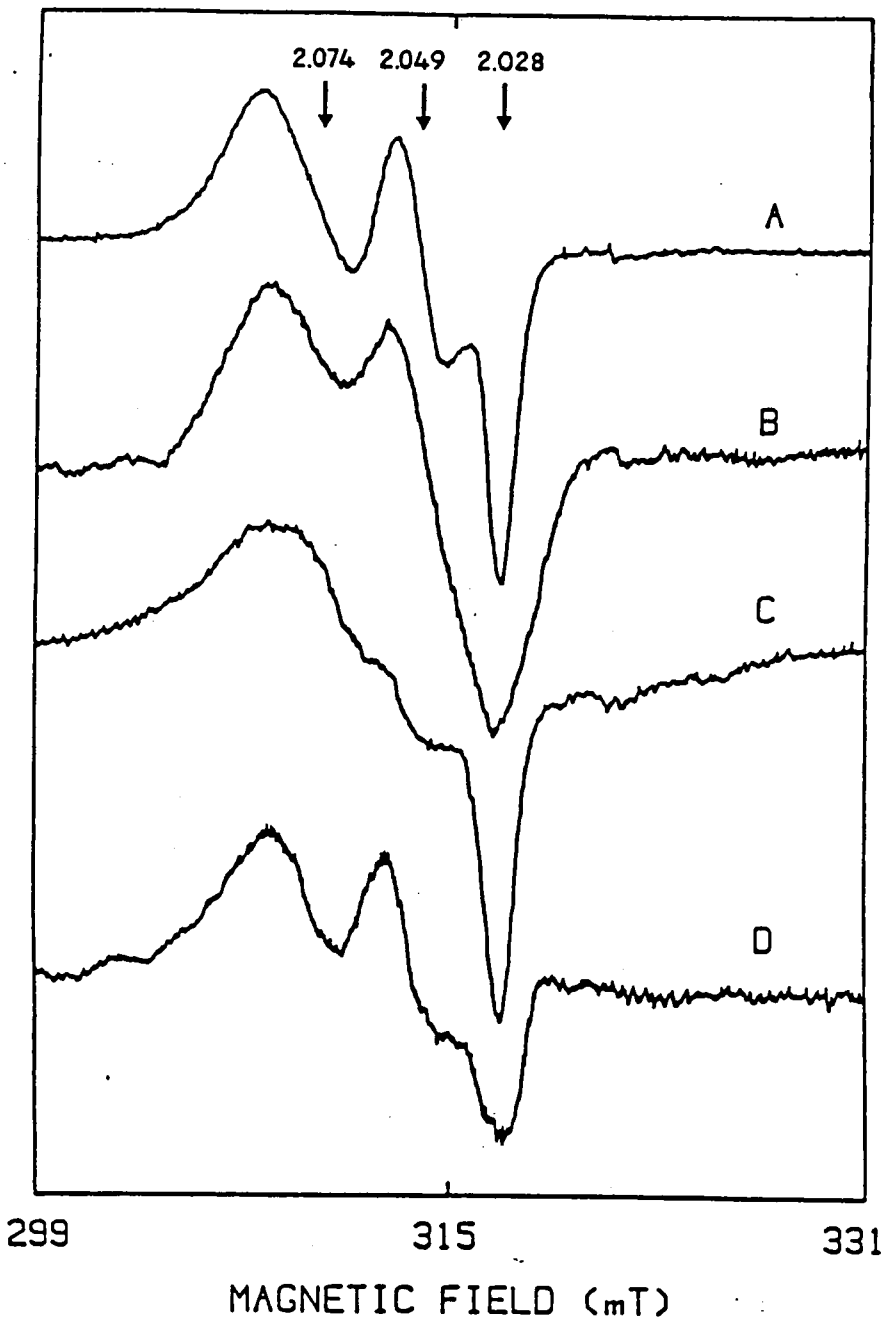

Figure 1. EPR spectra of the CO dehydrogenase complex from Methanosarcina thermophila. Enzyme preparations in 50 mM TES (pH 7.0), 10 mM $MgCl_2$, 0.33 M NH_4Cl and 10% (v/v) ethylene glycol. Spectra were recorded at 113 K and 20 mW power (except where indicated). The gain was 20,000. (A) enzyme at 5.2 mg/ml preincubated with ^{12}CO.
(B) ^{57}Fe enriched enzyme at 4.2 mg/ml preincubated with ^{12}CO. (C) ^{61}Ni enriched enzyme at 12.2 mg/ml preincubated with ^{12}CO. The power was 10 mW. (D) enzyme at 5.2 mg/ml preincubated with ^{13}CO. The field scale corresponds to a microwave frequency of 8.988 GHz. Arrows indicate g-values.

resulted in changes in the lineshape of the EPR signal although the degree of broadening was not as substantial as for ^{57}Fe and ^{61}Ni substitution. While individual hyperfine interactions were not resolved under these conditions, the results indicated Fe, Ni and C were present in these paramagnetic species, the carbon was derived from CO, and the signals arose following reduction of a Ni-Fe-C center, similar to that described forthe clostridial enzyme catalyzing acetyl-CoA synthesis.

The EPR signal of the as-purified, CO-reduced, enzyme was unchanged following replacement of the CO atmosphere with N_2. However, changes in the EPR signal were observed following addition of acetyl-CoA to the CO-reduced enzyme contained under N_2 (Fig. 2D). Treatment with acetyl-CoA resulted in disappearance of the feature observed at g = 2.074 (Fig. 2D), yielding a paramagnetic species of near axial symmetry which was simulated (Fig. 2E) using g_1 = 2.057, g_2 = 2.049 and g_3 = 2.027. We have designated the EPR signal observed in the presence of acetyl-CoA as signal II. Replacement of acetyl-CoA with equimolar concentrations of either acetyl-phosphate, CoASH or DTT failed to generate signal II. Addition of acetyl-CoA to the as-purified CO dehydrogenase complex under 1 atmosphere of CO had no effect on the EPR signal.

Computer subtraction of the EPR signal observed in the presence of acetyl-CoA (signal II) from that of the untreated CO-reduced enzyme yielded a second species of nearly axial symmetry that was simulated using g_1 = 2.089, g_2 = 2.078 and g_3 = 2.030 (Fig. 2B) and is referred to as signal I. Good agreement was obtained between the experimental spectrum for the CO-reduced untreated enzyme and the simulated spectrum obtained by adding 23% of signal I to signal II using the computer (Fig. 2C). These results indicated the signal from untreated enzyme was a composite of two

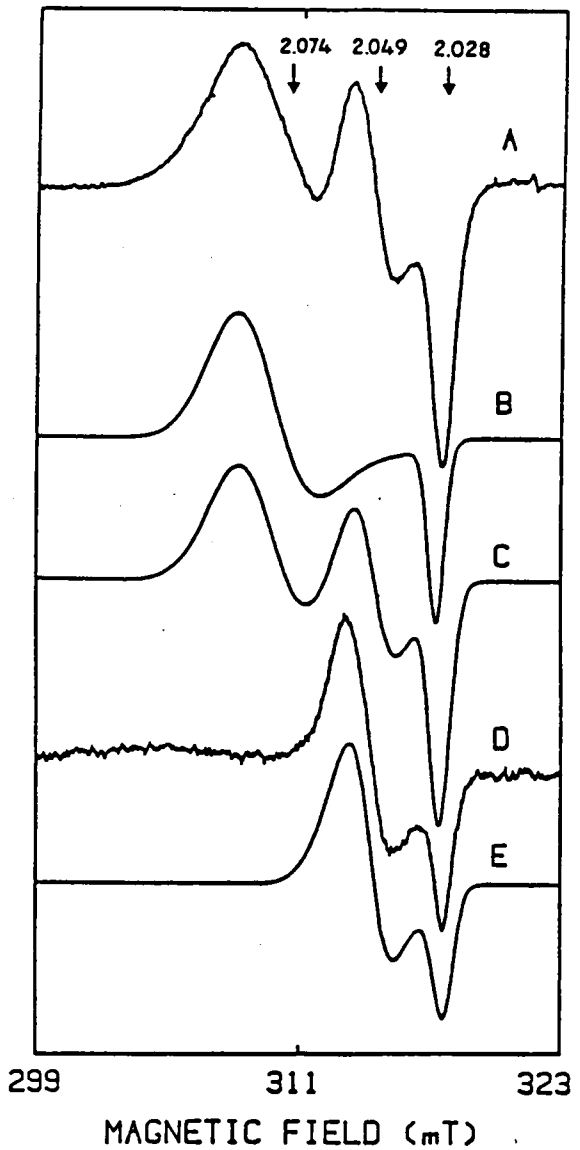

Figure 2. Experimental and simulated EPR spectra of the CO dehydrogenase complex from Methanosarcina thermophila. Enzyme preparations in 50 mM TES, pH 6.8, 10 mM $MgCl_2$, 0.33 M NH_4Cl and 10 % (v/v) ethylene glycol. Experimentally obtained spectra were measured at 113 K and 20 mW power. The gain was 20,000 except where indicated. (A) enzyme at 5.2 mg/ml incubated with ^{12}CO. (B) simulation of signal I using the following EPR parameters: g_1 = 2.089, g_2 = 2.078, g_3 = 2.03, w_1 = 1.5, w_2 = 1.5, w_3 = 0.45. (C) simulation of composite signal with EPR parameters obtained from E and B. (D) ^{12}CO-reduced enzyme at 2 mg/ml preincubated with 20 mM acetyl-CoA. The gain was 50,000. (E) simulation of spectrum in D using the following EPR parameters: g_1 = 2.057, g_2 = 2.049, g_3 = 2.027, w_1 = 1.18, w_2 = 0.73, w_3 = 0.45. Referred to as signal II. The field scale corresponds to a microwave frequency of 8.988 GHz. Arrows indicate g-values.

overlapping species.

Examination of the microwave power saturation characteristics of signals I and II suggested different saturation properties for the two species. The results of the saturation studies performed at 113 K are shown in Fig. 3. As the microwave power was increased, signal II saturated more readily with a $P_{½}$ of 90 mW while signal I was more resistant to saturation with a $P_{½}$ of 150 mW. The saturation behavior of the g_3 feature of the experimental spectrum, which is a composite of signals I and II, was fit well by a composite curve consisting of the two independently saturating components: 70% of the signal exhibiting a $P_{½}$ of 150 mW and 30% exhibiting a $P_{½}$ of 90 mW.

Cell extracts of acetate-grown M. thermophila contained high specific activities of acetate kinase and phosphotransacetylase suggesting involvement of these enzymes in a major catabolic pathway (Table 1). Furthermore, acetate kinase and phosphotransacetylase activities were 20- and 18-fold higher in acetate-grown cells when compared with methanol-grown cells (Table 1). When component B was substituted for coenzyme A as a substrate for the phosphotransacetylase, no activity was detected in extracts or with the partially purified enzyme using either assay method (data not shown). These results suggest that acetyl-CoA is the activated form of acetate involved in the pathway of acetate conversion to methane in M. thermophila.

DISCUSSION

The CO-reduced CO dehydrogenase complex, as purified from M. thermophila, showed paramagnetic properties which suggest a Ni-Fe-C spin coupled complex. The EPR properties were similar to the as-purified CO dehydrogenase from C.

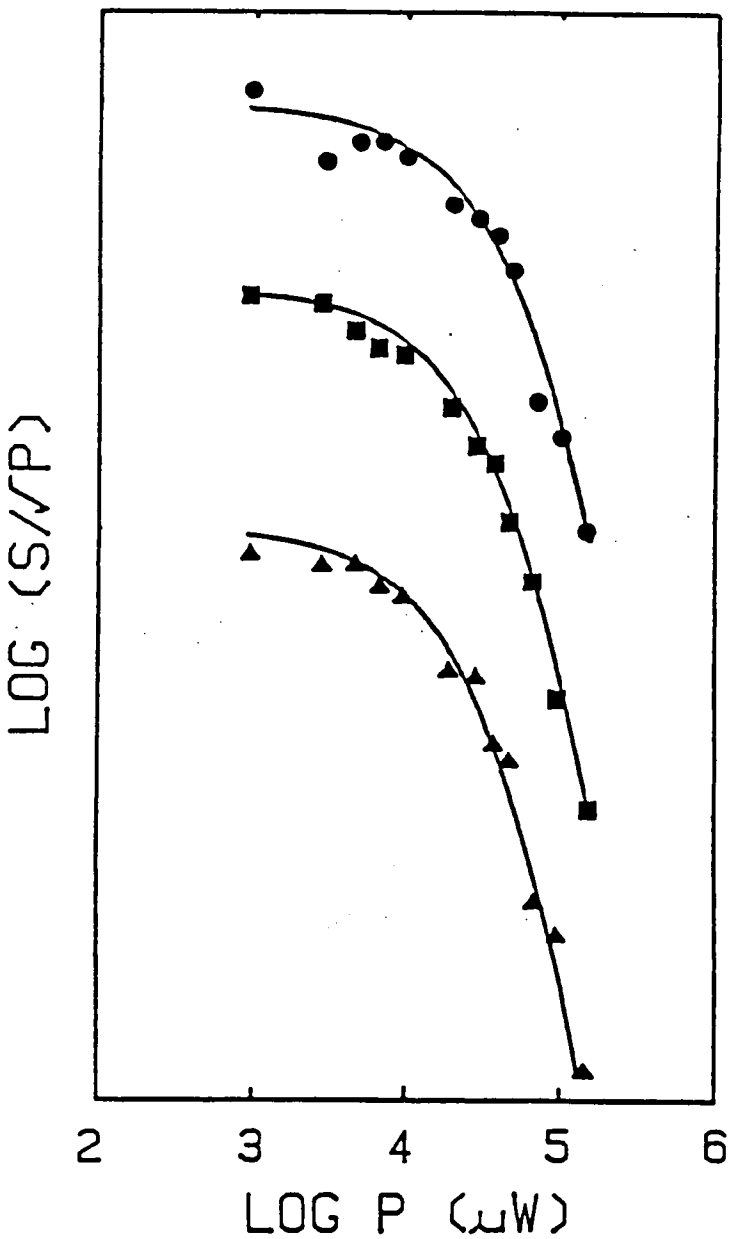

Figure 3. Microwave power saturation behavior of the Ni-Fe-C center in the CO-reduced CO dehydrogenase from Methanosarcina thermophila. Untreated CO-reduced enzyme, 5.2 mg/ml, in 50 mM TES buffer containing 10 mM $MgCl_2$, 0.33 M NH_4Cl and 10 % (v/v) ethylene glycol, pH 6.8. EPR spectra were recorded at 113 K using 0.4 mT modulation amplitude. Experimental points, corresponding to signal I (▲), signal II (●) were fit by equation 1. For the spectral feature comprising signal I and II (■), equation 1 was modified to allow for two relaxing components with parameters corresponding to 70% signal I and 30% signal II.

TABLE 1. Acetate kinase and phosphotransacetylase activities in acetate-grown or methanol-grown cells of Methanosarcina thermophila.

