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CHARACTERIZATION OF ELECTRON TRANSFER ACTIVITIES ASSOCIATED WITH ACETATE DEPENDENT METHANOGENESIS BY Methanosarcina barkeri MS

presented by

John Michael Kemner

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CHARACTERIZATION OF ELECTRON TRANSFER ACTIVITIES ASSOCIATED WITH ACETATE DEPENDENT METHANOGENESIS BY *METHANOSARCINA BARKERI* MS

By

John Michael Kemner

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ABSTRACT

CHARACTERIZATION OF ELECTRON TRANSFER ACTIVITIES ASSOCIATED WITH ACETATE DEPENDENT METHANOGENESIS BY *METHANOSARCINA BARKERI* MS

By

John Michael Kemner

The mechanism of electron transfer between the C1 and C2 carbons of acetate during acetotrophic methanogenesis was studie in *Methanosarcina barkeri* MS. Membranes of acetate-grown cells contained hydrogenase activity, multiple iron-sulfur centers, and a putative rubredoxin-like center. EPR spectroscopy demonstrated reduction of iron-sulfur centers and rubredoxin by hydrogen or CO. Optical spectroscopy demonstrated that a *b*-type cytochrome was reducible by hydrogen and oxidized by methyl CoM. The hydrogenase was purified from membranes of acetate-grown *Methanosarcina barkeri* MS. The aerobically purified enzyme was reactivated under reducing conditions in the presence of H_2. The enzyme showed a maximal activity of 120 ± 40 μmol $H_2 \cdot min^{-1} \cdot mg^{-1}$ with methyl viologen as an electron acceptor, and an apparent K_M for hydrogen of 5.6 ± 1.7 μM. The enzyme is a nickel-iron-sulfur protein consisting of an αβ heterodimer with subunits of 57,000 and 35,000. Coupling studies showed that the hydrogenase mediated the reduction of cytochrome *b* in a solubilized protein preparation, but did not couple with autologous F_{420} or ferredoxin. Acetate-grown cells of *Methanosarcina barkeri* MS had levels of hydrogenase and hydrogen-consuming ability similar to those found in hydrogen-grown cells. A large (13 kDa) ferredoxin which coupled to hydrogenase and CO dehydrogenase was purified from acetate-grown cells. The soluble fraction of acetate-grown

cells contained both F_{420}-reactive (hydrogenase I) and F_{420}-nonreactive (hydrogenase II) activities. These activities were separated and characterized. A reconstituted soluble protein system composed of purified CO dehydrogenase, F_{420}-reactive hydrogenase II fraction, and ferredoxin produced H_2 from CO oxidation at a rate of 2.5 nmol/min·mg protein. Hydrogenase II was immunologically and biochemically identical to the membrane-bound hydrogenase. Membrane-bound hydrogenase II coupled H_2 consumption to the reduction of CoM-S-S-HTP and the chemiosmotic synthesis of ATP. The differential function of hydrogenase I and II are ascribed to ferredoxin-linked hydrogen production from CO and cytochrome *b*-linked H_2 consumption coupled to methanogenesis and ATP synthesis, respectively.

for myself

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ABBREVIATIONS

CoM	2-mercaptoethanesulfonic acid; coenzyme M
CoM-S-S-CoM	homodisulfide of CoM
CoM-S-S-HTP	Mixed disulfide of CoM and HTP
kDa	kiloDalton
H_4MPT	tetrahydromethanopterin
HTP	7-mercaptoheptanoylthreonine phosphate
EPR	Electron paramagnetic resonance spectroscopy
F_{420}	7,8-didemethyl-8-hydroxy-5-deazariboflavin; factor 420
MOPS	3-(N-morpholino) propanesulfonic acid
M_r	relative mass
SDS-PAGE	sodium dodecyl sulfate polyacrylamide electrophoresis
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1

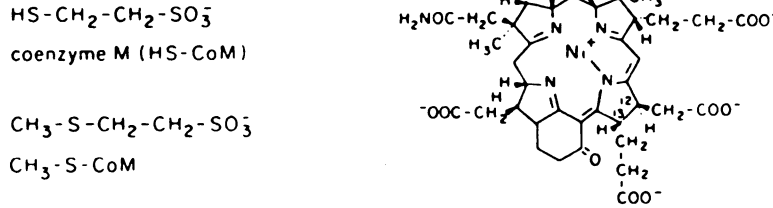
LITERATURE REVIEW AND OBJECTIVES

Methanogenic diversity

Methanogens are strictly anaerobic bacteria distinguished by their ability to catabolically reduce simple carbon compounds to methane. Methanogens are classified as members of the domain *Archaea*, which, along with certain thermoacidophiles, hyperthermophiles, and extreme halophiles, is considered to be a prokaryotic domain distinct from *Bacteria* (eubacteria) (119,121). These kingdoms were proposed on the basis of comparison of 16S ribosomal RNA sequences by Woese and Fox (120). This hypothesis has been supported by studies showing differences in other biochemical features such as cell wall and membrane lipid composition (2, 29, 119, 131). A number of novel coenzymes found in methanogenic archaea participate in the biochemistry of methane formation (95, 31) (Fig. 1). In addition, the membranes of these organisms contain unusual ether-linked isoprenoid lipids (Fig 2.) (29). Although unified on a biochemical basis by the formation of methane, there is considerable diversity among methanogens. Almost every known bacterial morphology is represented, and methanogens have been isolated from mesophilc, thermophilic, hyperthermophilic (ca. 100°C), and hypersaline habitats (for an overview see 3, 9, 20, 44, 53, 72, 94).

The more than 68 described species of methanogenic archaea are currently classified into three orders, seven families, and twenty genera (44) (Table 1). Cellular morphologies represented include regular and irregular rods and cocci, pseudosarcina, platelike forms, and the spiral-shaped rod *Methanospirillum hungatei*. Most known methanogens grow at near neutral pH, and thermophilic species are widely distributed throughout the genera (44). Members of the genus *Methanopyrus* are some of the most hyperthermophilic prokaryotes known, growing at temperatures at 110°C (72). The order Methanobacteriales, including *Methanobacterium* spp., contains those methanogens which possess a

Fig. 1. Structures of unusual coenzymes of methanogenesis (taken from [95]).



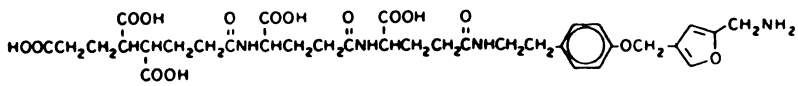
$HS-CH_2-CH_2-SO_3^-$

coenzyme M (HS-CoM)

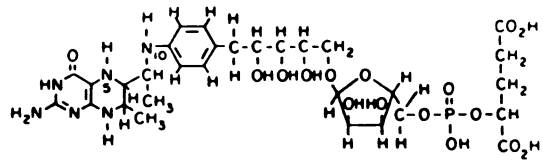
$CH_3-S-CH_2-CH_2-SO_3^-$

$CH_3-S-CoM$

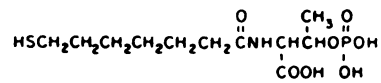
Factor F_{430}



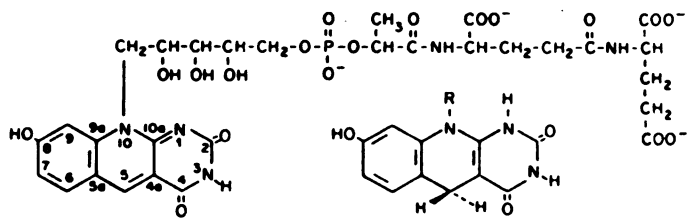
methanofuran



tetrahydromethanopterin (H_4MPT)



7-mercaptoheptanoylthreonine phosphate
(HS-HTP)



Oxidized

Reduced ($F_{420}H_2$)

coenzyme F_{420}

Fig. 2. Membrane lipids present in *Methanosarcina barkeri* (adapted from [29])

Table 1. Classification of representative methanogenic archaea.[a]

Organism	cytochromes[b]	opt. growth temp. (°C)	substrates used: H_2:CO_2	acetate	formate	methyl compounds
Order Methanomicrobiales						
Fam. Methanomicrobiaceae						
Methanomicrobium						
mobile		40	+	-	+	-
Methanolacinia						
paynteri		40	+	-	-	-
Methanospirillum						
hungatei		30-37	+	-	+	-
Methanogenium						
thermophilicum		55-60	+	-	+	-
Fam. Methanocorpusculaceae						
Methanocorpusculum						
aggregans		35-37	+	-	+	-
Fam. Methanosarcinae						
*Methanosarcina**						
barkeri		30-40	+	+	-	+
acetivorans		35-40	-	+	-	+
thermophila		50	-	+	-	+
mazei		30-40	+	+	-	+
*Methanolobus**						
tindarius		37	-	-	-	+
*Methanococcoides**						
methylutens		30-35	-	-	-	+
Methanohalophilus						
zhilinae		45	-	-	-	+
Methanohalobium						
evestigatus		n.r.[c]	-	-	-	+
*Methanosaeta**						
concilii		35-40	-	+	-	-
*Methanothrix**						
soehngenii		35-40	-	+	-	-
Methanopyrus						
kandleri		98	+	-	-	-

[a]Condensed from (44).
[b]Genera reported to contain membrane *b* or *c*-type cytochromes indicated by (*).
[c]Not reported

Table 1. (cont'd.)

Organism	optimum growth temp. (°C)	substrates used H_2:CO_2	acetate	formate	methyl compounds
Order Methanobacteriales					
Fam. Methanobacteriaceae					
Methanobacterium					
formicicum	37-45	+	-	+	-
thermoautotrophicum	65-70	+	-	-	-
Methanobrevibacter					
ruminantium	37-39	+	-	+	-
Methanospaera					
stadtmanae	37	(grows only on H_2 + methanol)			
Fam. Methanothermaceae					
Methanothermus					
fervidus	83	+	-	-	-
Order Methanococcales					
Fam. Methanococcaceae					
Methanococcus					
voltae	35-40	+	-	+	-
jannaschii	85	+	-	-	-

pseudomurein cell wall, a structural polymer differing from peptidoglycan. Other methanogens have proteinaceous (e.g., *Methanococcus*) or heteropolysaccharide (e.g., *Methanosarcina*) cell envelopes (53). *Methanospirillum hungatei* and *Methanothrix soehngenii* have an unusual thin fibrillar outer sheath (126, 127).

Ecology

Methanogens occupy a terminal niche in the mineralization of organic matter. These organisms are restricted to growth on a number of simple substrates (i.e., H_2, CO, formate, acetate, methanol, methylamines, and secondary alcohols) which represent the degradation products of more complex organic material entering anoxic environments (118, 122). Methanogens are found in a wide variety of anoxic habitats, including freshwater and marine sediments (53, 104, 130), animal digestive tracts (18, 81, 101), hydrothermal vents (54, 72), waste treatment digestors (21, 129), and even human dental plaque (8). Methanogens may also be found in syntrophic association with other microorganisms, where they function to consume H_2 or formate (22, 25, 111, 123).

As an end product of fermentative and acetogenic bacteria, acetate is an important precusor for methane in anaerobic ecosystems. In environments such as rice paddies, lake sediments, and anaerobic waste digestors, the majority of methane arises directly from acetate (24, 82, 103, 110). Biogenic methanogenesis from sources such as these is a significant source of atmospheric methane (100); thus methanogenesis from acetate is a significant process in global carbon and methane cycles. Mineralization of acetate by acetotrophic methanogens functions to remove complex carbon entering anaerobic environments, and contributes to the maintenance of a neutral pH (128). Currently, only two genera have been described which use acetate as a substrate, namely *Methanosarcina* and

Methanothrix (the latter generic nomenclature is currently a matter of debate with regard to replacement with *"Methanosaeta,"* see [88]). *Methanothrix* spp. are restricted to acetate as a sole substrate for methanogenesis (51, 89). *Methanosarcina barkeri* is one of the most catabolically versatile methanogens, which in addition to acetate, is able to catabolize methanol, H_2, methylamines, or carbon monoxide (Table 2.)

Biochemistry of methanogenesis

Despite considerable morphological and habitat diversity, methanogenic archaea use simple substrates, and do not degrade polysaccharides, proteins, amino acids, or sugars. The majority of methanogens isolated use hydrogen to reduce CO_2 to methane. Barker proposed in 1956 a general mechanism for methanogenesis (4). In this model, he suggested that carbon dioxide was reduced to methane on one carbon carriers in a series of two-electron reductions. Acetate and methanol were predicted to enter at the methyl oxidation level. With refinements, this remains the working model of methanogenesis.

A central C_1 carrier was identified when Wolfe and Taylor discovered the first of the unique coenzymes involved in methanogenesis, coenzyme M (106). Methyl-CoM serves as a one carbon carrier and is the direct precursor for methane formation by methyl reductase, an enzyme universally present in methanogens (31, 36, 96). This enzyme contains a nickel tetrapyrrole prosthetic group, F_{430} (30, 47). The direct donor of reducing equivalents for methyl-CoM reduction is the thiol cofactor, 7-mercaptoheptanoylthreonine phosphate (HTP) (61, 85) Methylreductase catalyzes the final step in methane formation:

$$CH_3\text{-CoM} + \text{HS-HTP} \rightarrow CH_4 + \text{CoM-S-S-HTP}\ (15, 37)$$

Table 2. Catabolic substrates of *Ms. barkeri* MS

Reaction	$\Delta G^{o'}$ (kJ/mol CH_4)	Reference
$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36	[108]
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-131	[108]
$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-107	[108]
$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	-51	[58, 108][a]
$4CH_3NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4$	-56.4	[63]

[a]calculated by summing the reactions for H_2 and CO_2 formation from CO and H_2 reduction of CO_2 to CH_4.

The mixed disulfide CoM-S-S-HTP is reduced by the enzyme heterodisulfide reductase (48, 90.). This enzyme has been purified from *Mb. thermoautotrophicum* (49), but its direct physiological electron donor is not currently known.

The carbon carriers and enzymes which participate in CO_2 reduction to methane have been extensively studied in the past decade, and this pathway has been elucidated in some detail. The initial step in CO_2 activation is reduction to the level of formate on the cofactor methanofuran (76, 77). The enzyme formylmethanofuran dehydrogenase in *Ms. barkeri* is a pterin-containing molybdo-iron-sulfur protein (57), and uses methanofuran-b as a cofactor (12, 79), This step is followed by transfer to a pteridine derivative, tetrahydromethanopterin (H_4MPT) (19, 28). This cofactor was initially identified by two laboratories as a yellow flourescent compound (27) and factor 342 (47); the complete structure was subsequently determined by van Beelen et al. (112). Formyl-H_4MPT is first converted to 5,10-methenyl-H_4MPT (32), and H_4MPT serves as the carbon carrier during reduction of the C_1 group to the methyl level in two 2-electron reductions (59). In *Ms. barkeri*, these reactions use F_{420} as a cofactor (59). F_{420} is a low potential 2 electron-carrying deazaflavin cofactor (35) widely distributed in methanogens. The methyl group is then transfered from H_4MPT to CoM by an enzyme-bound corrinoid intermediate and a methyl-corrinoid transferase (115). Recent reports provide evidence that the methyltetra-hydromethanopterin:CoM methyltransferase of *Methanosarcina* strain Gö1 is a membrane-bound corrinoid-dependent enzyme that translocates sodium ions across the cytoplasmic membrane (6, 7).

Catabolism of other methanogenic substrates proceeds by different initial biochemical steps, but shares features of the pathway of CO_2 reduction to methane. Formate is oxidized by formate dehydrogenase, which produces reducing equivalents and C_1 units for reduction to methane. The metabolism of

formate has been studied primarily in *Methanobacterium formicicum*, which posesses an F_{420}-linked formate dehydrogenase (97). This organism posesses a formate hydrogenlyase system which produces H_2 and CO_2 from formate (5). Entry of formate into the methanogenic pathway is currently a matter of discussion. It has been proposed that formate enters directly and is reduced to methane (5), or, alternatively, formate is first split to H_2 and CO_2 and subsequently converted to methane (124).

The methyl group of methanol is transferred to methyl CoM by two transferases. Methyltransferase I converts methanol into an enzyme-bound methyl-corrinoid intermediate (14, 114), and this methyl group is then transferred to CoM by the action of methyltransferase II (113). Reducing equivalents are generated by the oxidation of one methanol per three reduced. The oxidation of methyl groups has been proposed to take place on H_4MPT in a process essentially the reverse of CO_2 reduction to methyl-H_4MPT (58). A sodium motive force has been suggested as the driving force for the endergonic oxidation of methanol to the level of formaldehyde (11). The transfer of methyl groups from trimethylamine to CoM occurs by a specific transferase in *Ms. barkeri* (84); the generation of reducing equivalents from methyl groups is presumed to occur by reactions analogous to the case for methanol metabolism (31).

Results of numerous studies have led to the conclusion that transmembrane electrochemical gradients play an important role in energy coupling in methanogenic archaea (see 43, 75). In the chemiosmotic model of energy conservation, protons are translocated across the cytoplasmic membrane during electron transfer, and this gradient is then utilized by a H^+-translocating ATPase to drive the phosphorylation of ADP. *Methanosarcina barkeri* has been shown to couple H_2/methanol metabolism to energy conservation by means of a

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proton motive force (11). In addition to a proton gradient, it has been shown that Na^+ can also serve as a primary coupling ion in *Methanococcus voltae* (33). *Ms. barkeri* generates an electrochemical sodium gradient during methanogenesis from methanol by a Na^+/H^+ antiporter, and by primary electron transport driven sodium extrusion during methanogenesis from H_2:CO_2 (83).

To date, little detail is known about the nature of putative membrane-bound electron transfer chains in methanogens; however, some membrane-associated oxidoreductases and electron carriers have been described. Immunolocalization of methyl reductase has shown that this terminal electron-consuming enzyme is localized at the cytoplasmic membrane in some methanogenic archaea (1, 87). A high molecular weight complex containing methyl reductase and dubbed the 'methanoreductosome' has been visualized by electron microscopy to be associated with the membrane in *Mc. voltae* and *Methanosarcina* strain Gö1 (50, 80). Heterodisulfide reductase is over 50% membrane-associated in *Ms. barkeri* MS, and 20% membrane-associated in *Mtx. soehngenii* (99). Membrane-bound corrinoids (26, 98), FAD (74), iron-sulfur centers (73) and cytochromes *b* and *c* (55, 69-71) have been also been reported for methanogenic archaea. The organization of these components into electron transfer chains is at present poorly understood, and is an area of ongoing research.

Acetate catabolism

Pioneering studies with acetate enrichment cultures by Buswell and Sollo (23) demonstrated that CO_2 is not reduced to methane and inferred that methane arises from the methyl group of acetate. Stadtman and Barker later confirmed this conclusion by showing that in *Methanosarcina* methane is derived primarily from the methyl group of acetate, and carbon dioxide from the carboxyl group

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(105). Pine and Barker showed that deuterium atoms on the methyl moiety were retained after reduction to methane (93). These results suggested that methanogenesis from acetate proceeds by a so-called 'aceticlastic' mechanism:

$$^{\dagger}CH_3{}^{*}COOH \rightarrow {}^{\dagger}CH_4 + {}^{*}CO_2$$

The further study of the biochemistry of acetate degradation accelerated in 1978 with the isolation of pure cultures of acetotrophic methanogens (102, 117), With the later development of a cell-free system in *Ms. barkeri* which converted acetate to methane (66), in vitro study of the biochemistry of methanogenesis from acetate became possible.

Activation and cleavage of acetate

In acetotrophic methanogens, acetate is activated to acetyl-CoA, which serves as a substrate for carbon monoxide dehydrogenase (CODH) (38). *Methanothrix soehngenii* uses acetate thiokinase in a one step reaction (62), whereas *Ms. barkeri* employs acetate kinase and phophotransacetylase in a two-step reaction. (39, 46, 66). EPR spectroscopic and biochemical evidence has been presented that CODH functions in *Ms. barkeri* to cleave acetyl-CoA into a methyl and carbonyl moiety, as well as oxidizing the carbonyl group (65, 68). The methyl group is transfered to the one-carbon carrier, tetrahydromethanopterin (H_4MPT) (40, 45), and subsequently to CoM (68). Corrinoid proteins may be involved in methyl transfer, as indicated by inhibitor and labeling studies (34, 41, 116, 125).

Energetics of acetate catabolism

Expenditure of a phosphodiester bond in the activation of acetate represents a considerable investment of energy in a relatively low energy-yielding reaction. The standard free energy available from the conversion of acetate to methane and carbon dioxide is given by:

$$[1]\ CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad \Delta G^{o'} = -36\ kJ/mol \qquad (108)$$

which is very near the free energy of hydrolysis of ATP:

$$[2]\ ATP + H_2O \rightarrow ADP + P_i \qquad \Delta G^{o'} = -31.8\ kJ/mol \qquad (109)$$

The energetics of activation are summarized below (109):

$$[3]\ acetate + ATP \rightarrow acetyl\text{-}P + ADP \qquad \Delta G^{o'} = +13\ kJ/mol$$

$$[4]\ acetyl\text{-}P + CoASH \rightarrow acetyl\text{-}CoA + P_i \qquad \Delta G^{o'} = -9\ kJ/mol$$

Combining the preceding equations gives the following expression for the energy available from the conversion of activated acetate (acetyl CoA) to methane and carbon dioxide:

$$[5]\ acetyl\text{-}CoA + H_2O \rightarrow CH_4 + CO_2 + CoASH \qquad \Delta G^{o'} = -72\ kJ/mol$$

At a thermodynamic efficiency of 50%, within the range for anaerobic bacteria (see 109), 1.1 mol ATP could be conserved per mol acetate. Stoichiometric production of one ATP/acetate would result in no net energy gain, and

2ATP/acetate would require an efficiency of 88%. However, a nonstoichiometric mechanism of energy conservation (e.g., electron-transport coupled phosphorylation) would allow for the conservation of fractional amounts of ATP.

A chemiosmotic mechanism was initially proposed by Krzycki and Zeikus to function in acetate catabolism of *Ms. barkeri* (66) (Fig. 3). Inhibitor and uncoupler studies with *Ms. barkeri* have shown that acetate catabolism is accompanied by the generation of a primary proton-motive force across the cytoplasmic membrane, which is consumed to produce ATP (91).

The role of cytochromes

Membrane-bound *b*- and *c*-type cytochromes are present in several members of the genus *Methanosarcina* (69-71), including *Ms. barkeri* strain MS (71). *Ms. barkeri* strain MS contains several low-potential (E_m = ca. -182 mV to -330 mV) *b*-type cytochromes as well as a smaller amount of *c*-type cytochrome. The amount of cytochrome *b* (0.27-0.5 nmol/mg membrane protein) was found to be present in amounts similar to anaerobic respiring bacteria. Among the methanogens which have been examined, the presence of cytochromes is limited to the genera *Methanosarcina, Methanothrix, Methanolobus,* and *Methanococcoides* (69-71, 55). On the basis of the substrates utilized by these methanogens (i. e., those which contain methyl groups), it has been suggested that cytochromes function in methyl group oxidation (55). However, cytochromes are present at significant levels in cells of *Ms. barkeri* strain Fusaro grown on H_2:CO_2 (69), where there is no requirement for oxidation of substrate methyl groups. Alternatively, cytochromes may function in electron transport during methanogenesis by participating in methyl group reduction to methane. The presence of cytochromes is restricted to members of the family Methanosarcinae (44), so these electron carriers may be a common catabolic feature of this group.

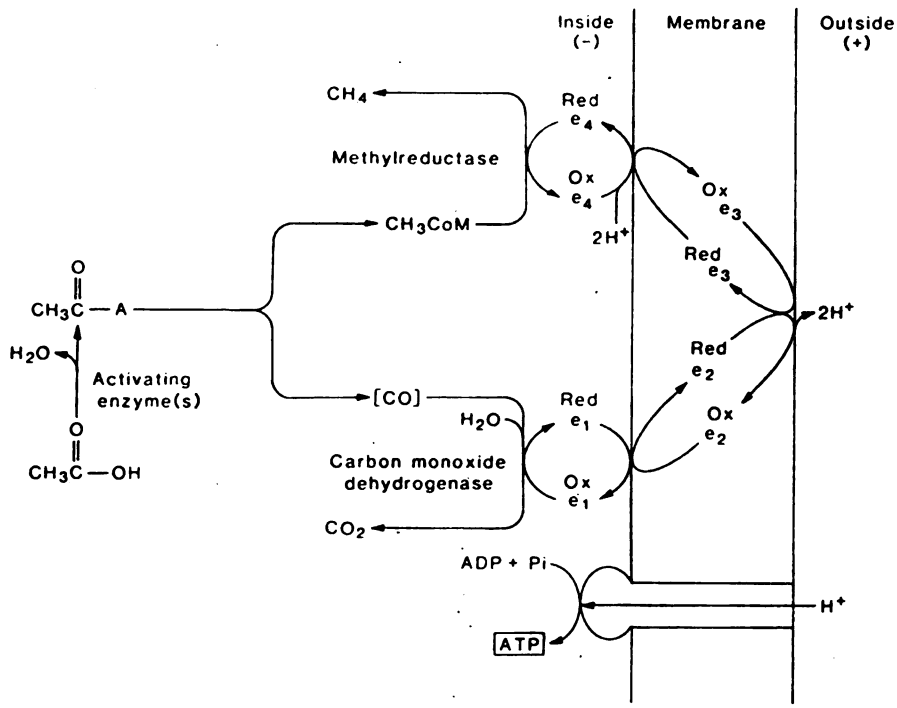

Figure 3. Hypothetical model for carbon and electron transformation and ATP synthesis during acetate catabolism by methanogens proposed by Krzycki and Zeikus (66).

In support of a role in methyl group reduction, a recent report suggests that the cytochromes in *Methanosarcina* strain Gö1 participate in energy conservation during electron transport from reduced F_{420} to CoM-S-S-HTP (56).

Hydrogen metabolism

Ms. barkeri produces a low steady state level of hydrogen, and consumes H_2, during acetate catabolism (64, 92). Hydrogen production during growth on acetate has also been observed in spp. of *Methanosarcina*, which consumed exogenously added H_2 to maintain partial pressures between 16 and 92 Pa (16, 78). Hydrogen production from acetate by *Ms. barkeri* was significantly enhanced by coculture with a H_2-consuming sulfate reducing bacterium (92). Competition for hydrogen by *Desulfovibrio vulgaris* resulted in a net oxidation of acetate; 30-33% of the reducing equivalents available from acetate were used to reduce sulfate to sulfide.

There are a number of lines of evidence that hydrogen is produced from carbonyl group oxidation during acetate catabolism. Hydrogen production from CO by the soluble fraction of cell extracts of acetate-grown *Ms. barkeri* has been shown (67), and this activity was shown to be dependent on ferredoxin (42). In cell extracts, it was found that Ti(III) could substitute for exogenous hydrogen; under these circumstances 1 mol H_2 was produced for every carbonyl group of acetyl CoA oxidized (41). Purified CODH itself has been shown to produce hydrogen at a low rate (0.28 μmol/min·mg) (10). Cells of *Ms. barkeri* produce H_2 and CO_2 from CO (86), and this activity was shown to couple with an increase of the proton motive force and phosphorylation of ADP (17).

OBJECTIVES

This research was conducted in order to understand the biochemistry of acetate degradation in relation to the electron transfer reactions associated with energy conservation. The research specifically focuses on the enzymes and cofactors involved in electron transfer between the C-1 and C-2 of acetate during methanogenesis by *Methanosarcina barkeri* MS, and coupling of this intramolecular redox event to chemiosmotic ATP synthesis. The following major objectives were undertaken:

(i) Investigate membrane-associated electron carriers and oxido-reductases, and identify their function in acetate catabolism by EPR and optical spectroscopic methods.

(ii) Purify and characterize the physical and catalytic properties of the membrane-bound hydrogenase present in acetate-grown cells.

(iii) Document routes of hydrogen production and consumption using purified and partially purified components from cell extracts.

(iv) Identify electron transfer reactions in a subcellular system that participate in chemiosmotic conservation of ATP.

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CHAPTER II

SPECTROSCOPIC AND ENZYMATIC EVIDENCE FOR MEMBRANE-BOUND ELECTRON TRANSPORT CARRIERS AND HYDROGENASE AND THEIR RELATION TO CYTOCHROME *b* FUNCTION IN *Methanosarcina barkeri*

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Spectroscopic and enzymatic evidence for membrane-bound electron transport carriers and hydrogenase and their relation to cytochrome *b* function in *Methanosarcina barkeri*

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1. SUMMARY

Membranes prepared from *Methanosarcina barkeri* cultured on acetate were examined for electron carriers using electron paramagnetic resonance (EPR) and optical spectroscopy. EPR analysis of membrane suspensions demonstrated multiple iron-sulfur centers of the 4Fe-4S type, a high-spin heme-like species and possibly rubredoxin. Optical spectroscopy demonstrated that a *b*-type cytochrome was reduced by molecular hydrogen and oxidized by methyl coenzyme M. A membrane-bound hydrogenase activity (14 μM $\cdot$ min^{-1} (mg protein)$^{-1}$) was detected. This suggests a putative role for cytochrome *b* and hydrogenase in electron transfer and methyl-group reduction during aceticlastic methanogenesis.

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2. INTRODUCTION

Various membrane-bound electron carriers have been discovered in methanogens, including cytochromes [1], a flavin [2] and iron-sulfur centers [3]. In addition, it has been suggested that cobamide-containing protein complexes might also function as electron carriers in *Methanobacterium* [4]. While a membrane-bound oxidoreductase and an electron transport chain are essential for current chemiosmotic models of energy transduction in methanogens, such a system has yet to be elucidated. This is particularly true for acetate catabolism to methane. The free energy available from the cleavage of acetate is roughly equivalent to the free energy of hydrolysis of ATP, which at cellular efficiencies would be insufficient to yield a high-energy phosphodiester bond via a stoichiometric reaction (i.e., substrate-level phosphorylation).

We have previously proposed the hypothesis that an electrochemical gradient is produced by channeling electrons through membrane carriers and concomitantly translocating protons across the membrane as a mechanism for conserving non-stoichiometric amounts of energy from each

acetate cleaved during methanogenesis [5]. We have demonstrated that aceticlastic methanogenesis in *M. barkeri* extracts was dependent on soluble enzyme, acetate, hydrogen and ATP. In addition, hydrogen production was demonstrated during growth of *M. barkeri* on acetate as the sole carbon and energy source [6,7] and cell extracts of acetate grown cells readily convert CO to H_2 [8]. The purpose of the present report is to examine the presence and role of membrane-bound redox centers and hydrogenase in oxidoreduction of intermediary metabolites derived from acetate catabolism. We present the first evidence in support of the involvement of cytochrome *b* and membrane-bound hydrogenase in both methanogenesis and electron transfer coupled to acetate catabolism by *M. barkeri*. In addition, we report the presence of several iron-sulfur redox centers detectable by EPR spectroscopy and discuss their relationship to hydrogenase and rubredoxin.

3. MATERIALS AND METHODS

All chemicals used were of reagent grade. Methyl coenzyme M; (CH_3-CoM; 2-[methylthio] ethanesulfonic acid, sodium salt) was prepared by the method of Romesser and Balch [9]. Chemical reductants and oxidants were placed in stoppered vials as a powder, flushed and evacuated with argon, and reconstituted with anaerobic 50 mM *N*-morpholinopropanesulfonic acid (Mops) buffer (pH 7.0).

3.1. Cell culture

M. barkeri strain MS (DSM800, acetate-adapted) was grown in 45-l carboys on 100 mM sodium acetate at 37°C, as described previously [10], except that twice the concentration of filter-sterilized vitamins was added after autoclaving. Cells of 15-day cultures were allowed to settle, and the spent medium above them displaced with N_2 gas. The cells were transferred to stoppered centrifuge tubes and washed 3 times in cold 50 mM Mops buffer by centrifugation at $8000 \times g$ for 15 min. Cells were stored at −70°C until use.

3.2. Preparation of extracts

All manipulations were performed anaerobically under a hydrogen headspace. Cells were suspended in 50 mM Mops (1 ml (g wet wt)$^{-1}$) and ruptured by passage through a French pressure cell at 138 MPa into stoppered centrifuge tubes. Cell debris was removed by centrifugation at $27000 \times g$ for 15 min. The membrane fraction was pelleted from the supernatant by centrifugation at $150000 \times g$ for 1 h. The gelatinous membrane pellet was resuspended with a Potter-Elheim homogenizer in 50 mM Mops buffer and recentrifuged at $15000 \times g$ for 15 min to remove particulate material. Membranes prepared for EPR analysis were washed first with buffer containing 10 mM EDTA to remove adventitiously bound metals, and then washed again with 50 mM Mops buffer. The final membrane suspension was prepared in a small volume of Mops buffer, transferred to stoppered vials, and flushed and evacuated with argon.

3.3. Spectroscopic analysis

Optical spectroscopy was performed using a Beckman DU-7 spectrophotometer using 1-ml glass cuvettes fitted with a rubber stopper under the indicated gas phases. EPR analyses were performed using a Varian E-109 spectrometer equipped with an Oxford ESR-900 liquid helium cryostat.

3.4. Enzyme assays

Hydrogenase and carbon monoxide dehydrogenase were measured by following methyl viologen dye reduction at 578 nm. Assays were performed in 100 mM Tricine buffer (pH 8.1) at 37°C. Cuvettes were allowed to equilibrate with the 100% headspace (either H_2 or CO) and the reaction was initiated by addition of supernatant or membrane preparation, and corrected for endogenous levels of dye reduction.

4. RESULTS AND DISCUSSION

Fig. 1A shows the EPR spectra of washed membrane preparations of *M. barkeri* after reduction by dithionite or oxidation by thionin. Reduced membranes possessed prominent features at apparent g values of 2.08, 2.0, 1.96, 1.94, and 1.89.