Growth substrate	Acetate kinase			Phosphotransacetylase		
	specific[a] activity	K_m(mM)[b]		specific[a] activity	K_m(mM)[b]	
		acetate	ATP		acetyl-phosphate	CoASH
acetate	2.42 ± 0.23	24	0.04	53.1 ± 14.2	0.65	0.18
methanol	0.12 ± 0.02	ND[c]	ND[c]	3.0 ± 0.5	ND[c]	ND[c]

[a] Expressed as µmoles product/min/mg cell extract protein. Values are the mean of three determinations, with standard error. Assays were initiated with 2.5 µl cell extract (10.9 - 13.8 mg protein/ml).

[b] Determined from double-reciprocal plots of initial velocity versus substrate concentrations using partially purified preparations of acetate kinase (50.2 units/mg) and phosphotransacetylase (451 units/mg). The range of substrate concentrations used were: acetate, 1-400 mM; ATP, 0.001-0.8 mM; acetyl-phosphate, 0.05-3.5 mM; and CoASH, 0.02-0.3 mM.

[c] Not determined

thermoaceticum (6) suggesting similar reaction centers in the two enzymes. The results also showed that,

like the clostridial enzyme, the EPR spectrum of the CO dehydrogenase complex from M. thermophila is a composite of two signals that were resolved by interaction of the enzyme with acetyl-CoA. However, differences between the clostridial and methanogenic enzymes are apparent. (i) In the presence of acetyl-CoA or CoASH the clostridial enzyme shows only one signal (signal 1) with g-values of 2.074, 2.074 and 2.028 (6). The second signal (signal 2), with g-values of 2.062, 2.047 and 2.028, is not experimentally observed separately from signal 1 and is only resolved by simulation (6). In contrast, treatment of the methanogen enzyme with acetyl-CoA resulted in one EPR signal (signal II) but with g-values similar to simulated signal 2 of the clostridial CO dehydrogenase; the other methanogen enzyme signal (signal I) was only obtained separately from signal II by simulation but was similar to the experimentally obtained pure signal 1 of the acetyl-CoA treated clostridial CO dehydrogenase. (ii) CoASH or acetyl-CoA influences the EPR signal of the clostridial enzyme; however, CoASH had no effect on the signal of the methanogen enzyme. (iii) Exchange of CO for a N_2 atmosphere above the CO-reduced methanogen enzyme solution was necessary for acetyl-CoA perturbation of the EPR signal; in contrast, acetyl-CoA influences the EPR signal of the clostridial enzyme under CO. These differences are unexplained but may reflect the ability of the enzymes to catalyze similar reactions albeit in opposite directions.

The influence of acetyl-CoA on the EPR signal of the M. thermophila CO dehydrogenase may result from direct interaction with the Ni-Fe-C center or by

allosteric changes induced by acetyl-CoA binding at an alternate site. Although the mechanism is unresolved, the results clearly show that acetyl-CoA binds to the methanogen enzyme and induces changes in the environment of the Ni-Fe-C center. This result and the presence of an acetate-inducible acetate kinase and CoASH-dependent phosphotransacetylase in cells suggest that acetyl-CoA is likely to be the activated form of acetate cleaved by the CO dehydrogenase complex from this organism. Acetate kinase and phosphotranscacetylase are also induced in acetate-grown cells of M. barkeri (19, 20).

The required absence of a CO atmosphere for binding of acetyl-CoA to the methanogen enzyme is unexplained. The extent to which CO was removed from the enzyme, or enzyme solution was not determined; thus, following addition of acetyl-CoA, it was unknown if the origin of carbon in the Ni-Fe-C center was other than from CO. Nonetheless, the results could explain why we were unsuccessful in attempts to demonstrate cleavage of the acetyl group of acetyl-CoA by exchange of the carbonyl carbon with atmospheric CO (unpublished results) as reported for the clostridial enzyme (5).

CO dehydrogenase is the third nickel-containing enzyme described among methanogenic bacteria following hydrogenase (21) and methyl-coenzyme M methylreductase (22, 23). Methanogen hydrogenases contain nickel paramagnetic resonance in certain oxidation states with characteristic g-values of 2.3, 2.23 and 2.02 (24). EXAFS studies indicate the nickel is liganded to 3 or 4 S atoms (25). Similar hydrogenases from other hydrogenotrophic and N_2-fixing organisms have been described. The methyl-coenzyme M methylreductase catalyzing the final reductive step

in methanogenesis, contains the nickel-tetrapyrrole, coenzyme F_{430}, which contains a paramagnetic Ni center with g-values greater than 2.1 (26). EXAFS reveals a Ni(II) center liganded to four pyrrole N atoms and one or two axial O or N atoms, but the actual oxidation state of nickel in the protein is believed to be Ni(I) or Ni(III) (27, 28).

The configuration of the Ni-Fe-C center of CO dehydrogenase in acetogenic clostridia and acetotrophic methanogens is unknown. It is unlikely that nickel in the M. thermophila CO dehydrogenase complex is present in coenzyme F_{430} since the enzyme does not exhibit strong absorption in the 430 nm region and g-values were different than previously observed for coenzyme F_{430} or coenzyme F_{430}-containing methyl-coenzyme M methylreductase (26). Recently, EXAFS studies indicate that nickel in the CO dehydrogenase from C. thermoaceticum is liganded to N or O and not S atoms (29). Similar studies are necessary with the methanogen enzyme to elucidate the nickel ligands.

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SECTION V. FERREDOXIN REQUIREMENT FOR ELECTRON TRANSPORT FROM THE CARBON MONOXIDE DEHYDROGENASE COMPLEX TO A MEMBRANE-BOUND HYDROGENASE IN ACETATE-GROWN Methanosarcina thermophila

SUMMARY

Cell extracts from acetate-grown Methanosarcina thermophila contained CO-oxidizing:H_2-evolving activity 16-fold greater than extracts from methanol-grown cells. Following fractionation of cell extracts into soluble and membrane components, CO-dependent H_2-evolution and CO-dependent methyl-coenzyme M methylreductase activities were only present in the soluble fraction but addition of the membrane fraction enhanced both activities. A b-type cytochrome(s), present in the membrane fraction, was linked to a membrane-bound hydrogenase. CO-oxidizing:H_2-evolving activity was reconstituted with: (i) CO dehydrogenase complex, (ii) a ferredoxin, and (iii) purified membranes with associated hydrogenase. The ferredoxin was a direct electron acceptor for the CO dehydrogenase complex. The ferredoxin also coupled CO oxidation by CO dehydrogenase complex to metronidazole reduction.

INTRODUCTION

The oxidation of CO is common among anaerobic bacteria able to metabolize one-carbon compounds. Several oxidize CO with protons as the electron acceptor (equation 1).

$CO + H_2O \text{ ---> } CO_2 + H_2 \quad \Delta G^{\circ\prime} = -20 \text{ kJ/mol}$ (equation 1)

Rhodopseudomonas gelatinosa will grow anaerobically in the dark with CO as sole carbon and energy source thus synthesizing ATP during oxidation of CO to CO_2 and H_2 (1). Whole cell suspensions of acetate-grown Methanosarcina barkeri synthesize ATP during conversion of CO to CO_2 and H_2 (2). These results imply that phosphorylation of ADP is coupled to electron transport catalyzed by a CO-oxidizing:H_2-evolving system in these organisms. A key enzyme involved in CO metabolism in anaerobes is CO dehydrogenase; determination of the physiological electron acceptors for CO dehydrogenases, and subsequent electron carriers, is fundamental to the study of energy yielding pathways in anaerobes metabolizing CO and other one-carbon compounds. A rubredoxin, flavodoxin and ferredoxins from Clostridium thermoaceticum are electron acceptors for the CO dehydrogenase catalyzing acetyl-CoA synthesis from a methylated corrinoid protein, CO, and CoASH (3).

Methanosarcina thermophila strain TM-1 is a thermophilic methane-producing archaebacterium capable of growth on acetate, methanol or methylamines. The pathway of methanogenesis from acetate in this organism involves transfer of the methyl group to coenzyme M (HS-CoM) followed by reductive demethylation of CH_3-S-CoM to methane and HS-CoM (4). It is proposed that, following activation of acetate to acetyl-CoA, carbon-carbon bond cleavage may be catalyzed by an enzyme complex with CO dehydrogenase activity; the carbonyl group of acetate may be bound as CO to a Ni-Fe center in the enzyme (5, 6). Oxidation of the proposed enzyme-bound CO would supply electrons for the demethylation of CH_3-S-CoM. The CO dehydrogenase

synthesized in acetate-grown M. thermophila is a complex of five subunits and contains Ni, Fe, Co, Zn and a corrinoid compound.

Acetate-grown M. thermophila contains CO-dependent methyl-coenzyme M methylreductase activity (7). The involvement of H_2 in electron transport from CO to CH_3-S-CoM has been postulated (7, 8). Here we show that the CO dehydrogenase complex reduces a ferredoxin which, together with membranes and associated hydrogenase, reconstitutes a CO-oxidizing:H_2-evolving system.

MATERIALS AND METHODS

Organism, culture conditions and preparation of cell extracts. M. thermophila strain TM-1 was cultured on acetate (9) or methanol (10) as described. Cell extracts were prepared anaerobically in a N_2 atmosphere as previously described (5) and stored in liquid N_2 until use.

Separation of membranes and soluble components. Sucrose density gradient centrifugation was used for fractionation of cell extracts. Buffer A consisted of 50 mM TES (pH 6.8) containing 10 mM $MgCl_2$ and 10% (v/v) ethylene glycol. Sucrose solutions with or without 1% (v/v) Triton X-100 were prepared anaerobically in buffer A and manipulations were performed in a Coy Anaerobic Chamber (Coy Laboratory Products, Ann Arbor, MI) containing N_2:H_2 (95:5). Sucrose solutions were layered in polycarbonate centrifuge tubes (16 x 78 mm) fitted with gas-tight screw caps. The gradient contained the following % (w/v) sucrose layers: 75%, 1.0 ml; 30%, 1.5 ml; 25%, 1.5 ml; and 20%, 3.5 ml. Cell extract (1.0 ml) with or without 1.5% (v/v) Triton X-100 was layered over the gradients and centrifuged in a Beckman 50 Ti fixed angle

rotor (90 min; 250,000 x g; 5°C). Fractions were collected anaerobically and stored in liquid N_2 until use.

Enzyme purifications. CO dehydrogenase complex was purified from extracts of acetate-grown cells as previously described (5) and pure enzyme was obtained in buffer A containing 0.33 M KCl.

Ferredoxin was purified from soluble proteins as described (11). The assay was by reconstitution of CO-oxidizing:H_2-evolving activity, and pure ferredoxin was obtained in buffer A containing 0.5 M KCl and was stored in liquid N_2 until use.

Enzyme assays. All assays were performed anaerobically using the general procedures as described (12). Buffer B was 50 mM MES (pH 6.0) containing 10 mM $MgCl_2$. All spectrophotometric assays were performed at 23°C using a Perkin-Elmer Lambda 4B spectrophotometer with the Cell Program/Scan/WPRG/Kinetics operating software package (Perkin-Elmer Corporation, Norwalk, CT).

(i) CO dehydrogenase. CO dehydrogenase activity was assayed as previously described (5) by monitoring the CO-dependent reduction of methyl viologen (ϵ_{603} = 11.3 mM^{-1} cm^{-1}). One unit of activity was the amount that reduced 1 μmol methyl viologen per min.

(ii) Hydrogenase. Hydrogenase assays were performed as previously described (13) with methyl viologen (ϵ_{603} = 11.3 mM^{-1} cm^{-1}) as the electron acceptor except the reaction mixtures contained: 50 mM Tris hydrochloride (pH 8.4), 20 mM methyl viologen, and 20 mM 2-mercaptoethanol. Assays were also performed with 40 μM coenzyme F_{420} (ϵ_{420} = 42.5 mM^{-1} cm^{-1}) substituted for methyl viologen.

(iii) CO-oxidizing:H_2-evolving system. Reaction mixtures (0.5 ml) contained: buffer B and cell extract (2.06 mg protein from acetate-grown cells, or 3.12 mg protein from methanol-grown cells) or purified components as indicated. The mixture was contained in butyl rubber stoppered 8.45 ml serum vials (Wheaton Scientific, Millville, NJ) with 1 atmosphere of N_2:CO (80:20), or 100% N_2. Reactions were initiated by placing the vials in an Orbit (Lab-line Instruments, Melrose Park, IL) reciprocating (120 rpm) shaker water bath at 57°C. H_2 was analyzed with a Varian 1400 thermal conductivity gas chromatograph equipped with a carbosieve B column (10 feet x 1/8 inch) operated at 45°C. N_2 was the carrier gas (40 ml/min). CO was separated from H_2 by 2 min and from CH_4 by 1.2 min.

(vi) Ferredoxin. Ferredoxin was assayed by complementation of CO-oxidizing:H_2-evolving activity. Typical reaction mixtures (0.5 ml) contained: buffer B, CO dehydrogenase complex (0.7 units), membranes (0.068 mg protein, 1.5 units hydrogenase), and ferredoxin as indicated. Ferredoxin was also assayed by coupling CO oxidation by the CO dehydrogenase complex to reduction of metronidazole which was followed by the decrease in A_{320} (ϵ_{320} = 9300 M^{-1} cm^{-1} (14)). One unit of activity was the amount that reduced 1 nmol of metronidazole per min.

(v) Methyl-coenzyme M methylreductase. Reaction mixtures (0.5 ml) contained: buffer B, 5 mM CH_3-S-CoM, 2 mM ATP, and 0.1 ml boiled cell extract. The assays were performed in butyl rubber stoppered 8.45 ml serum vials that contained 1 atmosphere of N_2 or CO. Reactions were initiated by placing the vials in an Orbit reciprocating (120 rpm) shaker water bath at 57°C. Methane was analyzed as previously described (7).

Analytical procedures. Absorption spectra were obtained at 23°C using a Perkin-Elmer Lambda 4B spectrophotometer and the Spectral Processing operating software package.

Protein concentrations of ferredoxin preparations were determined with BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) by the micro method of Pierce as described (11). All other protein concentrations were determined by the method of Bradford (15) using Bio-Rad protein dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as the standard.

Chemicals. Bovine serum albumin (fraction V), metronidazole, ATP (type II from Yeast), methyl viologen, Triton X-100, MES, TES, and 2-mercaptoethanol (type I) were obtained from Sigma Chemical Co., St. Louis, MO. Sucrose (reagent grade) was obtained from Fisher Scientific Co., Pittsburgh, PA. Coenzyme F_{420}, purified from Methanobacterium formicicum (16), was a gift from S. Baron. CH_3-S-CoM (a gift from M. Nelson) was synthesized as previously described (16) and isolated as the ammonium salt.

RESULTS

Fractionation of cell extract. Discontinuous sucrose density gradient ultracentrifugation was used to separate membranes from soluble proteins in extracts of acetate-grown cells (Fig. 1A). After centrifugation, the membranes were located in a translucent band (fractions 3 and 4) directly above the 70% sucrose layer. Whole cells and cell debris formed a pellet well separated from the membranes. Soluble proteins were concentrated in fractions 14 - 18. Hydrogenase activity was present in both

membrane and soluble fractions. In the presence of Triton X-100, membrane proteins and associated hydrogenase activity were redistributed to the soluble fractions which indicated a membrane-bound hydrogenase (Fig. 1B). One band of hydrogenase activity was visible after gel electrophoresis of membrane proteins (data not shown). Hydrogenase assays of the membrane fractions showed this enzyme reduced both methyl viologen (35 units/mg protein) and coenzyme F_{420} (2.4 units/mg protein). The reduced minus oxidized difference spectrum of intact membranes (Fig. 2) showed maxima at 559, 529 and 428 nm characteristic of b-type cytochromes. Identical results were obtained when a H_2 atmosphere replaced dithionite as reductant showing that hydrogenase was linked to reduction of b-type cytochromes (data not shown).