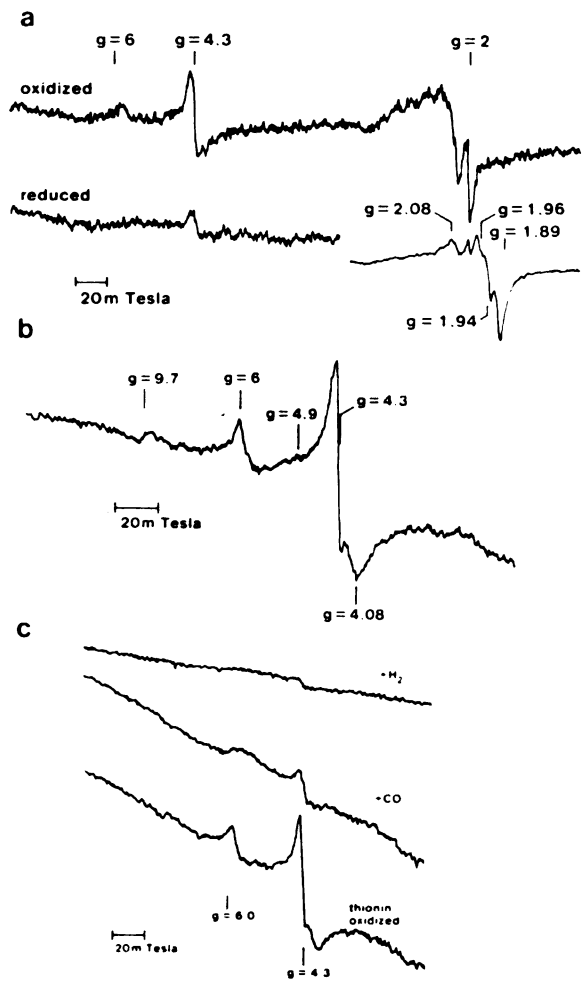

Fig. 1. EPR spectra of oxidized and reduced membranes of *M. barkeri*. (A) Normal spectrum. Membranes were prepared as described in MATERIALS AND METHODS and then either oxidized with thionin until the sample was light blue, or reduced by the addition of sodium dithionite. Samples were then frozen in chilled iso-octane at −50 °C. Spectra were recorded at 10 K, a microwave power of 10 mW and a frequency of 9.24 GHz, modulation amplitude of 1 mT, time constant of 0.128 s, and scan time of 100 mT·min^{-1}. (B) Expanded spectrum of thionin-oxidized membranes at low magnetic field. EPR parameters were as in above, except that the modulation amplitude was 1.6 mT and the microwave power was 20 mW. (C) Reduction of high-spin heme and g = 4.3 feature by CO and H_2. Membranes were first anaerobically oxidized with thionin, then incubated at room temperature under 0.5 atm CO or H_2 for 5 min then frozen.

The location and line-shape of these features is typical of iron-sulfur clusters, and this spectrum is probably composed of two or more overlapping signals. At least three different clusters were detectable upon examination of spectra from 8 K to 25 K, as evidenced by their different apparent g values (data not shown). No iron-sulfur signals were detectable at temperatures above 40 K and microwave power of 20 mW. The intensity of the feature at g = 1.89 decreased by 75% when the temperature of the cavity was raised from 9 K to 20 K. These observations suggest that the majority of the signal arises from several Fe_4S_4 clusters.

Spectra of oxidized membranes consistently showed two sharp features near g = 2. The higher field feature was observed up to 80 K, as expected for a 'radical' signal. The second feature (at g = 2.02) could only be observed between 10 K and 35 K. The general position of this feature is suggestive of a trinuclear cluster, but since three iron-sulfur clusters generally cannot be observed above 20 K, assignment of this feature is uncertain. At very low field, several interesting features can be seen (see Fig. 1B for an expanded spectrum of this region). At g = 6 a feature is readily apparent which is suggestive of a high-spin heme. Typically, *b*-type cytochromes are not high-spin [11]. In addition, a very strong feature at g = 4.3 is present. While the latter signal is usually ascribed to adventitiously bound iron, the sharpness of the spectral feature, together with the presence of the small feature at g = 9.7, suggest the presence of rubredoxin [12]. Similar behavior at this temperature was described for rubredoxin isolated from *Pseudomonas oleovorans* [12].

Fig. 1C details an experiment where physiological electron donors were tested for their ability to reduce the putative rubredoxin signal or the high-spin heme. Both signals were substantially decreased in the presence of either reductant, indicating the reduction of high-spin ferric to ferrous iron and the consequent loss of signal. Both CO and H_2 were also able to reduce thionin-oxidized membrane-bound iron-sulfur clusters (data not shown).

It is evident that the membrane of *M. barkeri* possesses numerous iron-sulfur clusters whose biochemical structure function attributes require further study. Hydrogenases are usually iron-sulfur proteins [13], and soluble hydrogenase has been purified from *M. barkeri* cell extracts [14]. Experi-

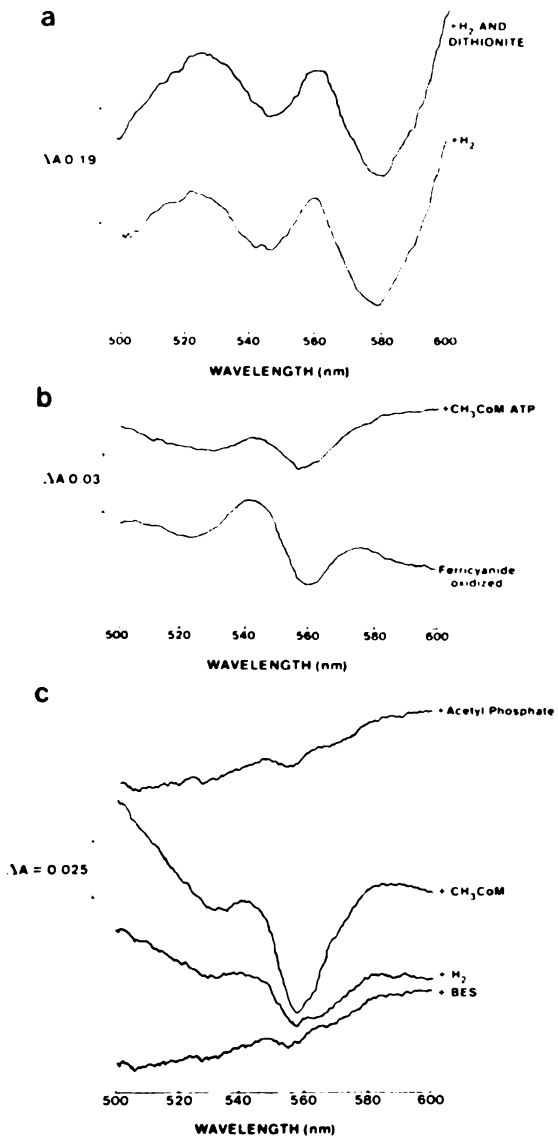

Fig. 2. Oxidoreduction of membrane-bound cytochrome *b* of *M. barkeri*. The curved spectral features that distort the cytochrome spectra are attributed to oxidoreduction of cobamide-containing species present in methanogens including *Methanosarcina* [21]. (A) Reduced minus oxidized spectrum preparation reduced by hydrogen and by sodium dithionite. Cuvettes contained 0.2 ml membrane preparation, 0.2 ml anaerobic 50 mM Mops buffer, and 50 μl high-speed supernatant (150000 × *g*). Membranes were used as prepared, the baseline was recorded with N_2 in the headspace, and reduced by addition of hydrogen and subsequently by sodium dithionite. (B) Oxidation by CH_3-CoM and ATP. Upper spectrum is membrane preparation with added ATP (10 mM) and CH_3-CoM (10 mM) vs. 'as is'. Oxidation was dependent on both ATP and CH_3-CoM, and was not contained by either reagent alone. Lower spectrum was taken after the addition of sodium ferricyanide. (C) Oxidation and reduction by sequential addition of methanogenic substrates and inhibitors. (1) Membrane preparation difference spectrum under nitrogen after addition of acetyl phosphate (10 mM). (2) Membrane preparation difference spectrum following addition of CH_3-CoM. (3) Same cuvette as above after headspace was flushed with hydrogen and allowed to equilibrate. (4) Same cuvette as above after addition of BES. Methane was detected in the headspace at the end of the experiment.

Table 1

Comparison of hydrogenase vs. CO dehydrogenase activities in *M. barkeri* fractions [a]

High-speed fraction	Specific activity (μmol·min^{-1}(mg protein)$^{-1}$	
	CO dehydrogenase	Hydrogenase
Soluble	12.0	8.9
Washed membranes	1.0	14.0

[a] Crude cell extracts were subjected to anaerobic ultracentrifugation at 150000 × *g* for 1 h, and CO or H_2 oxidation was assayed by methyl viologen reduction.

ments were performed to ascertain whether *M. barkeri* contained a membrane-bound hydrogenase. Table 1 shows that hydrogenase activity was higher in the washed membrane pellet fraction than in the supernatant fraction when crude extracts of *M. barkeri* were subjected to ultracentrifugation. CO dehydrogenase served as control, and its activity was much higher in the soluble fraction.

Optical spectra of membranes from *M. barkeri* strain MS (data not shown) demonstrated the presence of one or more *b*-type cytochromes and CO induced a similar spectral shift of the cytochrome *b*, as previously documented for *M. barkeri* strain Fusaro [1,15].

Fig. 2A demonstrates that membrane-bound cytochrome *b* was reduced to an equal extent by either dithionite or the addition of hydrogen. Since hydrogenase of methanogens has been shown to couple to soluble electron carriers such as F_{420}-deazoflavin, the present results cannot discriminate between an indirect or direct reduction of cytochrome *b* by hydrogenase, but clearly the cytochrome is reduced by a physiological reductant.

We have previously provided evidence that

carbon monoxide dehydrogenase, methyl CoM and methyl reductase were required for aceticlastic methanogenesis [16]. Fig. 2B shows that the membrane-bound *b*-type cytochrome was not only oxidizable by ferricyanide but could also be oxidized upon addition of ATP, CH_3-CoM and the high-speed supernatant fraction. Cytochrome *b* oxidation was totally dependent on both ATP and CH_3-CoM; it was not oxidized by the soluble enzyme fraction alone. The extent of the enzyme mediated oxidation of cytochrome *b* was approx. one-third of that observed upon chemical oxidation by ferricyanide. Apparently cytochromes can donate electrons either directly to the methyl reductase complex or indirectly to membrane-bound electron carriers connected to this enzyme system.

We have found that acetyl phosphate replaces the acetate and ATP requirement for cell-free aceticlastic methanogenesis in *M. barkeri* [16]. Experiments were designed here to test whether methanogenic intermediates involved in aceticlastic methanogenesis would result in oxidoreduction of cytochrome *b* (Fig. 2C). The addition of acetyl phosphate alone to the membrane preparation did not result in net oxidation of the *b*-type cytochrome. However, it did replace the ATP requirement for oxidation of the cytochrome *b* by CH_3-CoM. The subsequent addition of hydrogen to the headspace of the cuvette resulted in an intermediate oxidation state of the cytochrome *b*. Apparently this was the result of competition between the H_2-reducing and CH_3-CoM oxidizing reactions for cytochrome. The subsequent addition of bromoethanesulfonic acid (BES), an inhibitor of methyl reductase, collapsed the steady state and resulted in complete reduction of the cytochrome.

Thus far, cytochromes have only been detected in methanogens that must oxidize methyl groups during growth, such as methanol or acetate [17,18]. *M. barkeri* strain Fusaro has been shown to contain two *b*-type cytochromes when grown on methylamines and an additional *b*-type cytochrome when grown on acetate [1]. Thus, it has been suggested that cytochromes function in methyl-group oxidation to CO_2 [17].

Alternatively, we propose here that cytochrome *b* may also function as an intermediary electron carrier in methyl-group reduction to CH_4. Thus, cytochromes may also be involved in energy conservation in methanogenic species like *M. barkeri* which catabolize acetate and methanol as well as H_2/CO_2. Since the energetics and redox relationships of acetate transformation to methane are quite different from that of H_2/CO_2 [19], it is plausible that species lacking acetate catabolism may have evolved separately and might not require cytochromes for energy conservation.

The model advanced by Krzycki and Zeikus [20] suggests that acetate catabolism proceeds via a system of membrane-bound electron carriers and oxidoreductases that translocate protons during electron transport linked to methanogenesis. ATP is then conserved by chemiosmotic coupling. The present work suggests that cytochrome *b* and hydrogenase are part of such a membrane electron transfer system. However, whether hydrogenase, iron-sulfur centers and cytochrome *b* which are present in the *M. barkeri* membranes also function in an electron transport coupled phosphorylation system during aceticlastic methanogenesis remains to be proven. Experiments in progress are aimed at purification and characterization of the membrane components detected here.

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CHAPTER III

PURIFICATION AND CHARACTERIZATION OF MEMBRANE-BOUND HYDROGENASE FROM *Methanosarcina barkeri* MS

ABSTRACT

Hydrogenase was purified from the membrane of acetate-grown *Methanosarcina barkeri* MS. The overall purification was 13-fold to >95% homogeneity with a yield of 9.3%. Solubilization of the enzyme was accomplished with Triton X-100 under anaerobic conditions and purification was carried out by hydroxylapatite, immobilized [Cu^{2+}], and anion exchange chromatography under aerobic conditions. The enzyme was reactivated under reducing conditions in the presence of H_2. The enzyme showed a maximal activity of 120 $\pm$ 40 μmol H_2 oxidized·min^{-1}·mg^{-1} with methyl viologen as an electron acceptor, a maximal hydrogen production rate of 45 $\pm$ 4 μmol H_2·min^{-1}·mg^{-1} with methyl viologen as electron donor, and an apparent K_M for hydrogen oxidation of 5.6 $\pm$ 1.7 μM. The molecular weight estimated by gel filtration was 98,000. SDS-polyacrylamide gel electrophoresis showed the enzyme to consist of two polypeptides of 57,000 and 35,000 present in a 1:1 ratio. The native protein contained 8$\pm$2 mol Fe, 8$\pm$2 mol S^{2-}, and 0.5 mol Ni/mol enzyme. Noncovalently bound flavin was not detected. Hydrogen oxidation was coupled to the artificial electron acceptors methyl and benzyl viologen, methylene blue, and phenosafranine. The membrane-bound hydrogenase activity mediated the reduction of cytochrome *b* in a solubilized protein preparation. The hydrogenase did not couple with autologous F_{420} or ferredoxin, nor with FAD, FMN, or NAD(P)+. The enzyme was inhibited by CO (1 atm) but not by CN^- (1 mM). The physiological function of the membrane-bound hydrogenase in hydrogen consumption is discussed.

INTRODUCTION

Hydrogen is a key substrate for methanogenic archaea, providing reducing equivalents for biosynthetic reactions as well as catabolic methane formation. The enzyme hydrogenase is central to this metabolism. Most methanogens can grow at the expense of molecular hydrogen, and hydrogenases have been purified from a number of different methanogenic archaea (for review see 25, 26). Members of the genus *Methanosarcina* are capable of growth on H_2/CO_2, as well as on methanol, methylamines, CO, and acetate. In two reports, F_{420}-reactive hydrogenase has been purified from the soluble fraction of methanol-grown *Ms. barkeri* strain MS (7) and Fusaro (8).

Hydrogen metabolism appears to play a role during unitrophic growth of *Ms. barkeri* MS on acetate. Cell suspensions of *Ms. barkeri* MS were shown to produce hydrogen in an acetate-dependent manner, and contain hydrogenase activity as measured by a tritium exchange assay (16). We have presented the first evidence of both membrane-bound and soluble hydrogenase activity in acetate-grown cells of *Ms. barkeri* MS (13), which is present at specific activities comparable to H_2/CO_2-grown cells (14). We have also demonstrated the reduction of membrane-bound redox centers by hydrogen, and subsequent methyl-CoM-dependent reoxidation of cytochromes (13). These findings imply that membrane-bound hydrogenase may function to channel reducing equivalents to an electron transport chain involved in methanogenesis and energy conservation.

The purpose of this study was to purify the membrane-bound hydrogenase and study its physical and catalytic properties in more detail, and to compare its properties to previously reported soluble hydrogenases. Parts of this study were presented in preliminary form (14).

MATERIALS AND METHODS

Chemicals, gases, and chromatographic media.

All chemicals used were reagent grade or better. DNAse I, gel filtration standards, FAD, FMN, $NAD(P)^+$, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate) and dyes were obtained from Sigma Chemical Company (St. Louis, MO). Triton X-100 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Gases were obtained from AGA Specialty Gas (Cleveland, OH). Bio-Gel HTP hydroxylapatite was purchased from Bio-Rad Laboratories (Richmond, CA). Chelating Sepharose Fast Flow, MonoQ, and Superose were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ).

Cell culture.

Methanosarcina barkeri strain MS (DSM 800) was grown in stirred 45 L aspirator carboys in the dark on phosphate buffered basal medium containing 100 mM sodium acetate at 37°C as previously described (16) except that twice the concentration of filter-sterilized vitamins was added after autoclaving. Exponentially growing cells were harvested anaerobically by allowing them to settle to the bottom of the carboy, and the medium above them displaced with N_2 gas. The cells were transferred to stoppered anaerobic centrifuge tubes and washed 3 times with cold anaerobic 50 mM MOPS buffer by centrifugation at 2500 X *g* for 20 min. Cells were stored at -70°C under H_2 until use.

Purification of membrane-bound hydrogenase.

All steps were performed at 4°C.

(i) Step 1. Preparation and solubilization of membrane fraction. Isolation of the membrane fraction was performed in sealed vessels or inside an anaerobic chamber (Coy, Ann Arbor, MI) filled with 5%/95% H_2/N_2. Thawed cells (160 g) were suspended at 1 mL/g wet weight in 20 mM sodium phosphate buffer pH 7.5 along with a few crystals of DNAse I. The cells were ruptured by passage through an Aminco French pressure cell (Urbana, IL) at 140 MPa into a stoppered vial. Cell debris were removed by centrifugation at 15,000 X *g* for 20 min. The membrane fraction was pelleted from the supernatant by centrifugation at 150,000 X *g* for 90 min; the supernatant was promptly poured off. The membrane pellets were dispersed with a Potter-Elvehjm homogenizer inside the anaerobic chamber and suspended in 310 mL of 20 mM sodium phosphate pH 7.5 buffer containing 2% vol/vol Triton X-100 (10 mg detergent:mg protein). This suspension was flushed and evacuated with N_2 to remove hydrogen and allowed to stand for 2 h before exposure to air. All subsequent steps were performed aerobically. The suspension was stirred for 14 h and recentrifuged at 150,000 X *g* for 90 min; the supernatant at this point contained the majority of hydrogenase activity.