CO dehydrogenase activity was distributed throughout the gradient (Fig. 1A) with little activity in membranes (1.6 units/mg protein) which suggested this enzyme was not tightly bound to the membranes after isolation. Native gel electrophoresis showed that CO dehydrogenase located in fractions 6 - 11 was greater than M_r 10^6 suggesting an aggregated form of the CO dehydrogenase enzyme complex previously reported (5). In the presence of Triton X-100, some CO dehydrogenase complex redistributed to the soluble fractions but most of the enzyme remained aggregated in fractions 6 - 11 (Fig. 1B).

Extracts of acetate-grown cells contained CO-oxidizing:H_2-evolving activity (61 ± 10 nmoles H_2/min/mg protein) 16-fold greater than extracts from methanol-grown cells (3.7 ± 0.8 nmoles H_2/min/mg protein). The CO-oxidizing:H_2-evolving system of acetate-grown cells was partially resolved by separation of membrane from soluble fractions (Table 1). Only the soluble fractions catalyzed CO-dependent H_2 evolution;

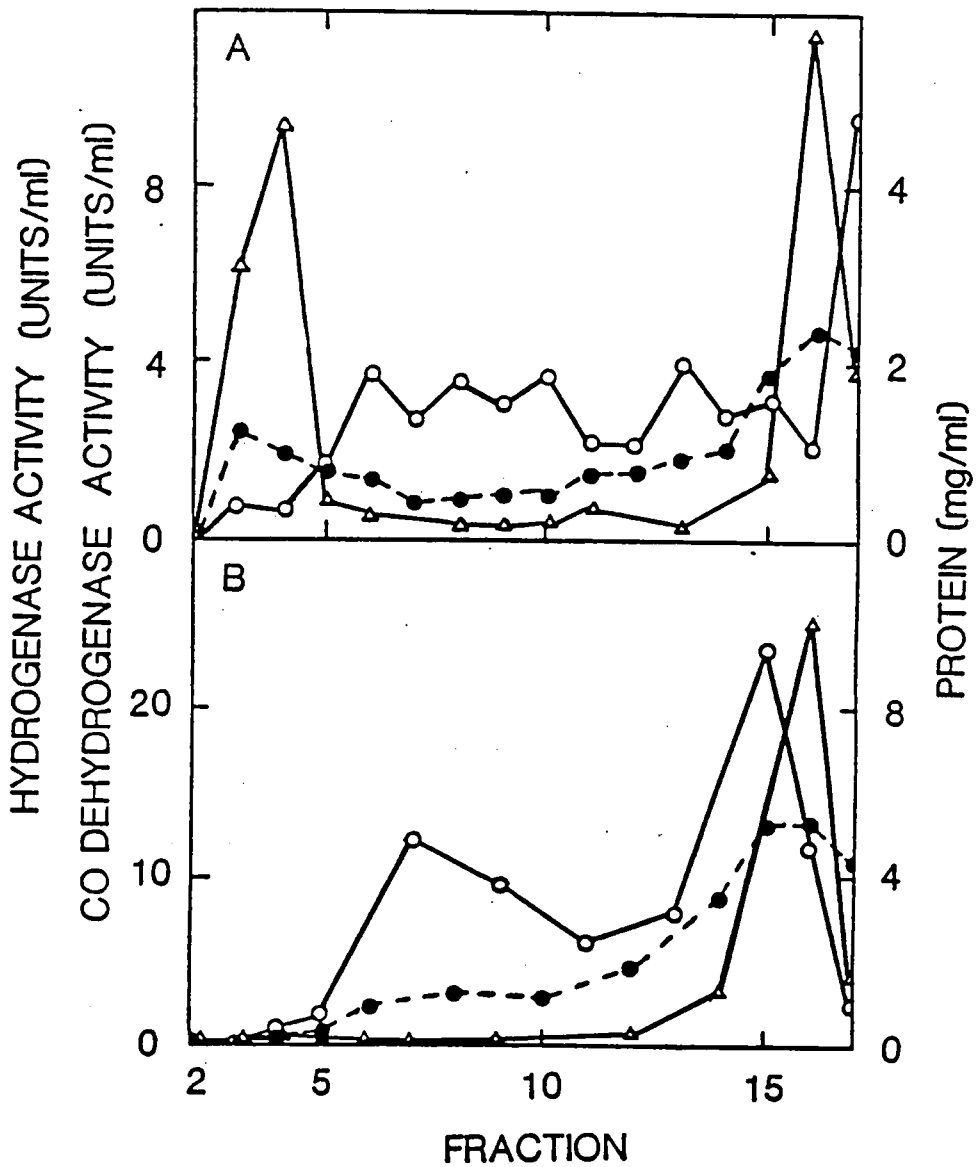

Figure 1. Fractionation of cell extracts by sucrose density gradient centrifugation. (A) Distribution of cell extract (15 mg protein) components following centrifugation without Triton X-100. (B) Distribution of cell extract (25 mg) components following centrifugation with Triton X-100. Fractions (0.5 ml) were numbered from the bottom of the tubes. Symbols: o, CO dehydrogenase activity; △, hydrogenase assayed with methyl viologen; •, protein.

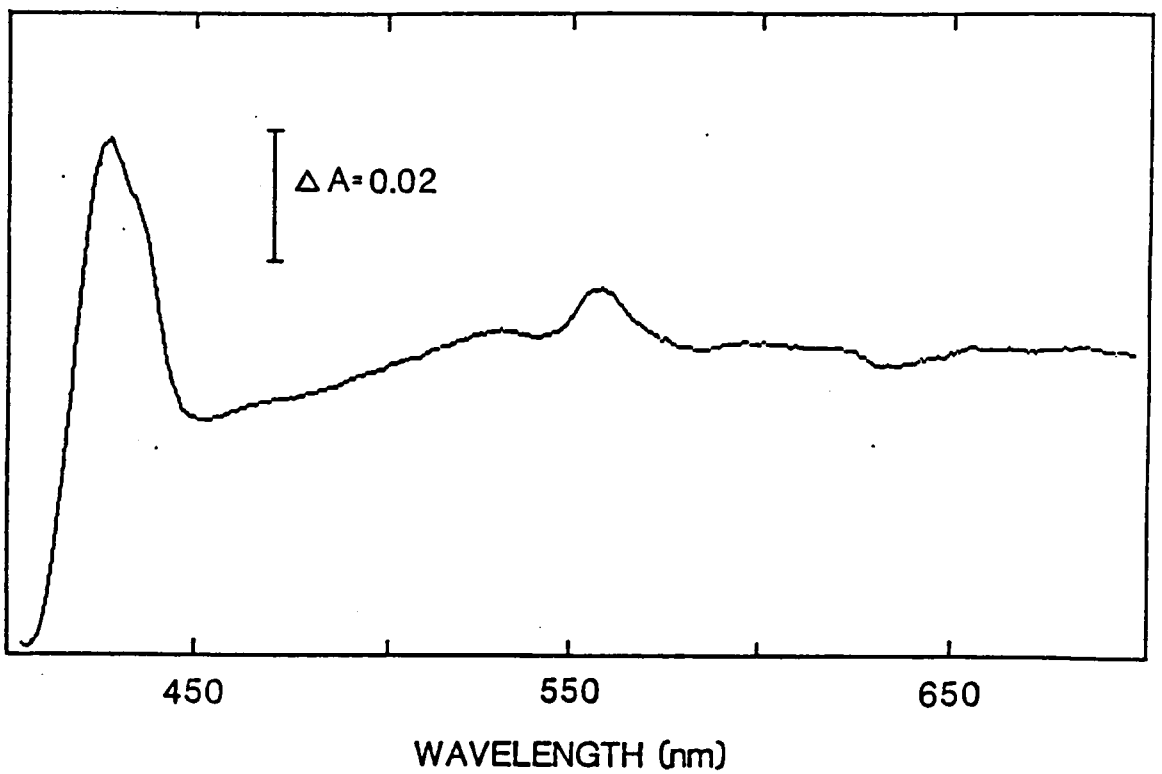

Figure 2. Reduced minus oxidized difference spectrum of the membrane fraction from acetate-grown cells. The membrane suspension (0.18 mg protein/ml), as described in Figure 1, was reduced with a few grains of sodium dithionite.

however, addition of membranes enhanced the rate. The CO-dependent methyl-coenzyme M methylreductase activity in membrane or soluble fractions was also enhanced by recombination suggesting both fractions were required for maximum activity (Table 1).

Reconstitution of a CO-oxidizing:H_2-evolving system. CO-oxidizing:H_2-evolving activity from acetate-grown cells was reconstituted (Table 2) with: (i) pure CO dehydrogenase complex, (ii) a ferredoxin purified from the soluble components (see below), and (iii) isolated membranes with associated b-type cytochromes and a hydrogenase. Figure 3 shows the dependence of the rate of H_2 evolutionon the concentrations of ferredoxin and CO dehydrogenase complex. The addition of 16 μM coenzyme F_{420} (final concentration) did not substitute for ferredoxin or enhance the rate of H_2 evolution in the presence of ferredoxin (data not shown). Although no hydrogenase activity was detected in the CO dehydrogenase complex or ferredoxin preparations, a low rate of CO-dependent H_2 evolution was catalyzed by combination of these two components (Table 2). This result may be attributed to the fortuitous reduction of protons by the CO dehydrogenase complex as reported previously for the CO dehydrogenase from M. barkeri in the presence of a clostridial ferredoxin (18).

Reduction of ferredoxin by the CO dehydrogenase complex. The purification, from soluble proteins of acetate-grown M. thermophila, was followed by complementation of CO-oxidizing:H_2-evolving activity as described (11). No other fractions during the purification stimulated CO-oxidizing:H_2-evolving activity. The ferredoxin was judged greater than 95% pure by spectral characteristics, and amino terminal sequence analysis

Table 1. Enzyme activities in membrane and soluble fractions of cell extracts from acetate-grown Methanosarcina thermophila.

Fraction	CO-dependent[a] H_2-evolution	CO-dependent[b] methylreductase
	(nmoles H_2)[c]	(nmoles CH_4)[c]
Membrane	ND[d]	2
Soluble	385	20
Membrane plus Soluble	775	165

[a] Assay mixtures contained 0.065 mg membrane protein and/or 0.43 mg soluble protein. See Materials and Methods.

[b] Assay mixtures contained 0.065 mg membrane protein and 0.21 mg soluble protein. See Materials and Methods.

[c] Amount of H_2 or CH_4 produced from the assay mixtures after 1 hour of incubation at 57°C.

[d] ND, none detected. Detection limit < 20 nmoles H_2.

Table 2. Reconstitution of CO-oxidizing:H_2-evolving activity with components purified from acetate-grown cells of Methanosarcina thermophila.

Components	H_2 evolution (nmoles/min)
Complete[a]	7.54
minus CO[b]	0[c]
minus ferredoxin	0
minus membranes	0.17
minus CO dehydrogenase complex	0

[a] Ferredoxin, 0.03 mg; membranes, 0.06 mg protein (2.3 units hydrogenase); CO dehydrogenase complex, 0.41 mg (26 units); N_2:CO (80:20) atmosphere. See Materials and Methods.

[b] N_2:CO (80:20) atmosphere replaced with 100% N_2.

[c] detection limit < 20 nmoles H_2

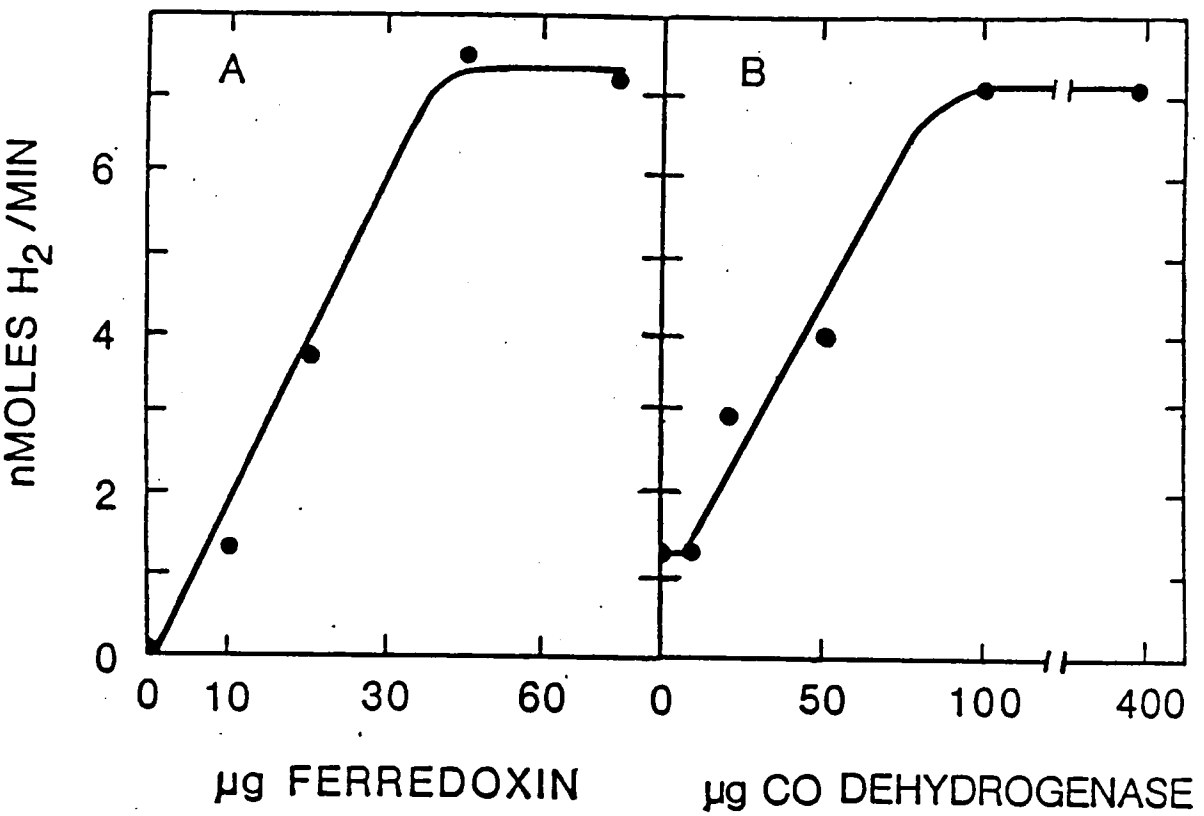

Figure 3. Dependence of CO-oxidizing:H_2-evolving activity on ferredoxin and CO dehydrogenase complex. The assay mixtures (0.5 ml) contained: buffer B, and 0.05 ml membrane suspension (0.065 mg protein, 2.27 units hydrogenase) as described in Figure 1. A. 0.15 mg CO dehydrogenase complex (60 units/mg) and the indicated amounts of ferredoxin were added to the standard reaction mixture. B. 0.06 mg ferredoxin and the indicated amounts of CO dehydrogenase complex (60 units/mg) were added to the standard reaction mixture.

of the first 39 residues (11).