(ii) Step 2: Hydroxylapatite chromatography. The ultracentrifugal supernatant from solubilized membranes was applied at 0.5 mL/min to a 2.6 X 17 cm hydroxylapatite column which had been equilibrated with 20 mM sodium phosphate buffer pH 7.5, 2% Triton X-100. The column was washed with 200 mL of the equilibration buffer and developed with a 500 mL linear gradient of 20-100 mM sodium phosphate. Fractions (10 mL) were collected and assayed for hydrogenase activity

(iii) Step 3. Affinity chromatography. Immobilized [Cu^{2+}] affinity chromatography was used as a subsequent purification step. A 2.6 X 9 cm column of Chelating Sepharose Fast Flow preequilibrated in buffer containing 50 mM Tris-acetate pH 7.5, 0.5 M NaCl, and 1% Triton X-100 was charged with 560 μmol Cu^{2+} applied as a 1% $CuSO_4 \cdot 5H_2O$ solution. Fractions containing hydrogenase activity from Step 2 were applied directly to the column, and the column was washed with 150 mL of equilibration buffer. Protein was eluted at 1 mL/min with a 250 mL descending pH gradient in the equilibration buffer from 7.5 to 4.0 (adjusted to pH 4.0 with acetic acid).

(iv) Step 4. Anion-exchange chromatography. Fractions containing hydrogenase activity from Step 3 were adjusted to pH 7.5 by addition of Tris base and concentrated to 5 mL using a flow dialysis cell (W. R. Grace & Co., Beverly, MA) fitted with a YM30 membrane (molecular weight cutoff 30,000). The protein was diluted to 50 mL with water, and applied to a Mono Q HR 5/5 column equilibrated in 20 mM Tris HCl pH 7.5, 1% Triton X-100. The column was developed with a 50 mL linear gradient of 0-0.4 M NaCl at a flow rate of 0.5 mL/min. Fractions containing hydrogenase activity were pooled and stored at -70°C.

Solubilization of hydrogenase and cytochrome *b*-containing system from membranes.

All steps were carried out anaerobically. Cells (40g) were lysed and membranes were pelleted as described above in a buffer containing 50 mM Tris HCl (pH 7.5). The membrane pellet was washed once in 50 mL of buffer, and suspended in 25 mL of buffer containing 2% Triton X-100 with a Potter-Elvehjem

homogenizer. After ultracentrifugation, the supernatant containing hydrogenase and cytochrome *b* was collected and kept under N_2 on ice until use.

Molecular weight determination.

The molecular weight of the hydrogenase was estimated using a Superose 12 gel filtration column equilibrated with 20 mM Tris HCl (pH 7.5) buffer containing 100 mM NaCl and 0.1% (vol/vol) Triton X-100. Standards for calibration were ferritin (443,000), β-amylase (200,000), alcohol dehydrogenase (150,000), aldolase (160,000), bovine serum albumin (66,000), and hen ovalbumin (43,000).

Hydrogenase assays.

Uptake hydrogenase activity was assayed by following methyl viologen dye reduction at 578 nm in 100 mM Tris HCl buffer (pH 7.8) at 37°C. In the standard assay, cuvettes were allowed to equilibrate with a 100% H_2 headspace. For kinetic studies, varying amounts of H_2 were injected into a N_2 headspace and the dissolved H_2 was calculated from solubility data (11). Sufficient sodium dithionite solution was added to produce a faint blue color and the reaction was initiated by injection of hydrogenase. Coupling to other carriers was conducted in the same buffer and monitored spectrophotometrically at the following wavelengths: $NAD(P)^+$ (340 nm), F_{420} (420 nm), FAD (450 nm), FMN (455 nm), ferredoxin (388 nm), benzyl viologen (600), methylene blue (670 nm), phenosafranin (521 nm). Autologous ferredoxin and F_{420} were isolated from the ultracentrifugal supernatant of acetate-grown cells as described in Chapter 4. Coupling to neutral red (426 nm) was carried out at pH 7.0 in 100 mM MOPS buffer. Production hydrogenase activity was assayed in 8 mL stoppered vials containing 1 mL 100 mM Tris HCl (pH 7.8), 10 mM methyl viologen, and 30 mM

sodium dithionite under N_2. After injecting enzyme, vials were incubated in a 37°C shaking water bath. Hydrogen evolution was quantitated by periodically removing samples of the headspace and assaying on a Packard gas chromatograph equipped with a thermal conductivity detector. Maximal linear rates were observed during the first 20 min.

Gel electrophoresis.

SDS polyacrylamide gel electrophoresis was carried out by the method of Laemmli (18) using a BioRad minigel apparatus. Prior to SDS-PAGE, protein in dilute solution was precipitated with an equal volume of cold 10% trichloroacetic acid and centrifuged for 10 min at 15,000 x g. The pellets were washed with 0.3 M Tris HCl (pH 7.5), suspended in denaturing sample buffer to a concentration of 1 μg/mL, and vortexed at room temperature until dissolved (<30 min). Gel scanning was performed with a Kodak BioImage Visage 110 scanning densitometer. Native linear gradient gel electrophoresis was performed as described (19) using a Tris-borate-EDTA buffer (pH 8.2). Electrophoresis was performed at room temperature at 80 V for 8 hr. Proteins were stained with Coomassie brilliant blue. Lanes to be stained for activity were excised and removed to a glove bag. The strips were soaked in 100 mM Tris HCl buffer (pH 7.8) containing 10 mM methyl viologen and sufficient sodium dithionite to turn the solution light blue. The gel strips were then placed in the airlock and flushed with H_2. A dark blue band of hydrogenase activity developed within 15 min. The stain was fixed by addition of 1 mg/mL 2,3,5-triphenyltetrazolium chloride, after which the band turned red. The gel was then removed from the glove bag and washed extensively with water.

Analytic methods.

Protein was determined with the BCA reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. Metals were analyzed using a Thermo Jarrell-Ash Polyscan 61E ICP-atomic emission spectrophotometer. Complexed iron was determined colorimetrically at 562 nm using the chelating agent Ferrozine as described by Fish (9). Acid-labile sulfide was determined by the semimicro method of Beinert (2) using a gravimetric Na_2S standard. Absorbance spectra were obtained with a Gilford Response II spectrophotmeter (Oberlin, OH).

RESULTS

Distribution of hydrogenase activities in acetate-grown *Ms. barkeri* MS.

We have previously reported that methyl-viologen linked hydrogenase activity in acetate-grown cells of *Ms. barkeri* MS distributes between the soluble and membrane fractions (13). Inspection of the data in Table 1 shows that hydrogenase specific activity is higher in the membrane fraction (8.0 U/mg) as opposed to crude activity (4.6 U/mg). It is also apparent that the membrane-bound activity represents 12% of the total methyl viologen-linked activity found in the cell lysate.

Purification of membrane-bound hydrogenase.

Methyl viologen-linked hydrogenase was purified from the membrane fraction by the scheme outlined in Table 1. The enzyme was solubilized with 2% Triton X-100; after this treatment typically ~80% of activity was found in the supernatant after ultracentrifugation. This fraction was adsorbed to a hydroxylapatite column and eluted with Na-phosphate as shown in Fig. 1. The majority of activity eluted in a peak corresponding to 50 mM Na phosphate. Use of hydroxylapatite as a first step improved the efficiency of subsequent chromatographic steps as analyzed by SDS-PAGE (not shown). The active fractions were pooled, loaded onto an immobilized [Cu^{2+}] affinity column, and eluted with a decreasing pH gradient as shown in Fig. 2. The binding of the enzyme to this column was specific for copper; immobilized Zn^{2+}, Co^{2+}, Ni^{2+}, or Mn^{2+} did not bind the hydrogenase. The yellow active fractions were adjusted to pH 7.5, desalted by flow dialysis, and subjected to anion exchange chromatography on MonoQ. The enzyme eluted at 100 mM NaCl, and was

Table 1. Purification of membrane-bound hydrogenase from *Ms. barkeri* MS

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	7700	35000	4.6	-	-
Membrane fraction	530	4200	8.0	100	1
Triton X-100 solubilization	400	3200	8.1	76	1.0
Hydroxylapatite	20	1000	50	24	6.3
Immobilized [Cu^{2+}] affinity chromatography	4.8	450	94	11	12
Mono Q	3.9	390	100	9.3	13

[a]Units of activity are expressed as µmol hydrogen oxidized/min @ 37°C using a methylviologen-linked assay

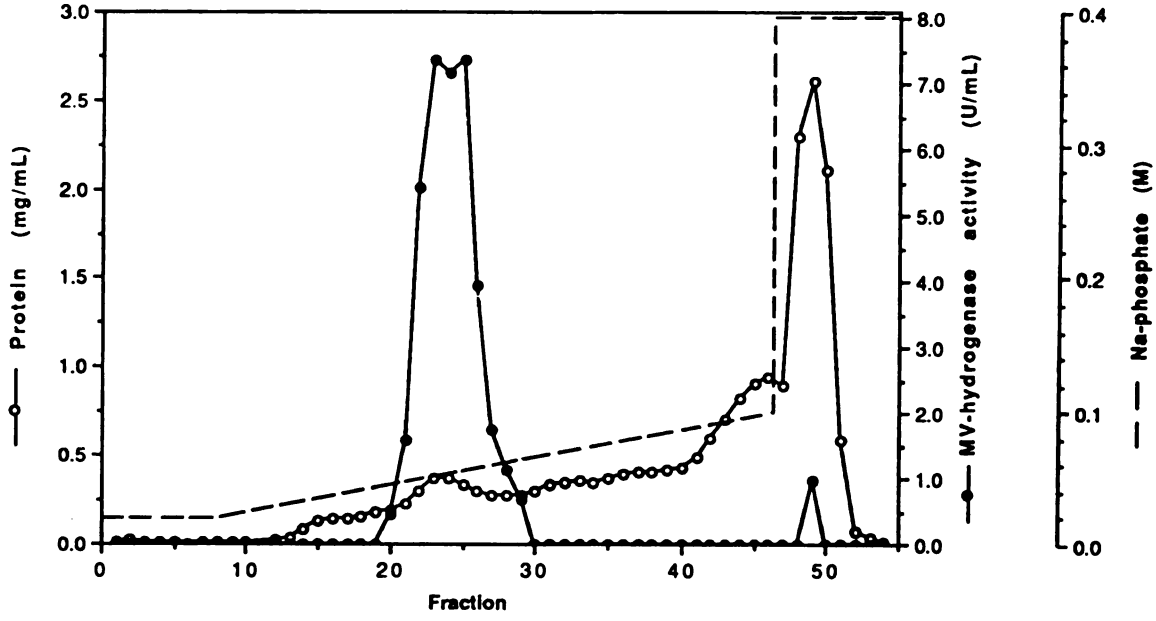

Fig. 1. Hydroxylapatite chromatography of membrane-bound hydrogenase of *Ms. barkeri* MS. 232 mL of Triton X-100 solubilized membrane protein (464 mg) was applied to a 2.6 X 17 cm hydroxylapatite column and washed with 200 mL of equilibration buffer. The column was developed with a gradient of 20-100 mM Na-phosphate at 0.5 mL/min, 10 mL/fraction.

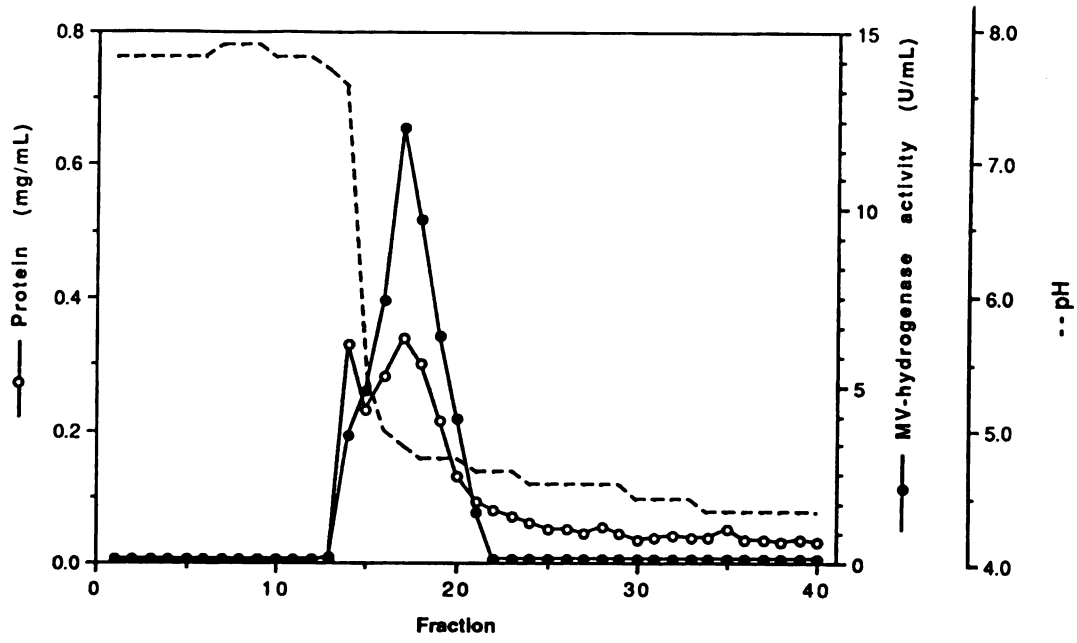

Fig. 2. Immobilized [Cu^{2+}] affinity chromatography of membrane-bound hydrogenase of *Ms. barkeri* MS. Active fractions from hydroxylapatite chromatography were pooled and loaded directly onto a 2.6 X 9 cm column of Chelating Sepharose Fast Flow charged with Cu^{2+}. Proteins were eluted with a descending pH gradient (7.5-4.0) at 1 mL/min, 5 mL/fraction.

judged as >95% pure by scanning densitometry of SDS-PAGE (Fig. 3). The overall purification was 13-fold with a yield of 9.3%.

Molecular properties.

Estimation of the molecular weight of the native enzyme by gel filtration on Superose 12 gave a value of 98,000. SDS-polyacrylamide gel electrophoresis of the protein showed the presence of two polypeptides with mobilities corresponding to 57,000 (α) and 35,000 (β) (Fig 3). Integration of the staining intensities of each band and correction for M_r gave an α/β ratio of 1.04, indicating that the subunits are present in a 1:1 ratio with a minimal molecular weight for the native enzyme of 92,000. Thus, the purified hydrogenase appears to be a heterodimer of $\alpha\beta$ configuration. Activity staining of a native linear gradient (4-20%) polyacrylamide electrophoretic gel showed that the purified hydrogenase migrates as a single broad band (Fig. 4). Excision of the active band and subsequent SDS-PAGE analysis showed the presence of the α and β subunit (not shown), indicating that the heterodimer is the minimally active form.

The absorbance spectrum of the purified hydrogenase showed a broad feature near 400 nm, suggesting the presence of iron-sulfur clusters (Fig 5). Colorimetric determination of iron and acid-labile sulfide gave values of 8 ± 2 mol Fe/mol enzyme and 8 ± 2 mol S^{2-}/mol enzyme. Determination of metals by plasma emission spectroscopy confirmed the presence of Fe and showed the presence of 0.5 mol Ni/mol enzyme. Other transition metals assayed simultaneously (Co, Cu, Mn, Mo, Ni, V, Zn) were not present in significant amounts. The sensitivity of the assay for selenium was too low to provide a determination. The presence of flavin was assayed spectroscopically after by precipitation of the enzyme with 5% trichloroacetic acid. No chromophores were

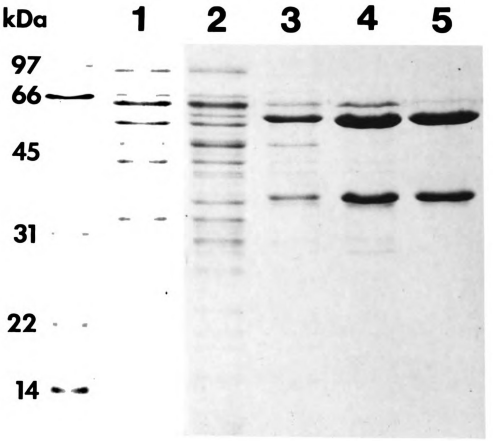

Fig. 3. SDS-PAGE analysis of purification of *Ms. barkeri* MS membrane-bound hydrogenase. Samples were separated in a 12% acrylamide gel and stained with Coomassie brilliant blue. Molecular mass standards are shown at the left of the figure. Lane 1, crude cell protein. Lane 2, membrane protein. Lane 3, hydroxylapatite pool. Lane 4, immobilized [Cu^{2+}] affinity chromatography pool. Lane 5, Mono Q pool. Each lane was loaded with 10 μg protein.

Fig 4. Activity staining of purified membrane-bound hydrogenase of *Ms. barkeri* MS. Samples were separated by native linear gradient (4-20%) gel electrophoresis. Lane 1, 50 μg crude protein stained for protein. Lane 2, 5μg purified membrane-bound hydrogenase stained for protein. Lane 3, 5μg purified membrane-bound hydrogenase stained for activity. Position of the bromphenol blue dye front is indicated by arrow.