The absorption spectrum of oxidized ferredoxin (Fig. 4A) showed two broad peaks centered at 295 nm and 395 nm. The spectrum was the same under N_2 or CO (data not shown). Addition of catalytic amounts of CO dehydrogenase complex to oxidized ferredoxin, under CO, resulted in reduction as evidenced by decreased absorbance in the 295 and 395 nm regions (Fig. 4B). These results show that the ferredoxin was a direct electron acceptor for the CO dehydrogenase complex.

Metronidazole is an artificial electron acceptor chemically reduced by ferredoxins (12). CO dehydrogenase complex alone did not reduce metronidazole; however, the ferredoxin from M. thermophila coupled CO oxidation by CO dehydrogenase complex to the reduction of metronidazole (Fig. 5) which provided a quantitative (Fig. 6) and convenient assay.

DISCUSSION

Here we report the reconstitution of a CO-oxidizing:H_2-evolving system from components of acetate-grown M. thermophila. The results show that a ferredoxin was required to couple the CO dehydrogenase complex to a membrane-bound hydrogenase which also established the direct electron acceptor for the CO dehydrogenase complex from this organism. The CO dehydrogenase complex contains a corrinoid protein (5). The involvement of this corrinoid protein in the mediation of electron transport to the ferredoxin was not investigated. The results showed a preponderance of b-type cytochromes in membranes of acetate-grown cells, though smaller amounts of c-type cytochromes could have been masked in the room temperature difference spectra. Previous results show two b-type cytochromes and a c-type cytochrome in membranes of

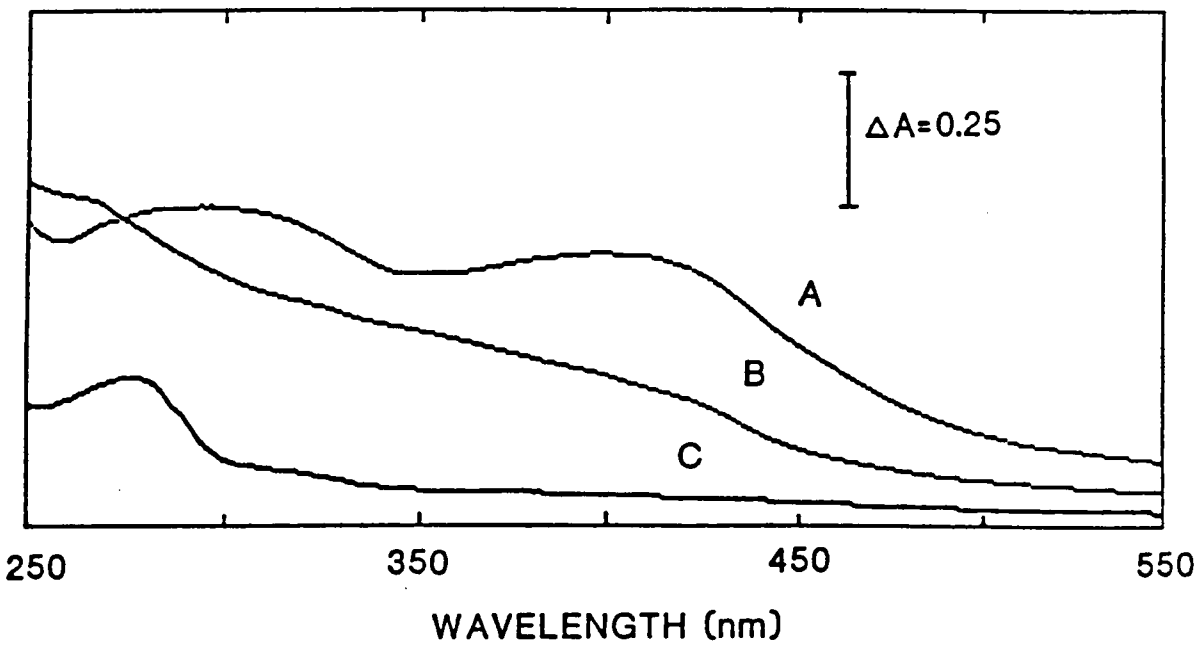

Figure 4. Absorption spectra of oxidized and reduced ferredoxin at 23°C. Sealed cuvettes contained a CO atmosphere. (A) ferredoxin (0.19 mg/ml) in buffer A, pH 6.8. (B) Same as in A except incubated for 5 min at 23°C after addition of 0.06 mg CO dehydrogenase complex (60 units/mg) in buffer A, pH 6.8 and subtraction of CO dehydrogenase complex absorbance (spectrum C). (C) 0.06 mg CO dehydrogenase complex in 0.55 ml buffer A, pH 6.8.

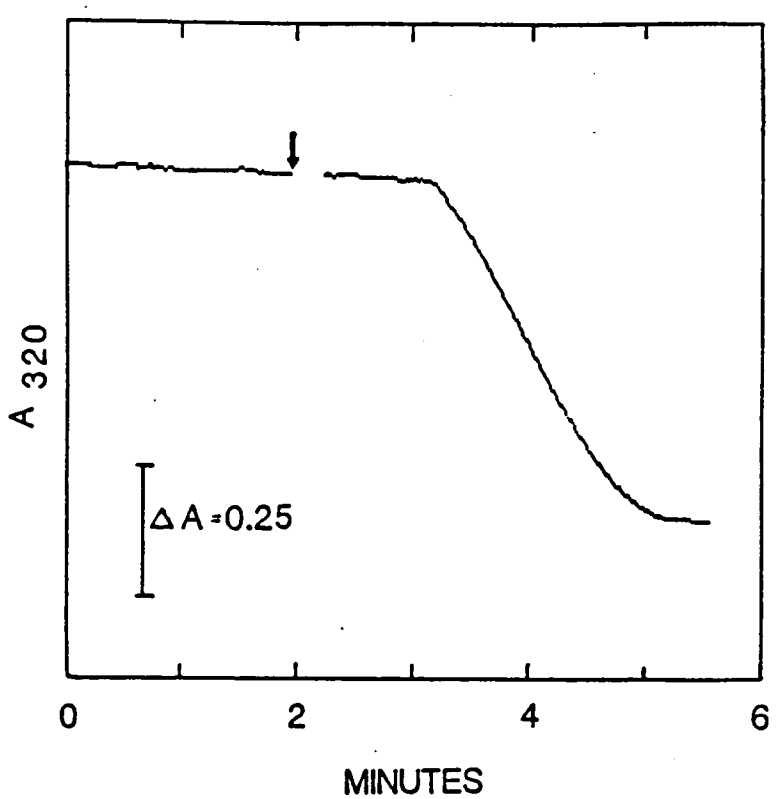

Figure 5. Reduction of metronidazole by ferredoxin and CO dehydrogenase complex. The reaction mixture (0.72 ml) contained: buffer A, 0.1 mM metronidazole, 0.05 mg CO dehydrogenase complex (60 units/mg). The reaction, contained in a sealed cuvette with a CO atmosphere, was initiated with 0.06 mg of ferredoxin (arrow).

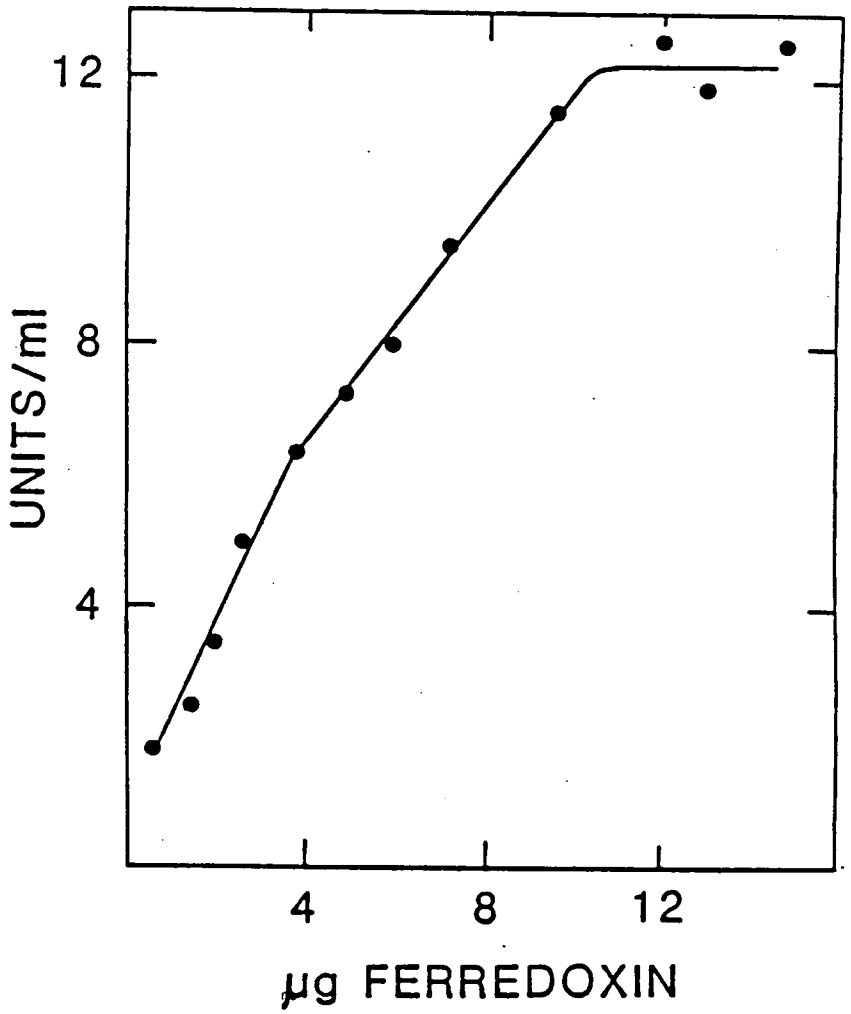

Figure 6. Dependence of the rate of metronidazole reduction on the concentration of ferredoxin. The reaction mixtures were as in Figure 5 except the amounts of ferredoxin were as indicated. Baseline activity before the addition of ferredoxin was not subtracted.

methanol-grown M. thermophila, and greater amounts of b-type cytochromes in membranes of acetate- compared to methanol-grown Methanosarcina barkeri (22). The membrane-bound hydrogenase of M. thermophila was linked to b-type cytochromes; however, we did not determine whether this electron carrier was also linked to the CO dehydrogenase complex. The hydrogenase was linked to F_{420} but this electron carrier was not required for CO-oxidizing:H_2-evolving activity; however, the results did not exclude that F_{420} was unspecifically bound to protein. Further research is necessary to define membrane-bound electron carriers and determine involvement in the CO-oxidizing:H_2-evolving system.

The location of the CO dehydrogenase complex and ferredoxin in M. thermophila requires further investigation. Disruption of cells by French pressure cell lysis may have solubilized the enzyme complex if it were peripherally associated with membranes of the intact cell. The CO dehydrogenase in Rhodopseudomonas gelatinosa is membrane-associated when cells are broken by osmotic lysis; however, French pressure cell disruption solubilized the enzyme (23).

The greater CO-oxidizing:H_2-evolving activity in acetate-grown compared to methanol-grown cells suggests a possible function in the pathway of acetate conversion to methane. It is interesting to postulate that ATP synthesis in acetate-grown cells is coupled to oxidation of enzyme-bound CO to CO_2 and H_2 by a membrane-associated CO-oxidizing:H_2-evolving system. The cytoplasmic reoxidation of H_2 by reduction of CH_3-S-CoM to methane theoretically could lower the partial pressure of H_2 and maintain a negative free-energy change in the oxidation of enzyme-bound CO. Several lines of evidence are consistent with this hypothesis: (i) acetate cleavage is located in

the particulate fraction of M. barkeri while the methyl-coenzyme M methylreductase is cytoplasmic (24), (ii) the phosphorylation of ADP in whole cells of acetate-grown M. barkeri is coupled to the oxidation of CO to CO_2 and H_2 by a chemiosmotic mechanism (2) which implies involvement of a membrane-bound electron transport chain, (iii) H_2 is produced and consumed during growth of M. thermophila on acetate (25), (iv) cell extracts of M. thermophila catalyze a highly active H_2-supported reduction of CH_3-S-CoM to methane (7). The membrane requirement for both CO-dependent H_2-evolution and CO-dependent methyl-coenzyme M methylreductase activity, reported here, is also consistent with this idea; however, the involvement of membranes in electron transport from CO dehydrogenase complex to the methylreductase can also be envisioned without the participation of H_2. The function of the CO-oxidizing:H_2-evolving system in the pathway of acetate conversion to methane, if any, is unknown. Other possible functions for the CO-oxidizing:H_2-evolving system include a mechanism for disposal of excess reducing potential generated from the oxidation of the methyl group of acetate to CO_2 for reductive biosynthesis. The reconstitution of a CO-oxidizing:H_2-evolving system with defined components should provide a basis for further studies on electron transport and the exact function of CO-oxidizing:H_2-evolving activity during growth of acetotrophic methanogens.

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SECTION VI. PURIFICATION AND CHARACTERIZATION OF A FERREDOXIN FROM ACETATE-GROWN Methanosarcina thermophila

SUMMARY

A ferredoxin, which functions as an electron acceptor for the CO dehydrogenase complex, was purified from acetate-grown Methanosarcina thermophila. It was isolated as a trimer having a native molecular weight of 16,000 and monomer molecular weight of 5,862. It contained 2.80 ± 0.56 Fe atoms and 1.98 ± 0.52 acid-labile sulfide. UV-visible absorption maxima were 395 nm and 295 nm with extinction coefficients of ϵ_{395} = 14,500 M^{-1} cm^{-1} and ϵ_{295} = 17,000 M^{-1} cm^{-1}. The A_{395}/A_{295} ratio was 0.88. The protien monomer contained 5 cysteines but no methionine, histidine, arginine and phenylalanine were detected. The protein was stable for at least 30 min at 70°C, but denatured during incubation at 85°C.

INTRODUCTION

Methanosarcina thermophila is an acetotrophic methane-producing archaebacterium. During the dissimilation of acetate to methane, we hypothesize that a multi-enzyme complex with CO dehydrogenase activity catalyzes carbon-carbon bond cleavage of acetyl-CoA (1, 2). We have proposed that the carbonyl group of acetyl-CoA is bound as CO at a Ni-Fe center in the enzyme complex (2). The reconstitution of a CO-oxidizing:H_2-evolving system in Methanosarcina thermophila is dependent on a ferredoxin which is also a physiological electron acceptor for the CO dehydrogenase complex in this organism (3). In this report we describe the purification and properties of this ferredoxin.

MATERIALS AND METHODS

Organism and culture conditions. Methanosarcina thermophila strain TM-1 (4) was cultured on acetate in a 10-liter pH auxostat as described previously (5).

Preparation of cell extract and separation of membrane and soluble components. Cell extracts were prepared anaerobically under a N_2 atmosphere as previously described (1). Breakage buffer consisted of 50 mM potassium TES (pH 7.0) containing 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 5% (vol/vol) glycerol, and 0.015 mg/ml of DNase I (Sigma, St. Louis, MO). Membranes and soluble components were separated by sucrose density gradient centrifugation as described (3). Fractions were collected anaerobically and stored in liquid N_2 until use.