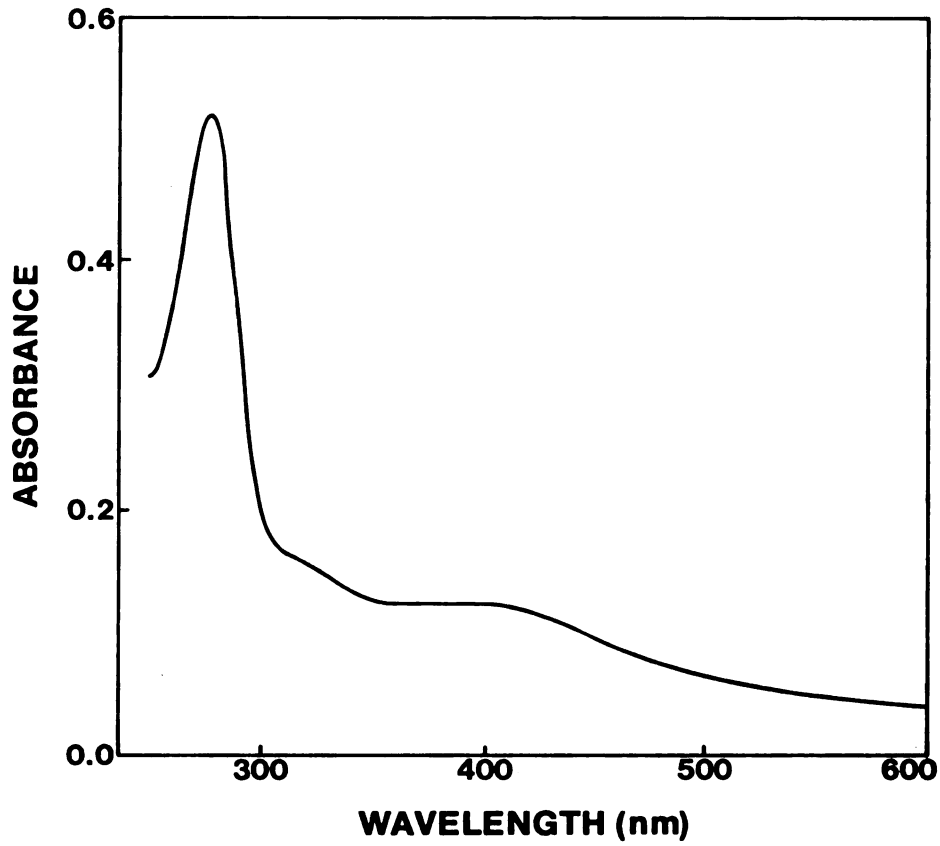

Fig. 5. Electronic absorption spectrum of membrane-bound hydrogenase of *Ms. barkeri* MS. Triton X-100 was removed from the purified enzyme by adsorption to DEAE-Sepharose and exchanging with a buffer containing 20 mM Tris/HCl (pH 7.5) and 5 mM CHAPS. The enzyme (0.3 mg/mL) was eluted with 200 mM NaCl.

detectable in the pellet or supernatant, indicating the absence of noncovalently bound flavin.

Catalytic properties.

The aerobically purified enzyme was isolated in a reversibly inactived form. Hydrogenase assays showed an initial lag phase, indicating reactivation under reducing conditions. Reactivation was accomplished by incubating samples of hydrogenase in the assay buffer at 37°C under hydrogen in the presence of 5 mM dithiothreitol or 5 μM sodium dithionite. Activity was followed by subsampling the reactivation mixture; maximal rates were observed at 10 min. Addition of KCl (0.5 M, 1.0 M) had no effect on activity, in contrast to activation conditions described for the F_{420}-reducing hydrogenase from *Ms. barkeri* strain Fusaro (8). Exposure of the reduced enzyme to air led to irreversible loss of activity.

The enzyme showed a maximal specific activity of 120 $\pm$ 40 μmol H_2 oxidized·min^{-1}·mg^{-1} with methyl viologen as an electron acceptor. Linear regression of three Eadie plots gave an apparent K_M for hydrogen of 5.6 $\pm$ 1.7 μM. Methyl viologen-linked activity was reversible and production hydrogenase activity from reduced methyl viologen was 45 $\pm$ 4 μmol H_2·min^{-1}·mg^{-1}. Both activities were inhibited by carbon monoxide, with consumption activity inhibited by 80% and production activity inhibited by 99% under 1 atm CO. Neither production nor consumption activity was inhibited by 1 mM NaCN. The enzyme also showed activity toward benzyl viologen (96 μmol·min^{-1}·mg^{-1}) phenosafranine (7.8 μmol·min^{-1}·mg^{-1}) and methylene blue (0.69 μmol·min^{-1}·mg^{-1}). The enzyme did not couple hydrogen oxidation to the reuction of neutral red, FAD, FMN, $NAD(P)^+$, nor the autologous cofactors F_{420} or ferredoxin.

Hydrogen-dependent cytochrome *b* reduction by a solubilized system.

Detergent-extracted material from the membrane of *Ms. barkeri* MS mediated the hydrogen-dependent reduction of endogenous cytochrome *b* (Fig. 6.). This preparation contained 2.7 U/mL of hydrogenase activity (sp. act. 4.1 U/mg) and 1.7 nmol cytochrome *b*/mL based on the reduced minus oxidized spectrum using $\Delta\varepsilon_{556\text{-}577nm} = 22\ mM^{-1}cm^{-1}$ (4). All of the chemically reducible cytochrome was reduced within 5 min after flushing the cuvette with hydrogen, as revealed by comparison to dithionite-reduced samples (data not shown). The specific rate of cytochrome *b* reduction was at least 0.23 nmol/min·mg protein. It is likely that these data underestimate the actual rate because of the small amount of substrate (i.e., 1.7 nmol cyt. *b*) and the rate of H_2 transfer into the reaction mixture. Attempts to separate the hydrogenase from cytochrome *b* by aerobic MonoQ or [Cu^{2+}]-IMAC chromatography were unsuccessful. Analysis of fractions showed loss of detectable cyt. *b*, indicating that the heme may be labile under these conditions.

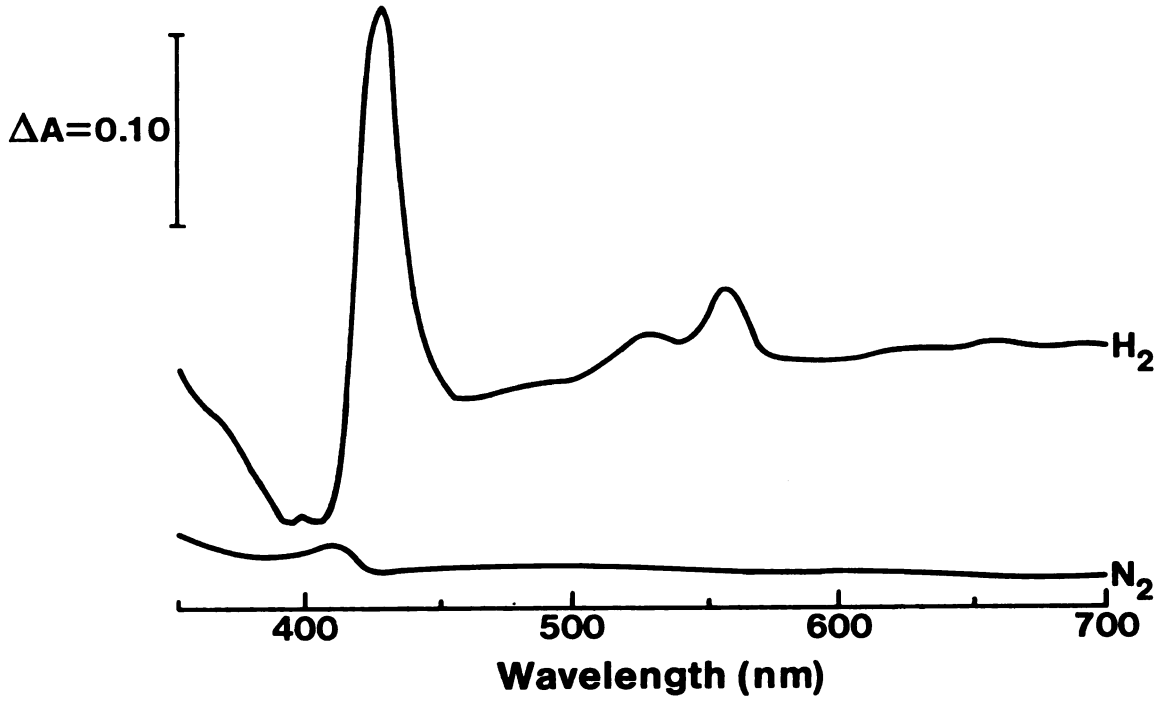

Fig. 6. Hydrogen-dependent reduction of endogenous cytochrome *b* by solubilized membranes of *Ms. barkeri* MS. Difference spectra were collected of 2% Triton X-100 solubilized membrane protein (1 mL, 1.5 mg protein) in anaerobic 50 mM Tris HCl buffer pH 7.5 in stoppered 1.4 mL cuvettes. The headspace of the indicated cuvette was flushed with hydrogen, all cuvettes were incubated at 37°C for 5 min. The reference cuvette was incubated under nitrogen. N_2, incubated under nitrogen versus reference. H_2, incubated under hydrogen versus reference. Absorbance maxima of the reduced cytochrome are 556 nm(α), 528 nm(β), and 428 nm (γ).

DISCUSSION

This study represents the first report of a hydrogenase purified from the cytoplasmic membrane of *Ms. barkeri* MS. The enzyme is a nickel-iron-sulfur [NiFe] hydrogenase, containing 0.5 mol Ni, 8±2 mol Fe, and 8±2 mol S^{2-} per mol enzyme. The iron and sulfur content is sufficient to coordinate 2 [3Fe-4S] or [4Fe-4S] centers. The enzyme couples with various dyes, but not with the known methanogenic electron carriers ferredoxin or F_{420}.

The membrane-bound enzyme is physically and catalytically distinct from soluble hydrogenases purified from strains of *Ms. barkeri* MS grown on methanol. A monomeric (60 kDa) hydrogenase was purified from *Ms. barkeri* DSM 800 (MS) (7) This enzyme was purified 550-fold from crude extract, contained a flavin prosthetic group, and coupled with F_{420}. A multimeric [48 kDa (α), 33 kDa(β), and 30kDa(γ)] hydrogenase of *Ms. barkeri* strain Fusaro was purified 64-fold from crude extract and showed coupling to F_{420} (8). This latter enzyme resembles three-subunit F_{420}-reactive hydrogenases purified from other methanogens (10, 12, 21, 30, 32). In contrast, this report represents the first characterization of an F_{420}-nonreactive hydrogenase from *Ms. barkeri*. The subunit composition of the membrane-bound hydrogenase in fact bears a closer resemblance to membrane-bound hydrogenases isolated from various bacteria, including *Desulfovibrio baculatus* (31) *Salmonella typhimurium* (29), *Rhizobium japonicum* (1), *Escherichia coli* (28), and the hyperthermophilic archaeon *Pyrodictium brockii* (24). These enzymes consist of a large and small subunit, and contain Ni. A membrane-bound F_{420}-nonreactive hydrogenase recently purified from *Methanosarcina* strain Gö1 also has a similar subunit pattern, i.e., 60 kDa and 40 kDa (5).

The location of this enzyme in the membrane suggests that electron acceptors for the enzyme may be membrane-associated redox cofators. We have previously shown that molecular hydrogen (presumably mediated by hydrogenase) is sufficient to reduce *b* cytochromes, iron-sulfur centers, and a rubredoxin-like center present in washed membranes of acetate-grown cells (13). In this same study, the *b* cytochromes were shown to be partially reoxidized by the addition of methyl-CoM. In this study, the membrane-bound hydrogenase was also shown study to mediate the reduction of the cytochrome *b* in a protein system solubilized from membranes. Thus, a possible role for the hydrogenase is to donate electrons to a membrane bound electron transport chain. This model is similar to respiration in sulfate-reducing bacteria, in which periplasmic hydrogenase(s) use low-poential cytochromes as electron acceptors (6). It is not clear from our studies whether reduction of the cytochrome by hydrogenase is direct or requires other cofactors or enzymes; nonetheless, the soluble system represents a starting point for the study of this reaction.

Hydrogen is continuously produced and consumed during growth of *Ms. barkeri* MS on acetate (16, 23). Whole cells (3, 22), and cell extracts (17) of *Ms. barkeri* MS form stoichiometric amounts of H_2 from CO, suggesting that hydrogen production may arise from oxidation of the carbonyl group of acetate. It is beneficial for the cell to utilize these hydrogen reducing equivalents in energy conservation. The standard free energy available from the reduction of methyl-CoM to methane is described by the following equation:

$$CH_3\text{-S-CoM} + H_2 \rightarrow CH_4 + \text{HS-CoM} \quad \Delta G^{o\prime} = -85\ \text{kJ} \qquad (8)$$

In this way, hydrogen produced by cytoplasmic reactions within the cell could be oxidized by a membrane-bound hydrogenase. This parallels metabolism in the

sulfate reducer *Desulfovibrio vulgaris* strain Madison, in which cycling of hydrogen produced from CO oxidation is suggested to provide chemiosmotic energy coupling (20). In support of a role for membrane-bound hydrogenase in energy conservation in *Ms. barkeri* MS, we have recently observed ATP synthesis coupled to hydrogen-dependent CoM-S-S-HTP reduction in a washed membrane fraction (unpublished data). It is also interesting to note that the pseudosarcina clumping morphology of *Ms. barkeri* would enhance such conservation. Cells with hydrogenase located in either the cytosol or membrane would be able to consume H_2 produced by neighboring cells. Further, the apparent K_M for hydrogen of the membrane-bound hydrogenase (5.7 μM) is of a similar magnitude as the overall apparent K_M for hydrogen oxidation by resting cells of *Ms. barkeri* (13 μM [27]), indicating that it could function in hydrogen consumption. In order to further clarify the roles of soluble and membrane-bound hydrogenases, studies are underway to compare and contrast these activities in acetate-grown *Ms. barkeri* MS.

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CHAPTER IV

REGULATION AND FUNCTION OF F_{420}-REACTIVE VERSUS F_{420}-NONREACTIVE HYDROGENASE IN ELECTRON TRANSFER AND ENERGY METABOLISM OF *Methanosarcina barkeri* MS

ABSTRACT

Acetate-grown cells of *Methanosarcina barkeri* MS were found to form methane from H_2:CO_2 at the same rate as hydrogen-grown cells. Cells grown on acetate had similar levels of soluble F_{420}-reactive hydrogenase, and higher levels of membrane-bound hydrogenase compared to hydrogen-grown cells. A large (13 kDa) ferredoxin which coupled to hydrogenase and CO dehydrogenase was purified from acetate-grown cells, and contained 3.8 $\pm$ 0.3 mol Fe and 3.3 $\pm$ 0.5 mol acid-labile sulfide per mol. The soluble fraction of acetate-grown cells contained both F_{420}-reactive (hydrogenase I) and F_{420}-nonreactive (hydrogenase II) activities. These activities were separated by differential binding properties to an immobilized Cu^{2+} column. Hydrogenase II did not react with ferredoxin, and was immunologically and biochemically identical to a cytochrome-linked membrane-bound hydrogenase previously purified from this organism. A reconstituted soluble protein system composed of purified CO dehydrogenase, F_{420}-reactive hydrogenase I fraction, and ferredoxin produced H_2 from CO oxidation at a rate of 2.5 nmol/min·mg protein. Membrane-bound hydrogenase II coupled H_2 consumption to the reduction of CoM-S-S-HTP and the synthesis of ATP. Hydrogen-dependent ATP synthesis was uncoupled by the protonophore TCS and inhibited by the H^+-translocating ATPase inhibitor DCCD. The differential function of hydrogenase I and II are ascribed to ferredoxin-linked hydrogen production from CO and cytochrome *b*-linked H_2 consumption coupled to methanogenesis and ATP synthesis, respectively.

INTRODUCTION

Hydrogen metabolism is a significant process in the catabolism of methanogenic archaea. Nearly all methanogens are capable of growth on H_2:CO_2 (28). *Methanosarcina barkeri* MS is one of the most catabolically diverse methanogens, capable of growth on acetate, methanol, methylamines, and CO, as well as H_2:CO_2. Hydrogen metabolism is associated with unitrophic growth of *Ms. barkeri* MS on acetate. Hydrogenase is present in cells grown on acetate (29), and hydrogen is both produced and consumed during growth on acetate in pure and coculture (37, 46).

During methanogenesis, acetate is activated via acetate kinase and phosphotransacetylase to acetyl-CoA, which serves as a substrate for the enzyme carbon monoxide dehydrogenase (CODH) (13, 32, 36). CODH then cleaves the acetyl group into a methyl and carbonyl group, and the carbonyl group is oxidized (18). Several observations indicate that hydrogen is produced as a consequence of carbonyl group oxidation. CO oxidation is linked to hydrogen production in whole cells (3, 44) and cell extracts (14, 35), implying that carbon monoxide dehydrogenase (CODH) can link to hydrogenase(s). CODH from *Ms. barkeri* uses ferredoxin, FAD, and FMN as an electron acceptor, but not F_{420} (15, 17).

Ms. barkeri contains two distinct hydrogenase activities. F_{420}-linked hydrogenases have been purified from the soluble fraction of methanol-grown *Ms. barkeri* strain Fusaro (12) and MS (11), which also couples with ferredoxin. We have purified and characterized a membrane-bound hydrogenase from acetate-grown *Ms. barkeri* MS which is F_{420}-nonreactive and is linked to cytochome *b* reduction (30).

The aim of this study was threefold: First, to characterize the regulation and function of F_{420}-reactive soluble hydrogenase (hydrogenase I) versus F_{420}-

nonreactive membrane bound hydrogenase (hydrogenase II); secondly, to reconstitute the protein components responsible for CO conversion to H_2, and thirdly, to assess the coupling of membrane-bound hydrogenase to H_2 consumption and ATP synthesis.

MATERIALS AND METHODS

Chemicals, gases, and chromatographic media.

All chemicals used were reagent grade or better and obtained from Sigma (St. Louis, MO) unless otherwise noted. Chelating Sepharose Fast Flow, MonoQ, PepRPC C2/C18, Q Sepharose Fast Flow, MonoQ, DEAE-Sepharose CL-6B, Sephadex G-50, and Superose were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). CoM-S-S-HTP (the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate) was a kind gift of Professor R. K. Thauer, Philipps-Universität, Marburg, Germany.

Cell culture

Methanosarcina barkeri strain MS (DSM 800) was grown in the dark as previously described (31) except that twice the concentration of filter-sterilized vitamins was added after autoclaving. The two trophic cultures described here have been maintained separately in continuous transfer by our laboratory for at least 10 years. Acetate-adapted cells were cultured in 45 L aspirator carboys at 37°C in phosphate buffered basal medium (PBBM) supplemented with 100 mM sodium acetate. Exponentially growing cells were allowed to settle, and the spent medium above them displaced with N_2 gas. Hydrogen-adapted cells were grown with shaking at 37°C in 1L sealed flasks (0.5 L PBBM) pressurized with 2 atm H_2:CO_2 (80%:20% vol:vol), which were periodically repressurized during exponential growth. Cells were harvested in an anaerobic chamber (Coy, Ann Arbor MI) filled with H_2:N_2 (5%:95% vol:vol) by collection on a Whatman #4 filter paper disc. The cells were then transferred to stoppered centrifuge tubes, washed 3 times with cold anaerobic 50 mM MOPS buffer pH 7.0 by centrifugation at 2500 X *g* for 20 min, and stored at -70°C until use.

Preparation of extracts

Cell extracts were prepared under anaerobic conditions by French pressure cell rupture as described (30). Membranes were pelleted from crude extract by ultracentrifugation at 150,000 X g for 90 min; the soluble fraction was collected as the upper 2/3 of the supernatant. The membrane pellet was washed by resuspending in 10 volumes MOPS buffer (50 mM, pH 7.0) and repelleting by ultracentrifugation. Washed pellets were resuspended in a small volume of MOPS buffer.

Membranes for ATP synthesis studies were prepared by a modification of the method of Deppenmeier et. al. used for obtaining membrane vesicles from protoplasts of strain Gö1 (7). Cells were lysed directly by French Press at 70 MPa in an anaerobic buffer containing 40 mM K-phosphate (pH 7.2), 20 mM $MgSO_4$, 0.5 M sucrose, 1 mM dithiothreitol, and 1 mg resazurin/mL as a redox indicator. Membranes were pelleted by ultracentrifugation as above, washed by resuspending in lysis buffer and repeating centrifugation, and finally suspended to a concentration of 5-10 mg/mL.

Cell suspension studies

Exponentially growing cells cultured on acetate or H_2:CO_2 were harvested anaerobically by removing samples with a syringe. The cells were transferred to N_2-flushed stoppered centrifuge tubes and washed three times by centrifugation at 750 X g for 10 min with 10 mL of reduced PBB medium without added vitamins or substrate. Suspensions were kept on ice until use. Reactions were started by injecting 1 mL of suspension into 13 mL anaerobic vials containing either a H_2:CO_2 (80:20 vol%) headspace or 0.1 mmol sodium acetate (final concentration 100 mM). Vials were incubated with shaking in a 37°C water bath.

Samples of the headspace (50 µL) were periodically removed by microsyringe and assayed for methane using a Gow Mac series 750 gas chromatograph (Bridegewater, NJ) equipped with a 6' x 1/8" Carbosphere column (Alltech, Deerfield, IL) and a flame ionization detector. For protein determination, 1 mL of cell suspension was pelleted using a microcentrifuge and washed with 1 mL H_2O. Cell pellets were digested in 0.5 mL 1.0 M NaOH at 100°C for 15 min, neutralized with 0.5 mL 1.0 M HCl, and centrifuged at 15,000 X g for 15 min. The supernatants were assayed for protein with the BCA reagent (Pierce), and bovine serum albumin standards which were digested in parallel.

Partial purification and resolution of hydrogenase I and II activities

Hydrogenase activities present in the soluble cell fraction were separated using immobilized [Cu^{2+}] affinity chromatography. Separation was carried out under aerobic conditions at 4°C after extracts were flushed and evacuated with N_2 to remove traces of H_2. 1 mL of supernatant (38-44 mg protein) was applied to a 1 x 10 cm column of Chelating Sepharose Fast Flow charged with 110 µmol Cu^{2+}. The column was then eluted with equilibration buffer (50 mM Tris acetate pH 7.5, 0.5 M NaCl), and approximately one bed volume (~8 mL) was collected and designated "unbound" (hydrogenase I). The column was subsequently washed with two additional bed volumes of equilibration buffer, and bound proteins were eluted with pH 4.0 buffer (adjusted with acetic acid). Approximately one bed volume was collected and designated "bound" (hydrogenase II).

The Cu^{2+} bound fraction which contained F_{420}-nonreactive hydrogenase activity (hydrogenase II) was further purified to near homogeneity. A larger scale separation was used as a first step. 19 mL of 150,000 X g supernatant (590 mg) was applied to a 2.6 X 9 cm column of Chelating Sepharose Fast Flow

charged with 560 μmol Cu^{2+}. The column was washed at 1 mL/min with 100 mL of equilibration buffer (pH 7.5), and the hydrogenase was eluted by batch desorption with 50 mL pH 4.0 buffer. The pH of the eluate was adjusted to pH 7.5 by addition of Tris base, and desalted by flow dialysis in an Amicon 8400 apparatus (W. R. Grace & Co., Beverly MA) fitted with a YM30 membrane (MW cutoff 30,000). The retentate (7.7 mg) was applied to a MonoQ HR5/5 column and eluted at 1 mL/min with a 40 mL linear 0-0.5 M NaCl gradient in 50 mM Tris HCl buffer (pH 7.5). Fractions containing hydrogenase were pooled and stored at -70°C until use.

Purification of CODH and electron carriers

Ferredoxin and F_{420} were isolated from the ultracentrifugal supernatant of acetate-grown cells. F_{420} purification was carried out at room temperature under aerobic conditions using foil-wrapped columns. Low molecular weight compounds were collected from 160 mL soluble cell extract (4300 mg protein) by flow dialysis using an Amicon PM10 membrane (MW cutoff 10,000). The ultrafiltrate was adsorbed onto a 2.5 x 14 cm column of Q Sepharose Fast Flow (bicarbonate form) equilibrated with deionized water. The column was eluted with a 500 mL linear gradient of 0-1.5 M ammonium bicarbonate at 1 mL/min; F_{420} eluted near the end of the gradient. Fractions showing absorbance maxima at 420 nm were pooled and lyophilized. The dry powder was dissolved in a minimum amount of 100 mM ammonium bicarbonate buffer and applied to a PepRPC C2/C18 reverse phase column. The column was eluted at 0.5 mL/min with a 20 mL linear gradient of 0-20 % methanol in 100 mM ammonium bicarbonate. F_{420} eluted near the middle of the gradient; these fractions were pooled and lyophilized. The resultant bright yellow powder was dissolved in 2

mL water, adjusted to pH 7.0 by addition of saturated K_2HPO_4, and stored at -70°C until use.

The procedure used for purification of the ferredoxin was a modification of Moura et. al. (43). Manipulations were performed inside an anaerobic chamber at room temperature. Soluble extract (74 mL, 2000 mg protein) was loaded onto a 2.6 X 18 cm column of DEAE Sepharose CL-6B and washed with 100 mL of a 50 mM Tris HCl pH 7.5 buffer containing 5 mM DTT. The column was eluted with a 500 mL linear 0-0.5 M NaCl gradient in the same buffer at 1 mL/min; ferredoxin eluted near the end of the gradient as a brown fraction showing absorbance at 390 nm. Prior to gel filtration, this fraction was concentrated by diluting twofold with buffer, adsorbing onto a 1.5 x 3 cm DEAE-Sepharose column, and desorbing with 0.5 M NaCl. The desorbed protein (~2mL) was loaded onto a 2.6 x 45 cm Sephadex G-50 column and eluted at 25 mL/hr. Fractions showing absorbance at 390 were pooled and adsorbed to a 2.6 x 10 cm DEAE-Sepharose column. The column was eluted isocratically in buffer containing 0.3 M NaCl at 1.3mL/min; the ferredoxin emerged as a broad band after several column volumes. This step was required to separate the ferredoxin from contaminating material showing absorbance at 257 nm. The pooled ferredoxin fractions were concentrated as described above and desalted on a G25 column, yielding 3 mg of protein. The purified ferredoxin showed an absorption ratio of $A_{390}/A_{280} = 0.79$, and was stored at -70°C under N_2 until use.

Carbon monoxide dehydrogenase (CODH) was purified from soluble extract as described (35). The final specific activity was 140 μmol/min·mg.

N-terminal sequencing of ferredoxin.

Prior to sequencing, cysteine residues of the ferredoxin were modified by S-pyridylethylation as follows: 11 μg of protein collected from a reverse-phase

column (see fig 4) was suspended in 50 µL 250 mM Tris HCl buffer (pH 8.5) containing 1 mM EDTA, 6M guanidine HCl, and 0.5% 2-mercaptoethanol. The protein was incubated at room temperature in the dark under argon for 2 hr, then 2 µL 4-vinylpyridine (Aldrich) was added. Incubation was continued for another 2 hr, and the protein was desalted by another round of reverse-phase chromatography. The N-terminal amino acid sequence of the first 20 residues was identified with an Applied Biosystems protein sequencer model 477A (Foster City, CA) with an on-line phenythiohydantoin analyzer. Sequencing was performed by the Macromolecular Structure, Sequencing, and Synthesis Facility at the Department of Biochemistry, Michigan State University.

Enzyme assays

Spectrophotometric assays for methyl viologen-linked hydrogenase and CO dehydrogenase were performed as described (30). Stoppered cuvettes were allowed to equilibrate with the headspace of either H_2 or CO and the reaction started by addition of enzyme. F_{420}-linked hydrogenase activity was assayed in 100 mM Tris HCl buffer, pH 7.8, as described (12), using 10 mM dithiothreitol to provide reducing conditions. Decrease in absorbance was monitored at 420 nm using a molar absorption coefficient of 45.5 $mM^{-1}cm^{-1}$ (9). FAD- and FMN-coupled activity was monitored in the same buffer by following decrease in absorbance at 450 nm and 445 nm, respectively (51). Ferredoxin-linked activity was assayed by following metronidazole reduction at 320 nm as described (15), except that ferredoxin was added to a concentration of 60 µM. Rates were calculated on the basis of one mol H_2 oxidized/mol of F_{420}, FAD, or FMN, and 1/2 mol H_2 or CO oxidized/mol methyl viologen or metronidazole. The K_{Mapp} for ferredoxin and F_{420} was determined in the hydrogen-oxidizing direction by linear regression of double reciprocal plots. Hydrogen production from reduced

methyl viologen was assayed as described (30). Gas concentrations in solution were calculated by interpolation of solubility data at 35°C and 40°C (24).

Reconstitution of CO-dependent hydrogen production activity.

Assays were performed in 13 mL N_2-flushed stoppered vials containing 1 mL 50 mM Tris HCl buffer (pH 7.5) with 0.5 M NaCl and 5 mM dithiothreitol. Additions were made with N_2-flushed microsyringes and vials were placed in a 37°C shaking water bath. The vials contained 0.13 mg CODH (18 Units), 0.13 mg ferredoxin, either 1.1 mg of the F_{420}-reactive hydrogenase fraction, 2.6 μg of purified membrane-bound hydrogenase (chapter 3), 1.9 μg of the F_{420}-nonreactive hydrogenase purified from the soluble fraction, or no hydrogenase, as indicated. Linear hydrogen formation rates were observed over the course of 3 hr.

Gel electrophoresis

Native linear gradient gel electrophoresis was performed as described by Lambin and Fine (39) using a Tris-borate-EDTA buffer. Staining for methyl viologen-linked activity and protein was performed as described (30). F_{420}-linked activity was visualized in gels by loss of fluorescence upon reduction of added F_{420} as described by (12). SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (40) using a BioRad minigel apparatus. SDS-PAGE of ferredoxin was perfomed in 16.5% acrylamide/10% glycerol gels by a modification of the method of Schägger et al. (48) using a low molecular mass standard kit from Sigma and following the manufacturer's instructions.

Hydrogen dependent CoM-S-S-HTP reduction and ATP synthesis by membranes.

Experiments were performed at room temperature in stoppered 10 x 75 mm test tubes containing either a nitrogen or hydrogen headspace as indicated. Assays contained 0.6 mL K^+-phosphate buffer as described above except that dithiothreitol was excluded. TCS (3,3',4',5-tetrachlorosalicylanilide, Kodak, Rochester NY) and DCCD (N,N'-dicyclohexylcarbodiimide) were added to tubes as ethanolic solutions and quickly dried under a stream of nitrogen before other additions were made. ADP was added to a concentration of 50 μM, and the buffer was reduced with a few microliters of a Ti(III)-nitrilotriacetate solution (Moench 1983). Membrane suspension (10-25 μL) was added to tubes and preincubated for 15 min. The reaction was started by addition of CoM-S-S-HTP. Disulfide reduction was quantitated by measuring the concentration of thiol in 10 μL samples using Ellman's reagent as described (23). The thiol content found at the start of the assay was subtracted from subsequent determinations. The Ellman reagent was standardized with HS-CoM (2-mercaptoethanesufonic acid sodium salt). ATP content was measured with the luciferase-luciferin system (Sigma). Samples (10 μL) were periodically removed from the reaction mixture and diluted 1:10 with H_2O at 100°C for 30 sec. Samples were kept on ice until assay (not longer than 60 min). ATP-dependent luminescence was quantitated with a DuPont 760 Luminescence Biometer (Dupont Instruments, Wilmington, DE). The luminometer was calibrated with a freshly prepared dilution of authentic ATP.

Oxidoreduction of the cytochrome *b* was studied in parallel experiments conducted at room temperature. Membranes were prepared anaerobically in 50 mM MOPS buffer as described above and suspended to a concentration of 2.4 mg protein/mL in N_2-flushed stoppered cuvettes. The headspace (0.4 mL) overlying

1 mL sample was flushed with hydrogen, which resulted in complete reduction of the cytochrome. The cuvettes were subsequently flushed with N_2; the cytochrome remained fully reduced under these conditions for at least 15 min. Additions of anaerobic CoM-S-S-HTP or CoM-S-S-CoM prepared as described (10) were then made by microsyringe. The extent of reduction of the cytochrome was monitored by measuring the difference absorption spectrum of the α band at 558 nm using $\varepsilon\Delta A_{558\text{-}577} = 22\ mM^{-1}\ cm^{-1}$ (5).

Immunologic techniques.

Antiserum was raised to the purified membrane-bound hydrogenase of *Ms. barkeri* (30) by injecting 100 μg of purified protein into New Zealand White rabbits, boosting two weeks later, and bleeding at 8 weeks. Western analysis performed after transferring proteins from 12% SDS-PAGE gels to PVDF membranes (Applied Biosystems, Foster City CA) using a Bio-Rad Trans-Blot SD Semi-dry electrophoretic transfer cell set at 20V for 25 min. The transfer buffer used was 48 mM Tris, 39 mM glycine, and 0.00375% SDS in 10% methanol (2). Membranes were blocked for 1 hr with 3% (w/vol) bovine serum albumin in a 50 mM Tris HCl pH 7.5 buffer containing 200 mM NaCl and 0.05% Tween 20. Blots were incubated with immune serum at a dilution of 1:10,000 for 1 hr. Immunostaining was performed using a goat anti-rabbit streptavidin/alkaline phosphatase kit (BRL, Gaithersburg, MD). Proteins were visualized on membranes by staining with Coomassie blue (0.1% in 40% methanol/10% acetic acid) and destaining with 50% methanol.

Molecular weight determination.

The molecular weight of proteins was estimated using a TSK G300SW gel filtration column (TosoHaas, Philadelphia PA) equilibrated with 50 mM Tris HCl

(pH 8.0) buffer containing 100 mM NaCl. Standards for calibration were β-amylase (200,000), yeast alcohol dehydrogenase (141,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), and α-lactalbumin (14,200).

Analytic methods.

Protein was determined with the BCA reagent (Pierce) using bovine serum albumin as a standard. Absorbance spectra were obtained with a Gilford Response II spectrophotometer (Gilford Instrument laboratories, Oberlin OH). Iron was determined by the method of Fish (16). Acid-labile sulfide was determined by the method of Beinert (1) using a gravimetric Na_2S standard.

RESULTS

Regulation of hydrogen metabolism and hydrogenases.

The influence of growth substrate on the rate of methanogenesis by resting suspensions of *Ms. barkeri* was examined (Table 1). Cells grown on hydrogen formed methane from acetate at only ~10% of the rate from H_2:CO_2, but cells grown on acetate formed methane at comparable rates from either acetate or H_2:CO_2. In addition, cells grown on acetate produced methane from H_2:CO_2 at the same rate as cells grown on H_2:CO_2.

The amounts of soluble F_{420}-linked hydrogenase (hydrogenase I), membrane-bound hydrogenase (hydrogenase II), and and carbon monoxide dehydrogenase (CODH) in extracts of cells grown on acetate versus H_2 were determined (Table 2). Cells grown on acetate had comparable levels of hydrogenase I when compared to hydrogen-grown cells, and higher levels of hydrogenase II. CODH was present in ~5-fold higher amounts in acetate-grown cells as opposed to H_2-grown cells. These results show substrate-dependent regulation of this enzyme as previously reported by Krzycki et al. (34).

Purification and characterization of ferredoxin.