Enzyme purifications. Buffer A contained 50 mM TES (pH 6.8), 10 mM $MgCl_2$ and 10% (v/v) ethylene glycol. The CO dehydrogenase complex was purified from extracts of acetate-grown cells as previously described (1) and pure enzyme was obtained in buffer A containing 0.33 M KCl. The purification of ferredoxin was as follows: All steps were performed at 23°C in the Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) . Sucrose gradient supernatants were filtered by Amicon pressure dialysis (Amicon Corp., Lexington, MA) with a YM-30 membrane, and the retentate was washed with 3 volumes of buffer A. (Although the native molecular weight of the ferredoxin is less than 30,000, the ferredoxin was not filtered through the membrane). The retentate was then fractionated by Fast-Protein-Liquid-Chromatography (FPLC) using a model GP-250 gradient programmer (Pharmacia, Piscataway, N.J.) and a Mono-Q anion exchange column (Pharmacia). The column was developed with a linear gradient of 0.0 to 1.0 M KCl in buffer A. Ferredoxin was the last major protein to

elute from the column; active fractions were pooled, diluted with buffer A, and further purified by batch elution from the Mono-Q column with 0.49 M KCl. As a final purification step, Mono-Q fractions containing ferredoxin were chromatographed using a Superose-12 (Pharmacia) gel filtration column (MW range = 1,000 - 300,000). Ferredoxin preparations with an A_{395}/A_{295} greater than 0.8 were considered to be pure. Pure ferredoxin was stored in liquid N_2 until use.

Enzyme assays: All assays were performed anaerobically using the general anaerobic procedures as described previously (6) at 23°C using a Perkin-Elmer Lambda 4B spectrophotometer with the Cell Program/Scan/WPRG/Kinetics operating software package (Perkin-Elmer Corporation, Norwalk, CT).

(i) CO dehydrogenase. CO dehydrogenase complex was assayed as previously described (1) by monitoring the CO-dependent reduction of methyl viologen (ϵ_{603} = 11.3 mM^{-1} cm^{-1}). One unit of activity was defined as the amount of enzyme that reduced methyl viologen at the rate of 1 umol per min.

(ii) Ferredoxin. Ferredoxin was assayed by complementation of CO-oxidizing:H_2-evolving activity as described (3). Typical reaction mixtures (0.5 ml) contained: 50 mM MES buffer (pH 6.0), 10 mM $MgCl_2$, CO dehydrogenase complex (0.7 units), membranes (0.068 mg protein, 1.5 units hydrogenase), and ferredoxin as indicated.

Spectroscopy. UV-visible absorption spectra were obtained at 23°C using a Perkin Elmer Lambda 4B spectrophotometer and the Spectral Processing operating software package. The thermal stability of ferredoxin was monitored by following the decrease in absorbance at 400 nm during incubation at 70°C or 85°C (7).

Amino Acid composition and N-terminal amino acid analysis.

Amino acid analysis was performed at AAA Laboratory (Mercer Island Washington). Ferredoxin samples were oxidized with performic acid for 4 hrs at 4°C by the method of Moore (8) and hydrolyzed in vacuo with 6N HCl at 115°C for 24, 48 and 72 h. Amino acids were analyzed using Dionex Analyzers (Dionex Corp., Sunnyvale, CA), Models D-500 and D-502.

N-terminal amino acids were sequenced at the U.Va. sequencing laboratory using an Applied Biosystems (Applied Biosystems, Inc., Foster City, CA) 470 A gas phase peptide sequencer and identifying the phenylthiohydantoin derivative (9) with an on-line Applied Biosystems liquid chromatograph.

Analytical procedures. The native molecular weight was estimated using a Sephadex G-50 (Sigma Chemical Co., St. Louis, MO) gel filtration column (1 cm x 17 cm) equilibrated with buffer A containing 0.15 M KCl. Molecular weight standards were chymotrypsinogen, 25,000 (Pharmacia); ribonuclease A, 13,700 (Pharmacia); myoglobin, 17,800 (Serva Feinbiochemica, Heidelberg, West Germany); horse heart cytochrome, 12,300 (Serva Feinbiochemica); Clostridium pasteurianum ferredoxin, 6,000 (Sigma).

Inorganic sulfide was determined by the micro method of Beinert (10) in 0.5 ml culture tubes fitted with serum stoppers (7 x 15 mm). Iron was determined by atomic absorbtion spectroscopy with a Perkin-Elmer Atomic Absorption Spectrophotometer Model 560. A standard curve was calibrated using dilutions of a certified atomic absorption iron standard (Fisher Scientific Co., Pittsburgh, PA). Ferredoxin solutions were maintained anaerobically until analyzed.

Protein concentrations of ferredoxin preparations were determined by the total weight of amino acids released during hydrolysis and compared to the values obtained by the micro method of Pierce (Pierce Chemical Co., Rockford, IL) using BCA protein assay reagent. A correction factor which included differences in the average residue molecular weight between ferredoxin and bovine serum albumin was employed in subsequent protein determinations by the Pierce method. All other protein concentrations were determined by the method of Bradford (11) using Bio-Rad protein dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as standard.

Chemicals. Bovine serum albumin (fraction V), methylviologen, MES, 2-mercaptoethanol (type I), TES, and DMPD were obtained from Sigma Chemical Co., St. Louis, MO. Sucrose (reagent grade) was obtained from Fisher Scientific Co., Pittsburgh, PA.

RESULTS

Physical and chemical properties. Ferredoxin was purified anaerobically from soluble proteins of acetate-grown M. thermophila. The N-terminal amino acid sequence revealed only one polypeptide chain and polyacrylamide gel electrophoresis showed no contaminating proteins. The native molecular weight, estimated by Sephadex G-50 gel filtration chromatography, was 16,000, and the minimum molecular weight, calculated from the amino acid composition (Table 1), was approximately 5,900, suggesting that the ferredoxin was isolated as a trimer. Analysis by atomic absorption spectroscopy showed 2.80 ± 0.46 (SD) (n = 7) Fe/mol of monomer. The acid-labile sulfide content

Table 1. Amino Acid Composition of M. thermophila ferredoxin

	Residues per monomer		
Amino acid	M. thermophila	M. barkeri (DSM 800)	M. barkeri (DSM 804)
alanine	6	7	7
arginine	0	0	0
aspartic acid	6	10	9
cystine/2	5	8	8
glutamic acid	9	11	10
glycine	5	4	5
histidine	0	0	0
isoleucine	2	3	4
leucine	2	1	2
lysine	2	2	2
methionine	0	0	0
phenylalanine	0	0	0
proline	2	3	3
serine	4	1	2
threonine	1	3	2
tryptophan	ND	ND	ND
tyrosine	0	0	0
valine	4	6	6
Total	48	59	60
minimum molecular weight	5,900	6,000	6,100

was 1.98 ± 0.52 (SD) (n = 3) S/mol of monomer. Estimations of acid-labile sulfide were consistently lower than those for iron; this probably resulted from losses of sulfide during analysis. Assuming an underestimation of the acid-labile sulfide molecules, these results are consistent with one 3Fe:3S core per monomer.

Spectral properties. UV-visible absorption maxima were at 395 nm and 295 nm (Fig. 1); the ratio of A_{395}/A_{295} was 0.88. Based on a molecular weight of 5,862, the extinction coefficients at 295 nm and 395 nm were 17,000 M^{-1} cm^{-1} and 14,500 M^{-1} cm^{-1}. This value is similar to the Desulfovibrio gigas ferredoxin II molar absorption coefficient at 415 nm of 15,700 M^{-1} cm^{-1} per 3Fe:3S chromophore (12). Following acidification of the ferredoxin solution with HCl to pH 3, the absorbance decreased in the 400 nm and 300 nm region indicating destruction of the Fe:S core (Fig. 1). Reduction with dithionite resulted in decreased absorbance in the 400 nm region (Fig. 1). No other chromophores were observed following acidification or reduction of the ferredoxin solution.

Amino acid composition and N-terminal analysis. Table 1 shows the total amino acid composition of ferredoxin from acetate-grown M. thermophila. Methionine, histidine, arginine and phenylalanine were absent, as was reported for ferredoxins isolated from two strains of M. barkeri (Table 1). The minimum molecular weight of the M. thermophila ferredoxin, determined from the amino acid composition, was 5,862. The protein contained 5 cysteines per monomer and a preponderance of acidic and hydrophobic amino acids. N-terminal sequence analysis of purified ferredoxin revealed only one polypeptide chain. The first N-terminal 39 amino acids are shown in Fig. 2.

Thermal stability. In the thermal stability assay, Clostridium pasteurianum

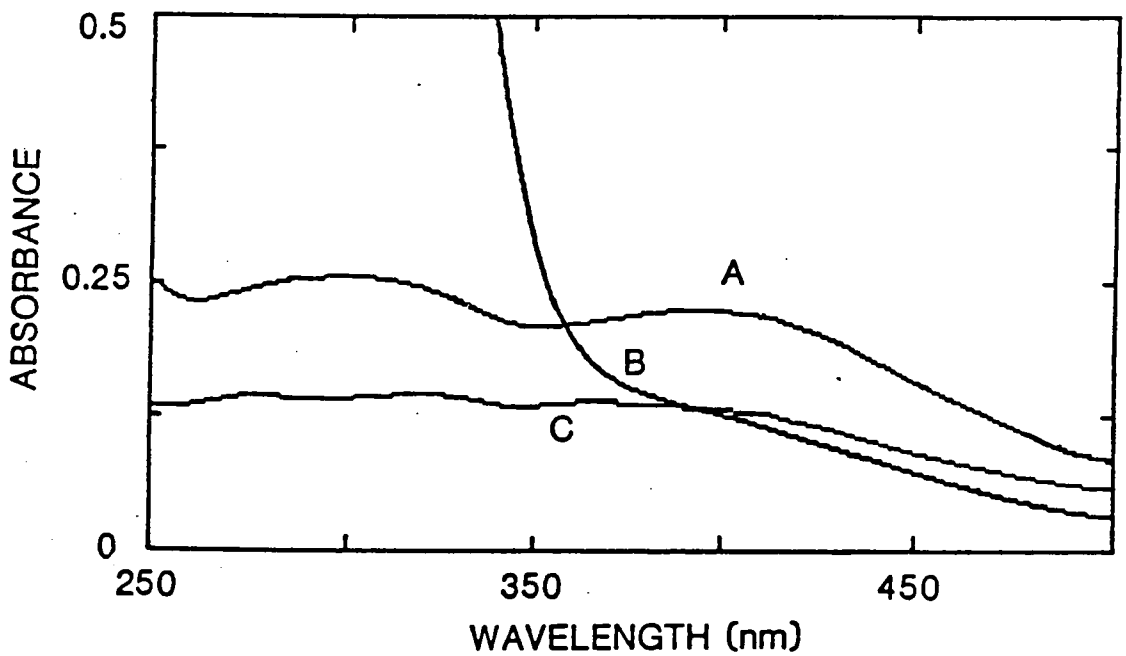

Figure 1. UV-Visible absorption Spectra of ferredoxin from M. thermophila . A. Spectrum of air-oxidized ferredoxin (0.1 mg/ml) in 0.05 M TES (pH 6.8), 10 mM $MgCl_2$, 0.5 M KCl and 10 % ethylene glycol. B. Spectrum of ferredoxin as in A after reduction with dithionite. C. Spectrum of ferredoxin as in A after acidification to pH 3 with concentrated HCl.

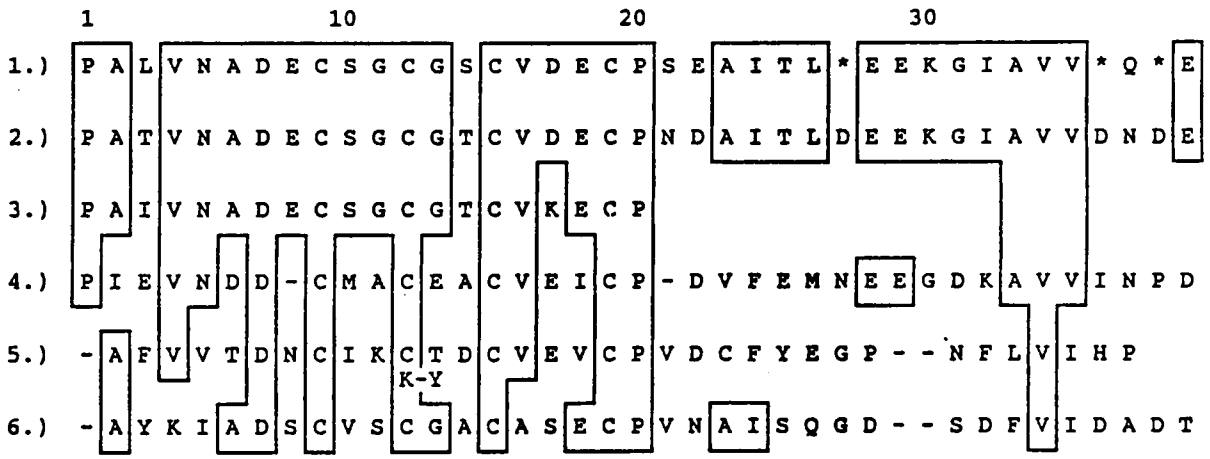

Figure 2. The N-terminal sequence of M. thermophila ferredoxin compared to other ferredoxin sequences. Numbering is according to M. thermophila ferredoxin and alignment for maximum identity. Ferredoxins are 1. M. thermophila 2. M. barkeri (DSM 800) (13), 3. M. barkeri (DSM 804) (14), 4. Desulfovibrio gigas (15), 5. Azotobacter vinelandii (16), 6. Clostridium pasteurianum (17).