The purified ferredoxin consisted of one major polypeptide, as evidenced by reverse-phase chromatography (Fig. 1) and SDS-PAGE (Fig. 2). Estimation of the molecular weight of the ferredoxin by SDS-PAGE showed a band at 13,000 Fig. 2, lanes 1 and 2). The band was broad and hazy, a property which has been associated with proteins having a high cysteine content (22). The presence of one polypeptide was confirmed after alkylation of the protein with iodoacetamide, which resulted in one sharp band (Fig. 2, lane 3)

Table 1. Influence of growth substrate on rate of methanogenesis from acetate versus H_2:CO_2 by cell suspensions of *Ms. barkeri* MS.

	Methane formation (nmol/min·mg protein) from:	
Growth Substrate	acetate[a]	H_2/CO_2[b]
acetate	57 ± 9[c]	46 ± 20
H_2/CO_2	4.1 ± 0.6	47 ± 6

[a] 100 mM

[b] 80:20 vol% in headspace at 1 atm

[c] Mean ± S. D. (n = 3).

Table 2. Influence of growth substrate on levels of CODH, hydrogenase I, and hydrogenase II in *Ms. barkeri* MS.

Growth substrate	SPECIFIC ACTIVITY (U/mg)[a]		
	Hydrogenase I[b]	Hydrogenase II[c]	CO dehydrogenase[d]
Acetate	0.5 ± 0.3	8.6 ± 3.7	10 ± 4
H_2-CO_2	0.29 ± 0.06	2.2 + 0.3	1.7 ± 0.8

[a] Units are defined as μmol CO or H_2 oxidized per minute at 37°C. Values are mean ± S.D. for at least three different batches of cells.

[b] F_{420}-linked activity measured in soluble fraction

[c] methyl viologen-linked activity measured in the membrane fraction

[d] methyl viologen-linked activity measured in the soluble fraction

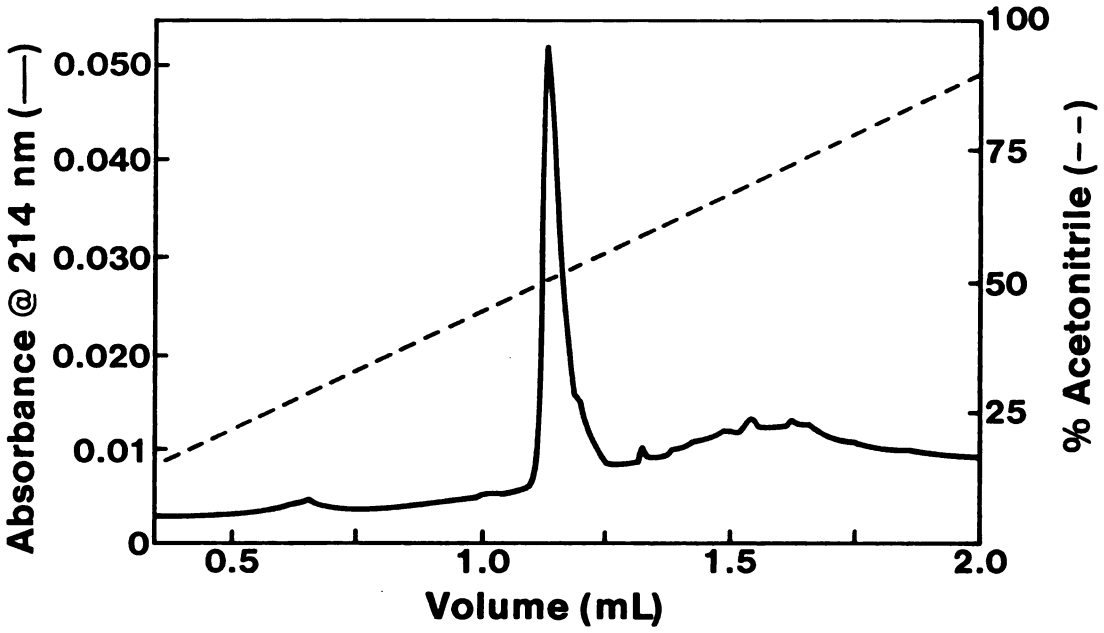


Figure 1. Reverse-phase chromatography of purified ferredoxin from acetate-grown *Ms. barkeri* MS. Ferredoxin (11 μg) was applied to a 1.0 x 50 mm Aquapore RP-300 microbore column (Applied Biosystems, Foster City, CA) equilibrated with 0.1% (vol/vol) trifluroacetic acid (TFA) in water. The protein was eluted at 50 μL/min with a linear 2 mL gradient of 0-100 % of a mobile phase consisting of 90% acetonitrile, 0.1% TFA in water.

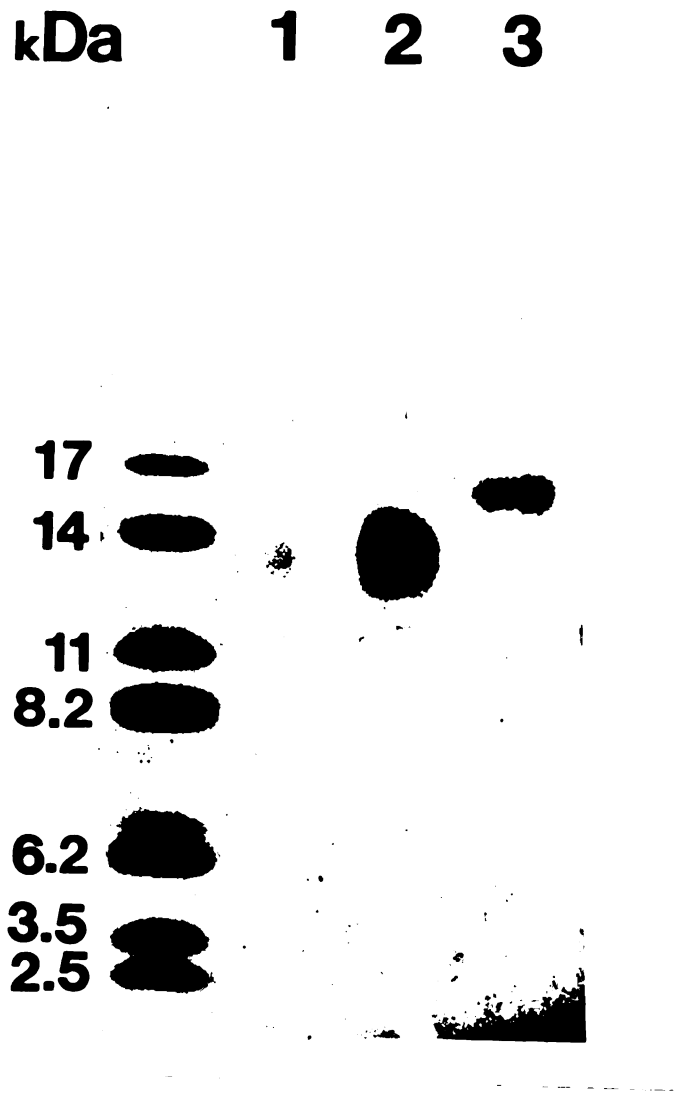


Figure 2. SDS-polyacrylamide gel electrophoresis of ferredoxin purified from acetate-grown *Ms. barkeri* MS. Proteins were separated in a 10% glycerol, 16.5% acrylamide gel. Lane 1, 25 μg ferredoxin. Lane 2, 100 μg ferredoxin. Lane 3, 25 μg ferredoxin after alkyation with iodoacetamide as described in (22). Molecular weight markers are glucagon plus polypeptide fragments derived from myoglobin.

which migrated at 16,000. The ferredoxin showed a weak staining with Coomassie Blue G. Accordingly, the protein content of samples assayed with the Coomassie-based Bradford reagent gave low protein values when compared with the BCA reagent assay (data not shown).

The electronic absorption spectrum of the ferredoxin showed maxima at 390 nm and 282 nm, with a shoulder at 312 nm (Fig. 3) Absorbance at 390 nm decreased upon addition of dithionite, indicative of iron-sulfur clusters. The N-terminal sequence of the ferredoxin showed a one amino acid difference from feredoxin previously purified from methanol-grown *Ms. barkeri* MS (Fig 4), i.e., the presence of valine at position 18 as opposed to a glutamic acid residue.

The ferredoxin was found to contain 3.8 $\pm$ 0.3 mol Fe and 3.3 $\pm$ 0.5 mol acid-labile sulfide per mol ferredoxin based on an apparent molecular weight of 13,000. Spectrophotometric analysis assuming an extinction coefficient of ε_{390} = 12,800 $M^{-1} \cdot cm^{-1}$ (53) indicated the presence of 1.3 iron-sulfur chromophpore/13 kDa polypeptide. Estimation of the native molecular weight gave a value of 28,000, suggesting that the ferredoxin is present as a dimer. The specific activity of purified CODH coupled to ferredoxin reduction was 4$\pm$2 μmol CO oxidized$\cdot min^{-1} mg^{-1}$.

Resolution of hydrogenase I and II activities.

Hydrogenase activity found in the soluble fraction of French pressure cell lysates of *Ms. barkeri* was separated into F_{420}-reactive (hydrogensase I) and F_{420}-nonreactive (hydrogenase II) fractions by passage over a [Cu^{2+}]-IMAC column (Table 3). F_{420}-reactive hydrogenase I did not bind to the column. The F_{420}-nonreactive hydrogenase II activity bound to the column, and was eluted by decreasing the buffer pH to 4.0.

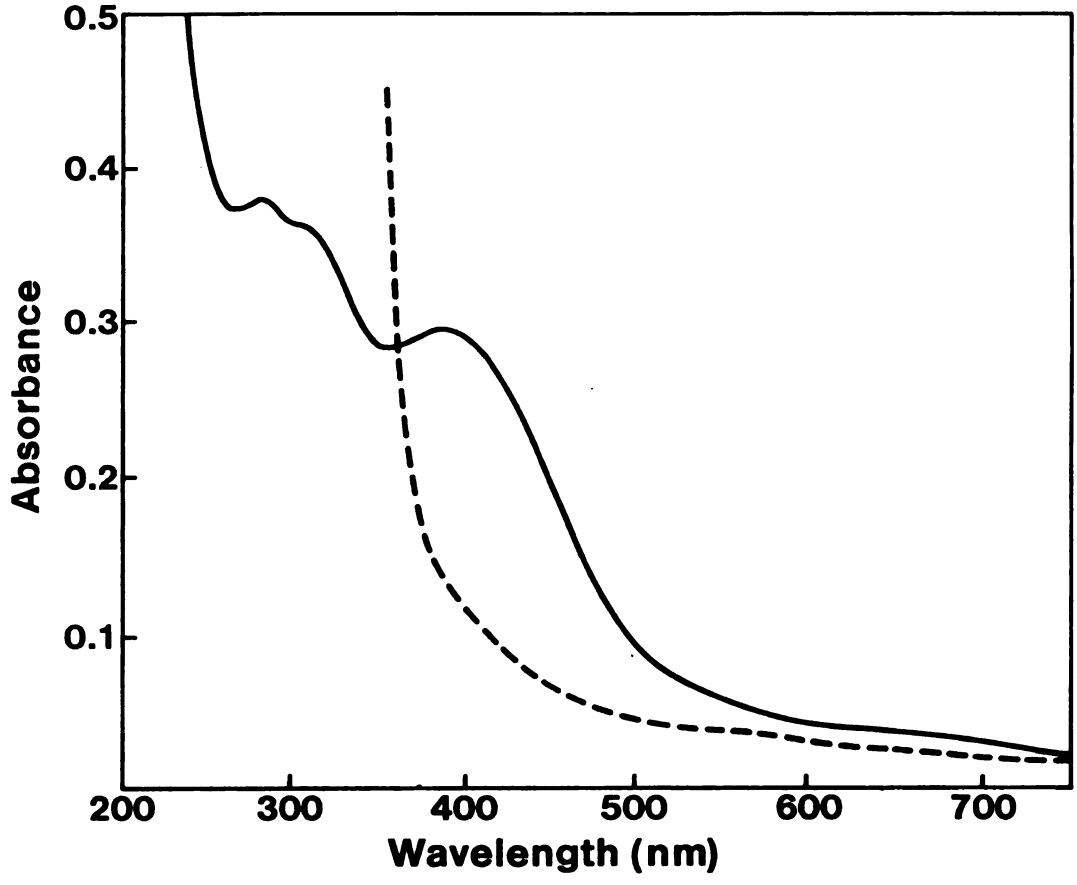


Figure. 3. Electronic absorption spectrum of ferredoxin from acetate-grown *Ms. barkeri* MS. Ferredoxin (0.25 mg/ml) was suspended in 50 mM Tris HCl buffer pH 7.5 in a sealed cuvette under under nitrogen (——) and after addition of sodium dithionite (- - -).

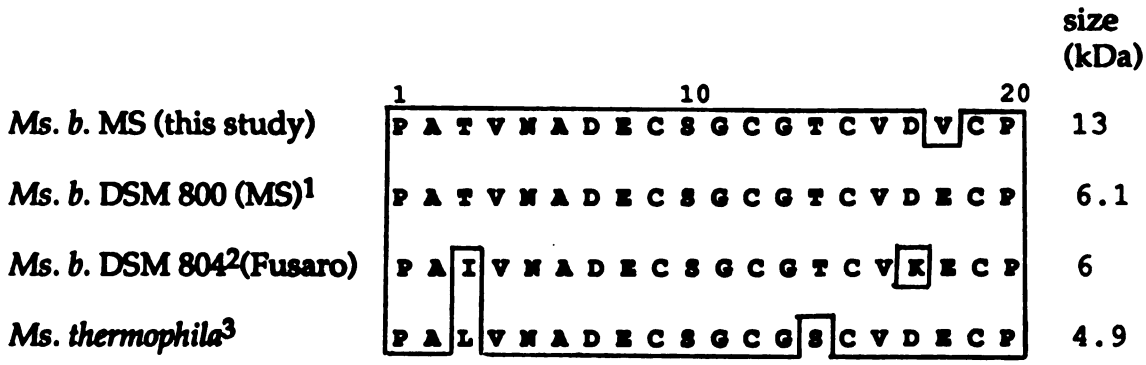


Figure 4. Comparison of the N-terminal sequence of *Ms. barkeri* MS ferredoxin with other *Methanosarcina* ferredoxin sequences. Boxed regions indicate consensus residues for this comparison. [1,2] Ferredoxin purified from methanol-grown cells (20, 21). [3]Ferredoxin purified from acetate-grown cells (53).

Table 3. Separation of hydrogenase I and II activities in soluble extract of acetate-grown *Ms. barkeri* MS by [Cu^{2+}]-IMAC.

Fraction	mg protein/ fraction	Units/fraction hydrogenase activity (yield)[a] MV-linked	F_{420}-reactive
Applied 150,000 X g supernatant	44	130	2.3
Unbound (hydrogenase I)	13	4.9 (3.8%)	1.8 (78%)
Eluted @ pH 4.0 (hydrogenase II)	1.0	41 (32%)	n.d.[b]

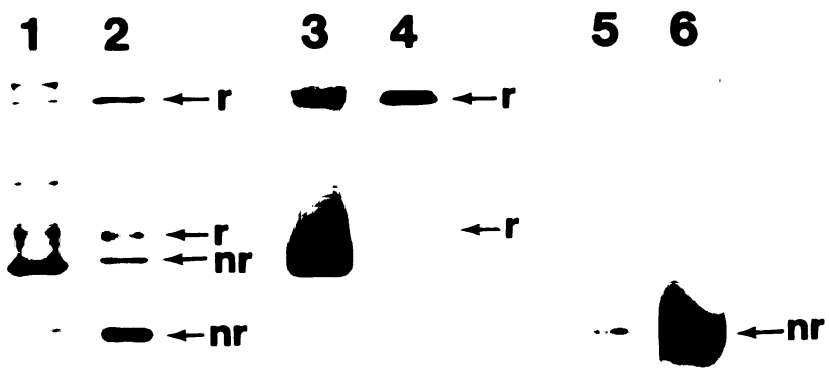
[a]Units are expressed as µmol hydrogen oxidized per minute using methyl viologen (MV) or 8-hydroxy-5-deazariboflavin (F_{420}) as electron acceptor. Yield is expressed as percentage of total units recovered. For details of separation, see text.

[b]Not detected.

Hydrogenase I and II fractions showed distinct patterns upon native gel electrophoresis (Fig. 5). Crude soluble cell extract revealed four prominent methyl viologen-reducing bands, two of them F_{420}-reactive (lane 2). The hydrogenase I fraction showed two bands of F_{420}-reducing activity (lane 4). The faster migrating band stained faintly with methylviologen, but was easily discernible by its F_{420}-reducing activity. A similar two-band pattern has been observed for homogenous F_{420}-reactive hydrogenase purified from methanol-grown *Ms. barkeri* Fusaro (12). The hydrogenase II fraction consisted of one band of F_{420}-nonreactive hydrogenase activity (lane 6).

Hydrogenase II was further purified to near homogeneity in one additional anion exchange chromatography step on MonoQ. The overall purification was 61-fold with a yield of 20% and a final specific activity of 170 μmol hydrogen oxidized·min^{-1}·mg^{-1} (methyl viologen linked). The enzyme showed the same physical and catalytic properties as membrane-bound hydrogenase previously purified from this organism (30). The enzyme did not couple hydrogen oxidation to F_{420}, FAD, FMN, $NAD(P)^+$, or ferredoxin. SDS-PAGE analysis of hydrogenase II showed two different polypeptides of the same apparent molecular weight as the membrane-bound hydrogenase (57 kDa and 35 kDa, Fig. 6). Estimation of the molecular weight of the hydrogenase II gave a value of 93,000, suggesting that the enzyme exists as a heterodimer as is the case for the purified membrane-bound hydrogenase. Antibodies raised to the membrane-bound hydrogenase reacted with the subunits from both hydrogenase II and membrane-bound hydrogenase (Fig. 6). By these criteria, the F_{420}-nonreactive hydrogenase II is indistinguishable from the membrane-bound hydrogenase.