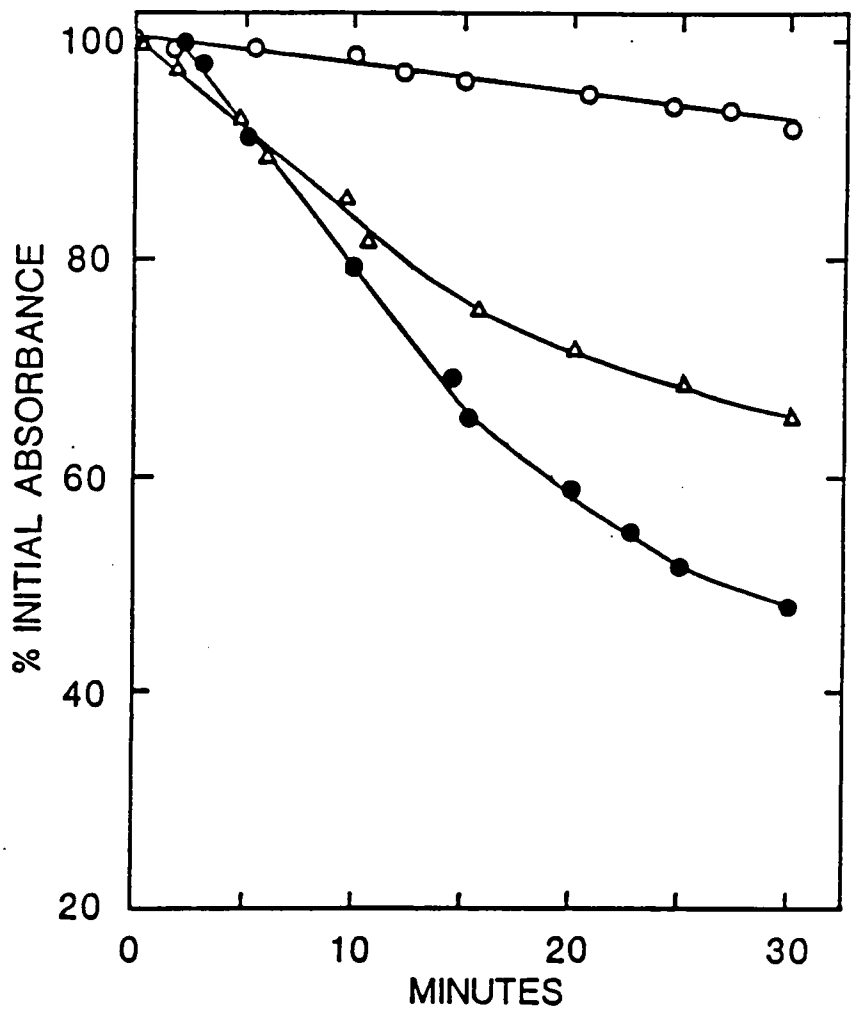

Figure 3. Thermal denaturation of M. thermophila and Clostridium pasteurianum ferredoxins. C. pasteurianum ferredoxin (0.1 mg/ml) and M. thermophila ferredoxin (0.12 mg/ml) in 0.05 M TES (pH 6.8), 10 mM $MgCl_2$, 0.5 M KCl and 10 % ethylene glycol were incubated anaerobically under N_2 at 70°C or 85°C as indicated and the absorbance at 400 nm was monitored. Symbols: o, M. thermophila ferredoxin incubated at 70°C; ∆, M. thermophila ferredoxin incubated at 85°C; •, C. pasteurianum ferredoxin incubated at 70°C.

ferredoxin rapidly denatured at 70°C; however, the ferredoxin from M. thermophila was relatively stable at 70°C for 30 min (Fig. 3) but rapidly denatured upon incubation at 85°C. Ferredoxin preparations were active when assayed after 15 min at 70°C, but inactive following incubation for 15 min above 80°C (data not shown).

DISCUSSION

In addition to the ferredoxin of M. thermophila reported here, two other ferredoxins have been described in two methanol-grown strains of Methanosarcina barkeri (13, 14). The ferredoxin from M. barkeri (DSM 804) (14), which couples H_2 evolution and the pyruvate dehydrogenase system, has been characterized and appears to be different from the ferredoxin described in this work based on the following criteria: i) The iron and acid-labile sulfide content of the M. thermophila ferredoxin is half of that obtained for the M. barkeri (DSM 804) protein, ii) the extinction coefficient at 410 nm of 36,500 M^{-1} cm^{-1} obtained for M. barkeri (DSM 804) ferredoxin (14) is nearly twice the value obtained for the M. thermophila protein, and iii) the M. thermophila ferredoxin contains only five cysteines per monomer compared to eight per monomer in the M. barkeri (DSM 804) protein.

The ferredoxin from acetate-grown M. thermophila shares some properties with the ferredoxin purified from methanol-grown M. barkeri (DSM 800). The iron and acid-labile sulfide content, extinction coefficients, thermal stability or physiological activity of the M. barkeri protein were not reported; however, EPR and Mossbauer spectroscopy indicate a 3Fe:3S core structure (18). The EPR signal of air-oxidized M. thermophila ferredoxin was consistent with the 3Fe:3S signal of the M. barkeri (DSM 800)

ferredoxin (data not shown). The amino acid compositions of the two ferredoxins were similar (Table 1) though the M. thermophila protein contained five cysteines per monomer compared to eight per monomer in M. barkeri (DSM 800) ferredoxin (13). Examination of the sequence of the first 39 N-terminal amino acids showed that the ferredoxins are 85% homologous. This value is high relative to the 24 % DNA homology between M. thermophila and M. barkeri (MS) (19) indicating the N-terminal sequence is highly conserved between the two species.

Since the spacing of the cysteines for the 4Fe:4S and 3Fe:3s ferredoxins are similar, it does not necessarily indicate the type of Fe:S core accommodated. Initial findings suggest that M. thermophila ferredoxin contains a 3Fe:3S core. Because strictly anaerobic purification procedures were used, it is unlikely that conversion of a 4Fe to a 3Fe core occurred during isolation of the protein, though in vivo interconversion of 4 Fe and 3Fe centers (20) is possible. Conversely, published results suggest the M. barkeri (DSM 804) ferredoxin coordinates 4Fe:4S centers (14) although the protein is 85 and 90% homologous with the first 20 amino acids of the M. thermophila and M. barkeri (DSM 800) proteins that may bind a 3Fe:3S center.

Azotobacter vinelandii ferredoxin contains a $C_{16}VE_{18}$ tripeptide which is thought to coordinate one Fe of the 3Fe:3S center through the cysteinyl and possibly glutamyl side chains (21). The 3Fe:3S ferredoxins of M. thermophila and M. barkeri (DSM 800) contain a corresponding tripeptide ($C_{15}VD_{17}$), albeit with a conservative substitution for glutamate. Given that the M. thermophila ferredoxin contains only five cysteines per monomer, we hypothesize that the $C_{15}VD_{17}$ tripeptide coordinates one Fe atom, and that the remaining four cysteines coordinate two Fe atoms, as in the A. vinelandii

3Fe:3S center (21); alternatively, the non-cysteinyl ligand may be an exogenous molecule such as water.

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SECTION VII. PRESENCE OF A CH_3-B_{12}:HS-CoM METHYLTRANSFERASE IN ACETATE-GROWN Methanosarcina thermophila

SUMMARY

Extracts of acetate-grown Methanosarcina thermophila catalyzed the conversion of acetate to methane at rates of 30-50 nmol/min/mg protein. CH_3-B_{12} was converted to methane by cell extracts at a rate of 19 nmol/min/mg protein. Mass spectrometry indicated that in the presence of the substrates, trideuterated acetate and CH_3-B_{12}, all of the methane formed was derived from CH_3-B_{12}. Extracts of acetate- and methanol-grown cells contained CH_3-B_{12}:HSCoM methyltransferase activity of 1.2 ± 0.5 U/mg protein and 1.5 ± O.3 U/mg protein respectively. The activity from acetate-grown cells was stable at 70°C for 5 minutes. The activity in extracts was fractionated by ammonium sulfate precipitation followed by phenyl-superose chromatography; the methyltransferase activity eluted as two peaks of activity from extracts of acetate- and methanol-grown cells. The partially-purified enzyme preparations from acetate- and methanol-grown cells gave a Km_{app} for HSCoM of 1.5 mM and a Km_{app} for CH_3-B_{12} of >7mM.

INTRODUCTION

The conversion of acetate to methane and CO_2 involves transfer of the methyl group intact to coenzyme M (2-mercaptoethanesulfonic acid). The methyl-coenzyme M is then reductively demethylated releasing methane; the electron pair necessary for this reduction are supplied from the oxidation of the carbonyl group of acetate to CO_2 (9).

In the conversion of acetate to methane in Methanosarcina thermophila and M. barkeri, the acetate is first activated to acetyl-CoA (6, 18). Little is known concerning the pathway of methyl group transfer from acetyl-CoA to HS-CoM.

Some methanogens are also able to use methanol as a substrate for methanogenesis and growth. In the presence of both methanol and acetate, a biphasic response is observed in which the methanol is utilized before acetate as a substrate for methanogenesis. (5, 11, 24). Since conversion of acetate and methanol to methane involves transfer of an intact methyl group, there may be similar mechanisms for the conversion of the methyl-moiety to methane in the two pathways. In methanol-grown M. barkeri, corrinoids function as methyl-group carriers prior to coenzyme M (22), and two enzymes catalyze the conversion of methanol to CH_3-S-CoM (21). The first, MT_1 (methanol:5-hydroxybenzimidazole (B_{12}-HBI) methyltransferase), is a corrinoid-containing enzyme that binds the methyl group of methanol to an enzyme-bound corrinoid cofactor (20). The second enzyme, MT_2 (Co-methyl-5-hydroxybenzimidazolylcobamide (CH_3-B_{12}-HBI):HS-CoM methyltransferase, transfers the methyl group from the enzyme-bound corrinoid of MT_1 to HS-CoM (21).

Corrinoids have also been implicated in methanogenesis from acetate though less is known concerning the enzymes involved. In M. barkeri, 5 uM propyl iodide, an inhibitor of corrinoid-containing enzymes, inhibits methanogenesis in extracts; this inhibition is relieved by exposure to light (4) due to photolysis of the Co-propyl bond. In another study, CH_3-B_{12}-HBI was isolated as an enzyme-bound intermediate during the conversion of acetate or acetyl-phosphate and ATP to methane in M. barkeri cofactor-free extracts (23).

The enzyme hypothesized to cleave acetyl-CoA during acetate conversion to methane in M. thermophila is the carbon monoxide dehydrogenase (CODH) complex (10). This enzyme complex contains an enzyme-bound corrinoid (19) believed to accept the methyl group following acetyl-CoA cleavage. The photolability of methylated corrinoids, and the inability to demonstrate acetyl-CoA cleavage with the purified enzyme, has made it difficult to demonstrate an enzyme-bound CH_3-B_{12} intermediate in purified enzyme systems. We report here that CH_3-B_{12} serves as an intermediate in the conversion of acetate to methane in extracts of M. thermophila, and that a CH_3-B_{12}:HS-CoM methyltranferase activity is present in extracts of acetate-grown and methanol-grown cells.

MATERIALS AND METHODS

Cell extract methanogenesis assay. For assays of methanogenesis in cell extracts, basic anaerobic techniques were used (13). Methanosarcina thermophila strain TM-1 (25) was cultured in basal medium in 3L round-bottom flasks (19). pH and acetate concentration were maintained by the addition of acetic acid (10M). Cell extracts were prepared anaerobically in a N_2 atmosphere as described previously (19). The breakage buffer was 50 mM sodium 3-[N-morpholino]propane-sulfonate (MOPS) (pH 6.8), 10 mM $MgCl_2$, DNase (10 mg/100 ml), and 5% (v/v) glycerol. Following breakage, the 12,000 xg supernatant (30 - 40 mg/ml) was placed on ice for a maximum of 2 hours prior to use in the reaction mixtures described below.

The reaction mixture (0.25 ml) prepared on ice in butyl rubber-stoppered 10 ml Wheaton amber serum vials, contained 0.1 ml cell extract protein (approx. 4 mg), 50

mM 2-[N-Morpholino]ethane sulfonic acid (MES) (pH 6.2), 25 mM $MgCl_2$, 48 mM acetate, 10 units creatine phosphokinase with 80 mM phosphocreatine, 27 units glucose oxidase with 4 mM glucose and 0.1 mM coenzyme A. The gas phase was N_2 and CO (8:1), N_2 and H_2 (8:1), or 100% N_2 as indicated. When present, the CH_3-B_{12} concentration was 12 mM, 2-bromoethanesulfonic acid concentration was 0.15 mM, and CH_3-S-CoM concentration was 24 mM. Vials containing CH_3-B_{12} were wrapped in foil to prevent photolysis. The reaction was initiated by placing the vials in a 45°C water bath. Aliquots (0.1 ml) were removed from the vial head space at 5 - 10 min intervals and analyzed for methane by gas chromatography as described (9). Protein concentrations of extract were determined using the Bradford protein assay (2) with bovine gamma globulin as standard.

Mass spectrometry. Aliquots (1.0 - 2.0 ml) of the head space from each vial were injected into a VG 7070E mass spectrometer operated at 70 eV with a source temperature of 200°C and 2000 resolution. Signals were recorded as total ion current integrated over 10 - 100 m/z. To classify methane species as deuterated or non-deuterated, the ion intensity of the major methane ion and the associated fragment ions were measured, summed and normalized. The published m/e and normalized abundances for the ions of deuterated and nondeuterated methane standards were used to calculate the percentage of each species present in the sample (7).

Mass culture conditions, and preparation of cell extracts. For characterization of CH_3-B_{12}:HS-CoM methyltransferase activity, M. thermophila was mass cultured on acetate or methanol as described previously (19). Cell extracts were prepared anaerobically in a N_2 atmosphere as described previously and stored in liquid nitrogen

until use (19). Protein was determined using the Bradford protein assay (2) with bovine serum albumin as standard.

CH_3-B_{12}:HS-CoM methyltransferase assay. All assays were performed anaerobically at 57°C unless otherwise noted. To assay for methyltransferase activity, the formation of $^{14}CH_3$-S-CoM from $^{14}CH_3$-B_{12} and HSCoM was monitored (17). Reaction mixtures (0.3 ml) contained 50 mM TES (pH 6.8), 10 mM $MgCl_2$, 5 mM HS-CoM, 5 mM [^{14}C]-CH_3-B_{12} (specific activity 494 cpm/umole). The reaction was initiated by the addition of cell extract protein or partially purified fractions as indicated. For cell extract and ammonium sulfate fractions, the reaction mixture also contained 0.25 mM 2-bromoethanesulfonic acid to inhibit the CH_3-S-CoM methylreductase reaction. The mixture was contained in butyl-rubber stoppered 10 ml Wheaton amber serum vials with 1 atm. of N_2. The vials were placed in a 57°C water bath and incubated for 5 minutes. The reaction was stopped by opening the vials and adding 2.0 ml of an aqueous slurry (50% w/v) of Biorad AG-50Wx4 cation exchange resin (H^+ form). The positively charged CH_3-B_{12} was bound to the resin, and the negatively charged HS-CoM derivatives remained in the supernatant. A sample (0.1 ml) of the supernatant was added to 2.5 ml Scinte verse scintillation cocktail and counted for radioactivity in a Beckman LS 8100 scintillation counter (Beckman Instruments Division, Fullerton, CA). The reaction mix did not quench any radioactivity from the [methyl-^{14}C]CH_3-B_{12} or [methyl-^{14}C]CH_3-S-CoM. The umoles of HS-CoM methylated were calculated from the specific activity of the [methyl-^{14}C]CH_3-B_{12} added, using a correction factor of 0.68 for the interior volume of beads not available for dilution. The product was confirmed as CH_3-S-CoM by thin layer chromatography (14) using a standard CH_3-S-CoM

preparation. One unit is equal to one umol of CH_3-S-CoM formed per minute.

Cell extract fractionation. All steps were performed aerobically at 4°C unless otherwise noted. Ammonium sulfate crystals were added to cell extracts to a final concentration of 0.50 saturation. Following centrifugation, a sample of the supernatant (approx. 3.5 mg protein) was injected onto a Phenyl Superose 5/5 column (Pharmacia LKB Biotechnology Inc.) equilibrated with Buffer A, 0.05 M sodium phosphate buffer (pH 6.8) containing 1.7 M ammonium sulfate, at 23°C. After baseline stabilization, a gradient of 1.7 M to 0.0 M ammonium sulfate in 0.05M phosphate buffer was applied at 0.5 ml/min using a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology Inc.) equipped with a model GP-250 gradient programmer. The major peak of methyltransferase activity was pooled following several phenyl superose separations and stored in liquid nitrogen until use.

Chemicals. Methylcobalamin was chemically synthesized and purified as described (3) to a specific activity of 3220 cpm/umole. The concentration and purity of the synthesized methylcobalamin was determined from the UV-Vis absorption spectrum. CH_3-S-CoM was a gift from Michael J. K. Nelson. 2-Bromoethanesulfonic acid (Na salt), TES, MOPS, and methyl cobalamine, phosphocreatine, creatine kinase, glucose oxidase, ATP and DNase were purchased from Sigma Chem. Corp. Enzyme-grade ammonium sulfate was purchased from Fisher.

RESULTS

Methanogenesis in cell extracts. Cell extracts routinely catalyzed the conversion of acetate to methane at rates of 30-50 nmol/min/mg protein (Table 1). This corresponds

to approximately 50% of the rate observed with whole cells under similar conditions (data not shown). Exogenous ATP was required for maximum rates and removal of the ATP-regenerating system caused a 50% decrease in the rate (data not shown).

CH_3-B_{12} was converted to methane at 66% of the rate observed with acetate conversion (Table I). Methanogenesis from CH_3-B_{12} did not require ATP or the regenerating system, but the reaction was inhibited by BrES indicating that CH_3-S-CoM is a direct intermediate during the conversion of CH_3-B_{12} to methane.

To determine if CH_3-B_{12} was preferred to acetate as a substrate for methanogenesis, trideuterated acetate and CH_3-B_{12} were included in the reaction mix, and the methane species formed were analyzed by mass spectrometry. Extracts incubated in the presence of both substrates synthesized only nondeuterated methane indicating that the CH_3-B_{12} served as the primary substrate for methanogenesis (Table 2).

CH_3-B_{12}:HS-CoM methyltransferase Activity in Cell Extracts. Cell extracts of acetate- and methanol-grown M. thermophila were assayed for CH_3-B_{12}:HS-CoM methyltransferase activity. Extracts of methanol-grown cells contained 1.5 ± 0.3 Units/mg protein, and extracts of acetate-grown cells contained 1.2 ± 0.5 Units/mg protein activity. The extracts and partially purified enzyme preparations could be handled aerobically without any effect on the assay results, though it was necessary to perform the assay anaerobically. The reaction progressed with time (Fig. 1). The addition of 5 mM ATP or 50 mM KCl, NaCl, $(NH_4)_2SO_4$, or $MgCl_2$ did not effect the methyltransferase activity (data not shown).

Thermal stability. The thermal stability of the CH_3-B_{12}:HS-CoM methyltransferase activity in extracts of acetate-grown cells was 70°C, and there was still substantial

Table 1. Methanogenesis in extracts of acetate-grown M. thermophila

Assay components	Maximum rate nmol/min/mg protein	Total yield CH_4 (nmol)
complete[1]	30	4500
complete +BrES	0[2]	5
complete -CO	0	41
complete -ATP	0	170
complete -CoA	13	2830
complete -acetate	2	100
+ CH_3-S-CoM	8	1000
+ CH_3-B_{12}	19	5430
+ CH_3-B_{12}, -ATP	17	4170
+ CH_3-B_{12}, + BrES	0	6
+ CH_3-B_{12}, -CO	4.8	1570

[1]Experimental conditions and composition of the reaction mixture are as described in Materials and Methods. Rates of methanogenesis were maximal within the first 20 minutes of incubation and were calculated from a linear portion of the curve in this time period.

[2]Detection limit < 2 nmol CH_4

Table 2. Methanogenesis in extracts[1] of acetate-grown M. thermophila from trideuterated acetate in the presence or absence of CH_3-B_{12}.

substrate	% methane formed deuterated	non-deuterated
CH_3-B_{12}	0	100
CD_3COO^-	100	0
CH_3-B_{12}, CD_3COO^-	0	100

[1]Extracts were incubated with either of the above two substrates as described in the Materials and Methods. The particular methane species were quantified using mass spectrometry as described.

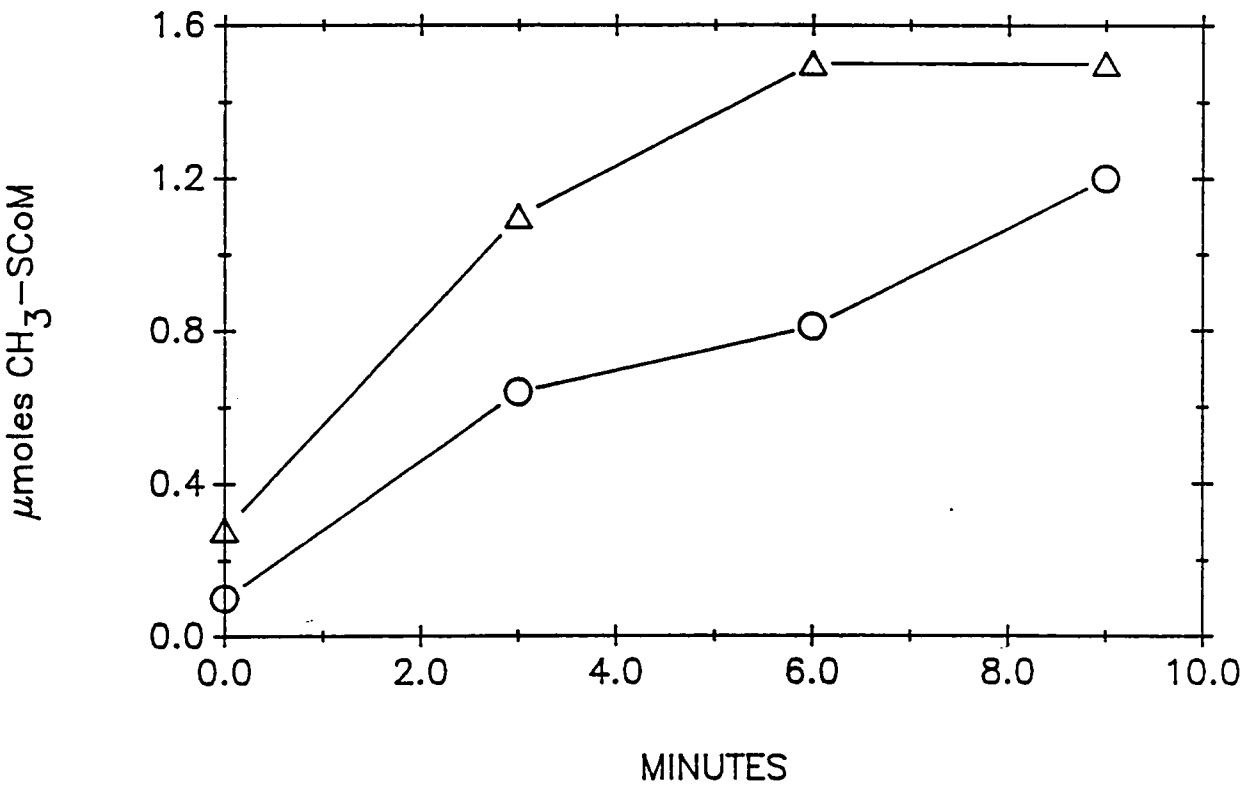

Figure 1. Time course for the CH_3-B_{12}:HSCoM methyltransferase assay in cell extracts of M. thermophila. Cell extract (0.71 mg protein) from methanol- and acetate-grown cells was assayed for CH_3-B_{12}:HSCoM methyltransferase activity in the standard assay. △,extracts of methanol-grown cells. ○,extracts of acetate-grown cells.

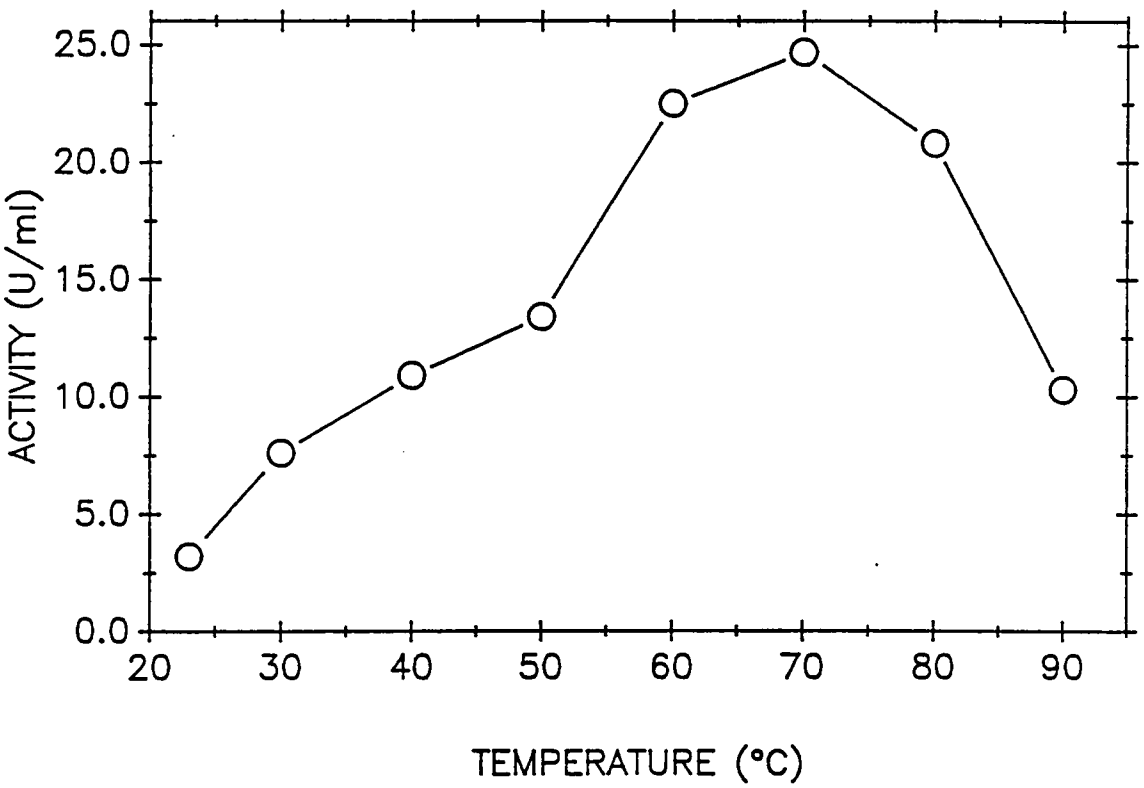

Figure 2. Thermal stability of CH_3-B_{12}:HSCoM methyltransferase activity in extracts of acetate-grown M. thermophila. Cell extract protein was assayed for methyltransferase activity at the indicated temperatures as described in the Materials and Methods. The activity (Units/ml) represents the methyltransferase activity in the cell extract preparation (21 mg/ml).

activity after incubation for 5 min at 90°C (Figure 2).

Fractionation of CH_3-B_{12}:HS-CoM methyltransferase activity. Extracts of both methanol- and acetate-grown M. thermophila were first brought to 50% ammonium sulfate concentration. Greater than 70% of the methyltransferase activity was found in the supernatant fraction resulting in a 2-3 fold increase in the specific activity. The soluble proteins were then fractionated using phenyl superose HR 5/5 chromatography (Fig. 3) with a decreasing ammonium sulfate concentration gradient. Fractions were assayed for methyltransferase activity. Figure 3, panel A shows the elution profile of protein and activity from the ammonium sulfate fraction of methanol-grown cells. The major peak of methyltransferase activity from methanol-grown cells was consistently located in fraction 17 with a minor peak of activity in fraction 26. Panel B shows the elution profile of protein and activity from the ammonium sulfate fraction of acetate-grown cells. The major peak of methyltransferase activity from acetate-grown cells was consistently found at fraction 28 with a minor peak of activity at fraction 17. Addition of 0.1% Triton x-100 to the column in buffer containing no ammonium sulfate did not release any additional methyltransferase activity.

Two peaks of CH_3-B_{12}:HSCoM methyltransferase activity were consistently observed following phenyl superose fractionation of several extract samples of either acetate- or methanol-grown cells. The ratio of the major to minor peak varied from 60/40 to 90/10 with different batches of extracts; however, the major peak of activity was consistently at fraction 17 for methanol-grown cells and at fraction 28 for acetate-grown cells. Ammonium sulfate fractionation and phenyl superose chromatography resulted in an approximate 10-fold purification of the enzyme from extracts with >50%

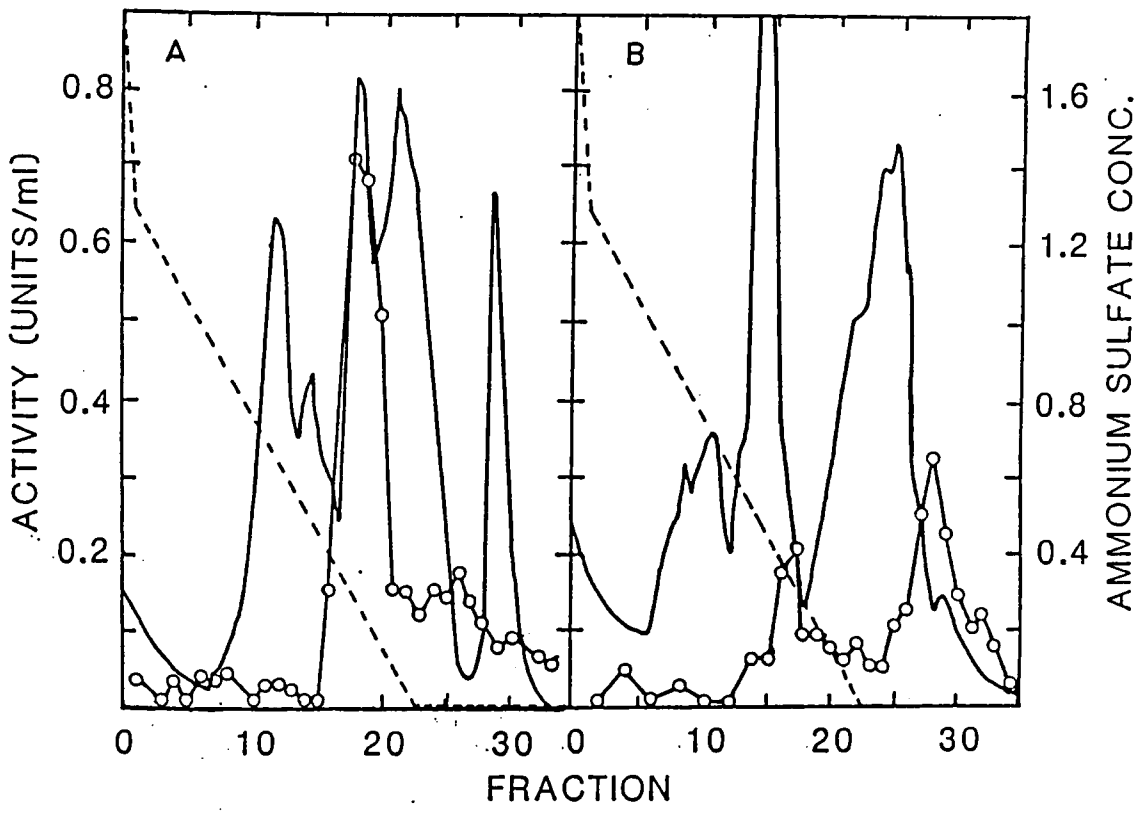

Figure 3. FPLC phenyl superose chromatography of proteins from ammonium sulfate-treated cell extracts of methanol- and acetate-grown M. thermophila. A) Extract of methanol-grown cells (7.6 mg protein) was brought to 50% ammonium sulfate saturation followed by removal of precipitated protein. A sample of the supernatant solution (3.65 mg protein) was chromatographed by phenyl superose chromatography (HR 5/5) at 0.5 ml/min with a decreasing ammonium sulfate gradient. Aliquots (0.1 ml) of fractions were assayed for CH_3-B_{12}:HSCoM methyltransferase activity in the standard assay. B) Extracts of acetate-grown cells (7.3 mg protein) were treated and chromatographed (3.46 mg protein) as described in A. o, methyltransferase activity. –, relative A_{280} absorbance. - - -, ammonium sulfate concentration.

recovery. Only the major peak of methyltransferase activity from each extract was pooled for further characterization. Attempts to further purify the enzyme using anion exchange chromatography (hydroxylapatite, DE52, or FPLC Mono Q) resulted in a loss of greater than 50% of the enzyme activity.

Each major phenyl superose methyltransferase pool from extracts of methanol- and acetate- grown cells was electrophoresed in a 12% nondenaturing gel; the gel was then sliced into 4 mm sections and assayed for CH_3-B_{12}:HSCoM methyltransferase activity. The major methyltransferase pool (fraction 17) from methanol-grown cells showed methyltransferase activity migrating with an Rm of 0.42 (Figure 4). The major methyltransferase pool (fraction 28) from acetate-grown cells showed methyltransferase activity migrating with an Rm of 0.58 (Figure 4).

Physiological Properties of the partially purified CH_3-B_{12}:HS-CoM methyltransferases. The partially-purified enzymes from both acetate- and methanol-grown cells were examined for various kinetic parameters. The apparent K_m for CH_3-B_{12} was greater than 7 mM for each enzymes. The kinetic assays employed concentration of CH_3-B_{12} to 10 mM, which is near the solubility limit; the maximal velocity for the reaction was not observed at these concentrations (Fig. 5). The apparent Km for HS-CoM was 1.5 mM for both enzymes (data not shown). The activity was not effected by the addition of 100 mM KCl, 100 mM $MgCl_2$ or 5mM dithiothreitol (data not shown).

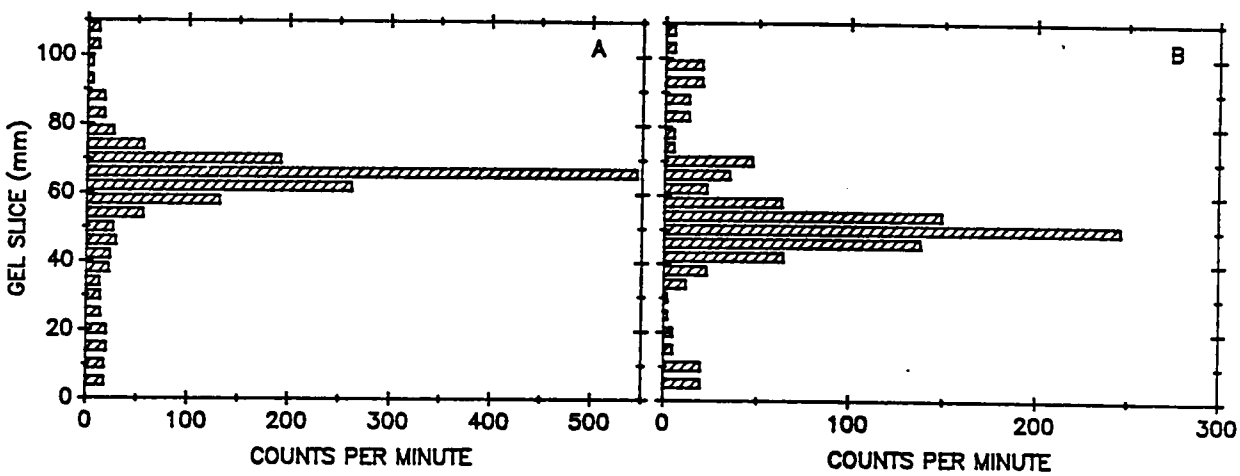

Figure 4. Migration of CH_3-B_{12}:HSCoM methyltransferase activity in a nondenaturing 12% polyacrylamide gel. A) A sample (0.03 mg protein) of the phenyl superose-purified methyltransferase from acetate-grown cells was electrophoresed in a 12% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was sliced into 4 mm sections and each slice assayed for methyltransferase activity in the standard assay. B) A sample (0.03 mg protein) of the phenyl superose-purified methyltransferase from methanol-grown cells was electrophoresed and assayed as in A.

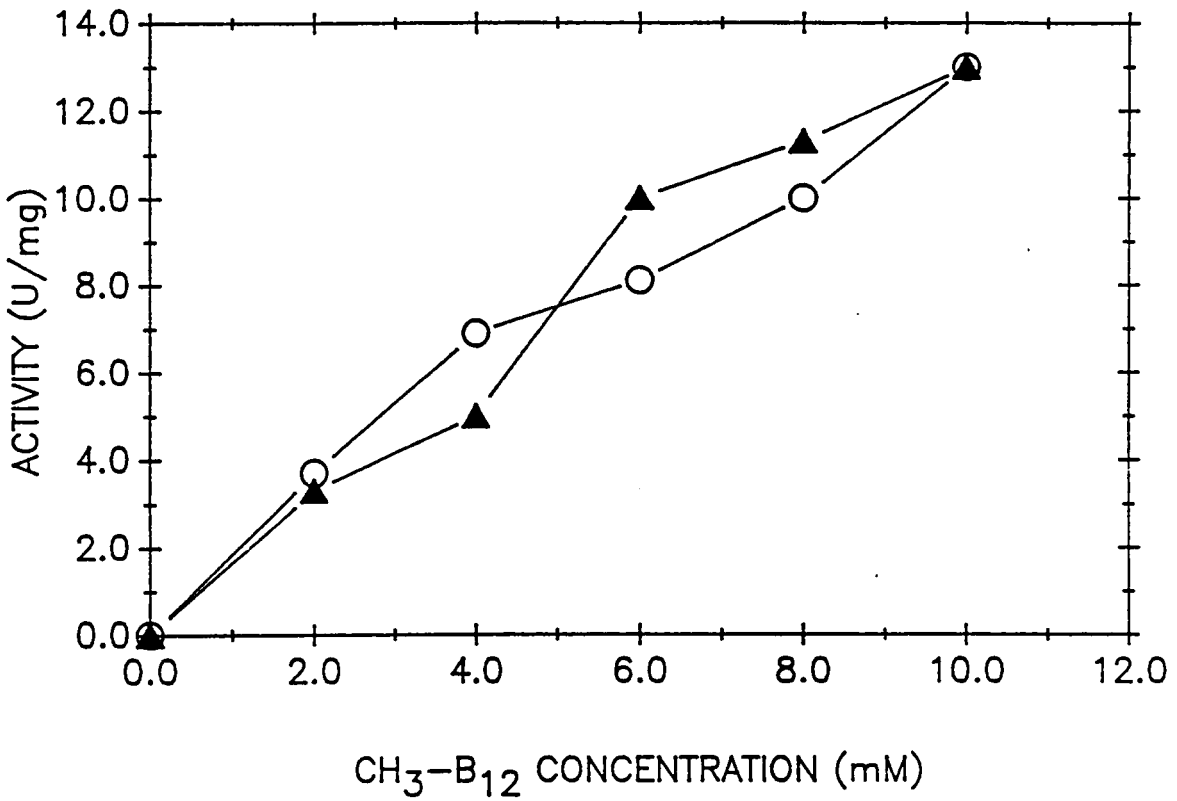

Figure 5. CH_3-B_{12}:HSCoM methyltransferase activity in relation to the concentration of CH_3-B_{12} in the assay. Phenyl superose-purified methyltransferase from extracts of acetate- (0.012 mg protein) (○) and methanol-grown cells (0.015 mg protein) (▲) was assayed for CH_3-B_{12}:HSCoM methyltransferase activity in the standard assay.

DISCUSSION

Corrinoid compounds are present in high concentrations in methanogenic bacteria (16), and are hypothesized to play a role in the methyl-transfer (22) and/or the electron-transfer (1) reactions of methanogenesis. Here we describe the formation of methane from CH_3-B_{12}, and the presence of a CH_3-B_{12}:HS-CoM methyltransferase activity in cell extracts of acetate-grown M. thermophila.

Cell extracts of acetate-grown M. thermophila catalyzed the conversion of acetate to methane in the presence of ATP and CO or H_2. This in vitro methanogenesis assay was also used to demonstrate methane formation from CH_3-B_{12}. Since BrES inhibited methane formation from CH_3-B_{12}, and in the presence of both CH_3-B_{12} and trideuterated acetate all of the methane was derived from CH_3-B_{12}, it can be concluded that CH_3-B_{12} is a more immediate precursor to methane than acetate, and CH_3-S-CoM is an intermediate in this conversion.

The ability of the cells to convert CH_3-B_{12} to methane in the absence of H_2 or CO may be due to the product of the demethylation reaction, $B_{12}r$, acting as the electron source for the CH_3-S-CoM methylreductase. Reduced cobalamin compounds have been shown to act as an electron source for the methylreductase reaction in M. thermoautotrophicum (1).

The CH_3-B_{12}:HS-CoM methyltransferase activity (1.2 umol/min/mg) in extracts is much greater than the rates of acetate conversion to methane in extracts (0.03-0.05 umol/min/mg), and is consistent with a role of the enzyme in the pathway. Though the apparent km for CH_3-B_{12} in the CH_3-B_{12}:HS-CoM methyltransferase enzymes was >7 mM, it is unlikely this is representative of physiological conditions. This high figure

may be due to the fact that the actual substrate for the enzyme is probably not CH_3-B_{12}, but rather 5-hydroxy(benzimidazoyl)cobamide, the corrinoid cofactor found in methanogens (12). Furthermore, the Km may be lower when the CH_3-B_{12} cofactor is bound to an enzyme; in methanol-grown cells this may be the methanol:B_{12}-HBI methyltransferase, and in acetate-grown cells it may be the corrinoid-containing CO dehydrogenase (19). Binding of the CH_3-B_{12}-HBI substrate by the CH_3-B_{12}-HBI:HS-CoM methyltransferase may be enhanced by the presence of the cofactor in another protein. Thus the CH_3-B_{12}:HSCoM methyltransferase enzymes from acetate- and methanol-grown cells may catalyze the same reaction, but interact with different proteins in the cell resulting in different chromatographic and electrophoretic behavior. The implication of corrinoids in methanogenesis and the presence of the CH_3-B_{12}:HS-CoM methyltransferase enzyme adds to our understanding of the carbon flow during acetate conversion to methane.

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SECTION VIII. SUMMARY AND DISCUSSION

In the past four years, research on the physiology of acetate conversion to methane has led to a better understanding of the process. The work presented in this dissertation established a physiological role for the CO dehydrogenase enzyme complex during acetate conversion. The major advances made in this study were the understanding of some of the compounds or proteins that interact with the CO dehydrogenase complex. A physiological substrate, acetyl-CoA, and electron acceptor, ferredoxin, were identified; and the methyl transfer protein subsequent to the CO dehydrogenase in the pathway was described. These major advances have allowed the proposal of a new pathway of acetate conversion (Figure 1).

Though the pathway of carbon flow has been studied extensively, the pathway of electron transfer is less well understood. A ferredoxin accepts the electron pair derived from the oxidation of the carbonyl group of acetate, but the subsequent electron carriers are not known. A possible method to identify such electron carriers is to utilize UV-Vis difference spectroscopy methods of the electron transfer components in the membranes, such as cytochromes. Further research is necessary in this area to comprehend the energy-conserving mechanisms used by these organisms.

Another area deserving intensive research is the reconstitution of methanogenesis from acetate in a defined enzyme system. Only recently was aceticlastic methanogenesis optimized in cell extracts. Most of the enzymes required for the carbon transfer reactions have been purified and characterized. However, the in vivo acetyl-CoA cleaving activity of the central enzyme, CO dehydrogenase, has not been demonstrated in vitro. In order to obtain acetyl-CoA cleavage by the enzyme, it will probably be

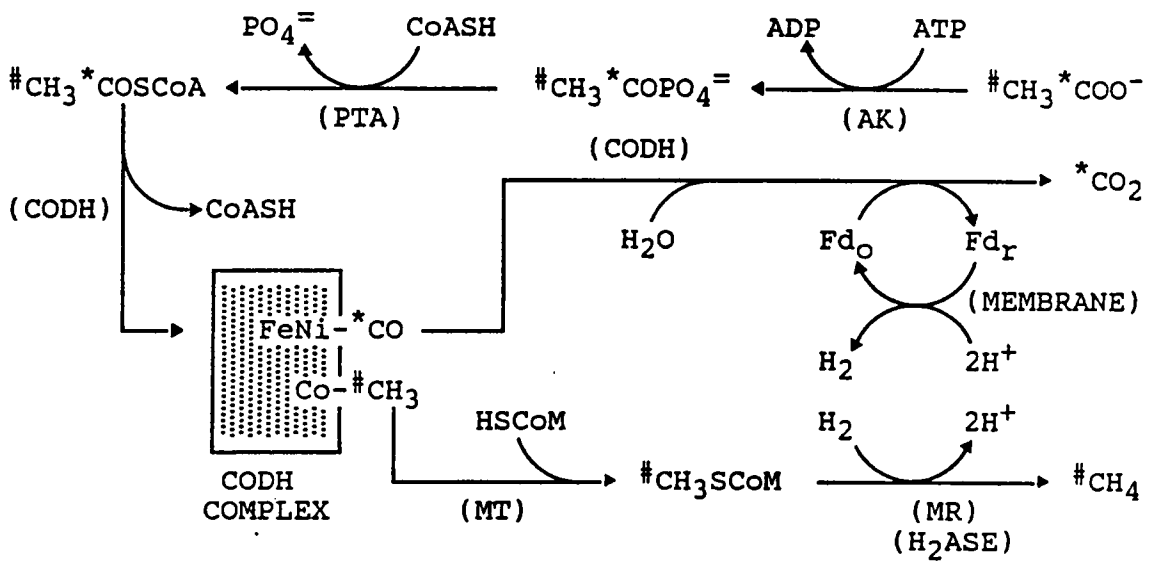

Figure 1. Proposed carbon and electron flow during acetate conversion to methane in M. thermophila. AK, acetate kinase; PTA, phosphotransacetylase; CODH,carbon monoxide dehydrogenase; FD,ferredoxin; H_2ase, hydrogenase; MT, methyltransferase; MR, methylreductase.

necessary to elucidate the function of all five protein components of the complex. For example, once the corrinoid protein is purified away from the complex, we may obtain information on the requirements for methyl transfer to the corrinoid site that will aid in acetyl-CoA cleavage. Thus, the key to reconstitution of methanogenesis from acetate in vitro is understanding the enzymatic process catalyzed by the CO dehydrogenase and the energy conserving mechanisms used in electron transport by this organism.

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