Figure 5. Native gel electrophoresis of hydrogenase I and II activities of *Ms. barkeri* MS. Samples were electrophoretically separated in a native linear 4-20% polyacrylamide gel. Lanes 1, 3, and 5 were stained for protein with Coomassie blue. Lanes 2, 4, and 6 were stained for methyl viologen-linked hydrogenase activity and fixed with triphenyl tetrazolium chloride. Bands indicated by "r" were F_{420}-reactive as determined in parallel experiments; bands indicated by "nr" were F_{420}-nonreactive. Lanes 1 and 2, 35 μg soluble cell extract. Lanes 3 and 4, 100 μg hydrogenase I (protein which did not bind to a $[Cu^{2+}]$-IMAC column at pH 7.5). Lanes 5 and 6, 5 μg hydrogenase II (protein which was eluted from the column by shifting the pH to 4.0). Arrow to the left indicates the position of the bromphenol blue dye front.


1
2
3
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5
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r
r
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nr
nr
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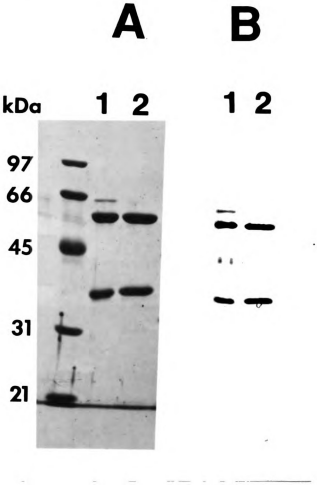
Figure 6. Western analysis of hydrogenase II and membrane-bound hydrogenase of acetate-grown *Ms. barkeri* MS. A. Protein staining of a PVDF membrane blot of a 12% SDS-PAGE gel. Molecular mass markers are shown at left. Lane 1, 1 μg purified membrane-bound hydrogenase (30). Lane 2, 1 μg purified F_{420}-nonreactive hydrogenase II. **B.** Immunostaining with polyclonal antibody raised to purified membrane-bound hydrogenase. Lane 1, 50 ng purified membrane-bound hydrogenase. Lane 2, 50 ng purified hydrogenase II.


A
B
kDa
1 2
1 2
97
66
45
31
21

Characterization of hydrogenase I function.

The F_{420}-reactive hydrogenase I fraction oxidized hydrogen at a specific activity of 0.14 μmol/min·mg protein with F_{420} as an electron acceptor, 0.052 μmol/min.mg with ferredoxin as an electron acceptor, and 0.38 μmol/min·mg with methyl viologen as an electron acceptor. Hydrogenase I activity was reversible, forming 0.55 μmol hydrogen/min·mg with methyl viologen as an electron donor. Coupling to FAD or FMN was not observed. Estimation of kinetic parameters using crude soluble anaerobic extract gave a K_{Mapp} = 6 μM for ferredoxin and a K_{Mapp} = 45 μM for F_{420}.

The role of hydrogenase I in hydrogen production from CO was investigated by recombining this fraction with purified components. The observation that ferredoxin couples to both CODH and hydrogenase I suggested that this carrier may function in electron transport between these two enzymes. CO-dependent hydrogen producing activity was successfully reconstituted with CODH, ferredoxin, CO, and the hydrogenase I fraction (Table 4). Hydrogen formation was dependent on each of the added components, and was not supported by the previously purified membrane-bound hydrogenase or hydrogenase II. Methane formation was not detected in these reactions.

Characterization of hydrogenase II function.

Hydrogenase II was found to be very similar to the previously purified membrane-bound hydrogenase (30) which linked to cytochrome *b* reduction but not F_{420} or ferredoxin. Membranes prepared from *Ms. barkeri* containing hydrogenase activity (8-13 U/mg protein) catalyzed the reduction of CoM-S-S-HTP with molecular hydrogen coupled to the synthesis of ATP from

Table 4. Reconstitution of CO-dependent hydrogen production activity with protein components isolated from acetate-grown *Ms. barkeri* MS.

Constituents	H_2 production (nmol/min·mg hydrogenase)
CO + CODH + ferredoxin + hydrogenase I	2.5 + 0.4[a]
Minus CO[b]	n.d.[c]
Minus CODH	n.d.
Minus ferredoxin	n.d.
Minus hydrogenase	n.d.
CO + CODH + ferredoxin + hydrogenase II	n.d.

[a] Mean $\pm$ S.D. (n = 3)

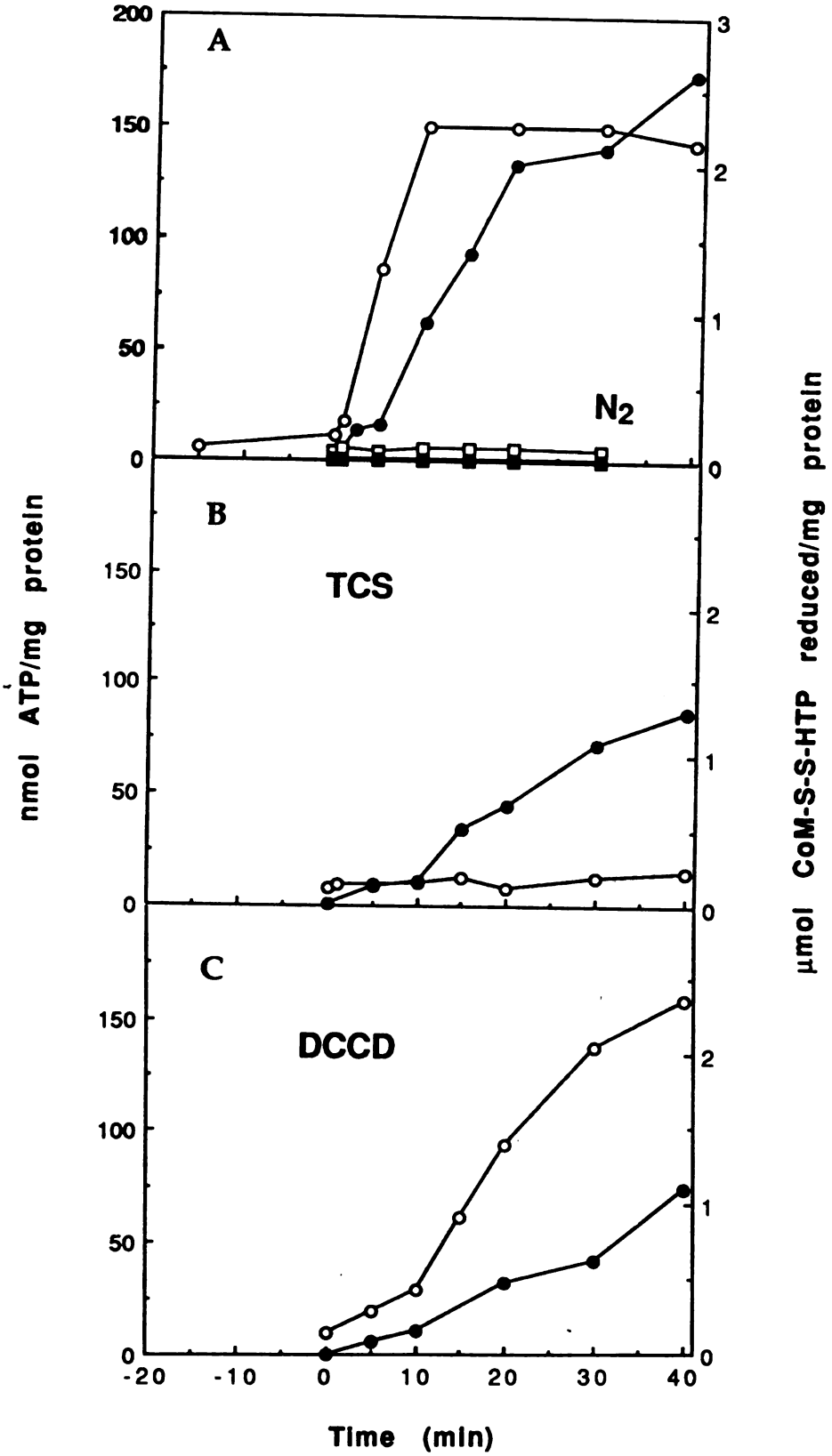
[b] 100% N_2

[c] Not detected (limit of assay: 0.1 nmol/min)

ADP + P_i. (Fig. 7A). The maximal rate of ATP formation was 14 nmol/min·mg protein. The corresponding rate of CoM-S-S-HTP reduction was 86 nmol/min·mg protein, or 1 mol ATP synthesized per 6 mol CoM-S-S-HTP reduced. Addition of the protonophore TCS completely abolished ATP formation but reduction of CoM-S-S-HTP continued at a reduced rate of 38 nmol/min·mg (Fig. 7B). TCS has been shown to dissipate the protonmotive force in whole cells of *Ms. barkeri* (3), which suggests that the ATP synthesis observed in the membrane preparation is dependent on a proton gradient generated by substrate turnover. DCCD is an inhibitor of the H^+-translocating ATPase of *Ms. barkeri* (25) Addition of DCCD inhibited ATP formation (4.0 nmol/min.mg) and CoM-S-S-HTP reduction (27 nmol/min.mg) to a similar extent (29% and 31%, respectively, Fig 7C). Addition of TCS at this point did not relieve inhibition of CoM-S-S-HTP reduction (data not shown), possibly because of its inhibitory effect as noted above. Taken together, these results suggest that hydrogen-dependent reduction of CoM-S-S-HTP by membranes is accompanied by proton translocation across the membrane, and that this gradient is used to drive phosphorylation of ADP.

The oxidation state of cytochrome *b* was monitored spectrophotometrically in parallel experiments. Addition of hydrogen resulted in compete reduction of the cytochrome *b* as previously reported (29). When the headspace of the cuvettes was replaced with N_2, the cytochrome remained in the fully reduced state. Subsequent addition of 25 µL of 10 mM CoM-S-S-HTP (250 nmol) resulted in reoxidation of ~2/3 of the cytochrome. This reaction was specific for CoM-S-S-HTP; neither CoM-S-S-CoM nor 25 µL aerobic H_2O resulted in reoxidation of the cytochrome.

Figure 7. ATP synthesis coupled to hydrogen-dependent CoM-S-S-HTP reduction by membranes of *Ms. barkeri* MS. Membranes were preincubated for 15 min in anaerobic phosphate buffer (pH 7.2) with 50 μM ADP and additions as indicated. Reactions were started by adding the heterodisulfide CoM-S-S-HTP (0.6 mM) at 0 min. **A.** ATP synthesis (○-○) and CoM-S-S-HTP reduction (●-●) in the presence of a hydrogen headspace; ATP synthesis (□-□) and CoM-S-S-HTP reduction (■-■) in the presence of a nitrogen headspace. **B.** Effect of the protonophore TCS (47 nmol/mg protein) on membranes incubated under hydrogen. **C.** Effect of the inhibitor DCCD (9.3 nmol/mg protein) on membranes incubated under hydrogen.


A
B
TCS
C
DCCD
N_2
nmol ATP/mg protein
µmol CoM-S-S-HTP reduced/mg protein
Time (min)
200
150
100
50
0
3
2
1
-20
-10
0
10
20
30
40

DISCUSSION

To our knowledge, this report is the first to document the regulation and function of F_{420}-reactive (hydrogenase I) and F_{420}-nonreactive (hydrogenase II) hydrogenases in acetate-grown *Ms. barkeri* MS. The presence of two hydrogenases, one F_{420}-reactive and one F_{420}-nonreactive has been observed in numerous methanogenic archaea (26, 33, 47, 50, 57). Recently a report has appeared describing four gene clusters, each potentially coding a distinct hydrogenase, in *Mc. voltae* (19). We have previously reported that the membrane-bound, F_{420}-nonreactive hydrogenase II from *Ms. barkeri* links either directly or indirectly to cytochrome *b* reduction and hydrogen consumption (30). We show here that the F_{420}-reactive hydrogenase I links to ferredoxin oxidation and hydrogen production.

It is interesting to note that hydrogenase levels found in acetate-grown cells are comparable to those in hydrogen-grown cells even after 10 years of continuous cultivation (> 1,800 generations) on acetate in our laboratory. In fact, acetate grown cells were found to form methane from H_2:CO_2 at the same rate as hydrogen-grown cells, indicating that these cells are capable of catabolic consumption of hydrogen. Thus, hydrogenase is constitutively expressed by *Ms. barkeri*, and serves a catabolic role during acetate catabolism. Other catabolic enzymes involved in methanogenesis are regulated according to growth substrate in *Ms. barkeri*, i.e, carbon monoxide dehydrogenase (35), carbonic anhydrase (27), and formylmethanofuran dehydrogenase and methylenetetrahydromethanopterin dehydrogenase (49).

This study was undertaken to describe hydrogen-forming and consuming reactions during methanogenesis from acetate by *Ms. barkeri* MS. Several lines of evidence indicate that hydrogen arises from CO oxidation: i) whole cells (3, 44)

and cell extracts (35) of acetate-grown *Ms. barkeri* MS form stoichiometric amounts of H_2 and CO_2 from CO, and ii) these activities are sensitive to CN^-, a potent inhibitor of carbon monoxide dehydrogenase (3, 35). Hydrogen formation from CO in extract was shown to be ferredoxin-dependent (15). Therefore, we tested whether ferredoxin could link electron transfer to hydrogen production in soluble protein system by recombining purified components. CO oxidizing:H_2 producing activity was successfully reconstituted with CODH, ferredoxin, and the F_{420}-reactive hydrogenase I fraction. Hydrogen formation from CO was not seen with the membrane-bound hydrogenase II. This is in contrast to a different system reported in *Ms. thermophila* which requires a membrane component for H_2 production from CO (54). This latter system resembles membrane-associated CO oxidizing:H_2 producing activity of the phototroph *Rhodopseudomonas gelatinosa* (55, 56).

The ferredoxin purified from acetate-grown cells which participates in both CO oxidation and hydrogen production is approximately twofold larger (~13 kDa) than ferredoxins purified from other *Methanosarcina* spp. (20, 21, 43, 53). This value places it at the upper size range for bacterial ferredoxins (4). This ferredoxin differs in size and sequence (at residue #19) from a 6.1 kDa ferredoxin purified from methanol-grown *Ms. barkeri* MS (43). It is possible that the observed differences may be due to differential expression of ferredoxin according to growth substrate. Genetic analysis has revealed the presence in the *Ms. thermophila* genome of two open reading frames, one which encodes the previously purified ferredoxin, and another which potentially encodes a second ferredoxin-like protein (6). The corresponding mRNAs of these genes were found to be differentially regulated with respect to substrate. The size of the ferredoxin is interesting in light of the recent purification of the so-called

polyferredoxin from *Mb. thermoautotrophicum*, a 44 kDa polypeptide which may coordinate as many as 12 [4Fe-4S] clusters (22, 52).

This study also provides evidence that hydrogen consumption by acetate-grown cells may be linked to energy conservation by hydrogenase-mediated electron transport phosphorylation. Membranes were shown to couple hydrogen oxidation and CoM-S-S-HTP reduction to the formation of ATP in a manner consistent with a chemiosmotic mechanism. The observation that the protonophore TCS abolished ATP synthesis indicates that a primary proton gradient was formed during electron transport from hydrogen to CoM-S-S-HTP. This is the first report of ATP synthesis by a subcellular fraction of a methanogen other than *Methanosarcina* strain Göl (7, 8). In addition, the cytochrome *b* in the membranes was reduced by hydrogen and oxidized by membrane-bound CoM-S-S-HTP, suggesting that the cytochrome may be part of a membrane-bound electron transport chain. The terminal step in methane formation is the reduction of CH_3-CoM with HS-HTP, thus the cytochrome would function in methyl group reduction. This is essentially the reverse of a role proposed for the cytochrome in methyl group oxidation (4). In the absence of specific inhibitor data, it is not possible to state conclusively that the cytochrome is directly involved in electron transfer from H_2 to CoM-S-S-HTP. However, the presence in *Ms. barkeri* of several cytochrome *b* species (38) suggests that cytochromes could serve multiple functions in this microbe.

We propose that the hydrogen producing and consuming activities may be cycling hydrogen in order to couple CO oxidation to methyl group reduction during acetate transformation. This would allow linkage of reducing equivalents generated from oxidation of the carbonyl group of acetate with a membrane-bound electron transport chain. A model for the role of hydrogenase I and II during acetate catabolism is presented in Fig. 8. In this scheme, CO

dehydrogenase cleaves acetyl CoA and oxidizes the carbonyl group, reducing ferredoxin. Reduced ferredoxin then serves as a substrate for hydrogenase I, forming molecular hydrogen. The hydrogen is then oxidized by the membrane-bound hydrogenase II, donating electrons to a proton-translocating electron transport chain. The membrane-bound electron carriers may include cytochrome *b* and a rubredoxin. The membrane-bound hydrogenase could either leave protons on the inner or outer side of the membrane, although the data presented here do not specifically address this point. The oxidation of CO to CO_2 and H_2 has been shown to couple to energy conservation (3); here, we show that H_2 consumption is linked to energy conservation as well. These may represent two discrete sites for proton translocation during methanogenesis from acetate. Further purification studies and analysis of the organization of the genes encoding the hydrogenases present in acetate-grown *Ms. barkeri* may provide more information about the role of these enzymes.

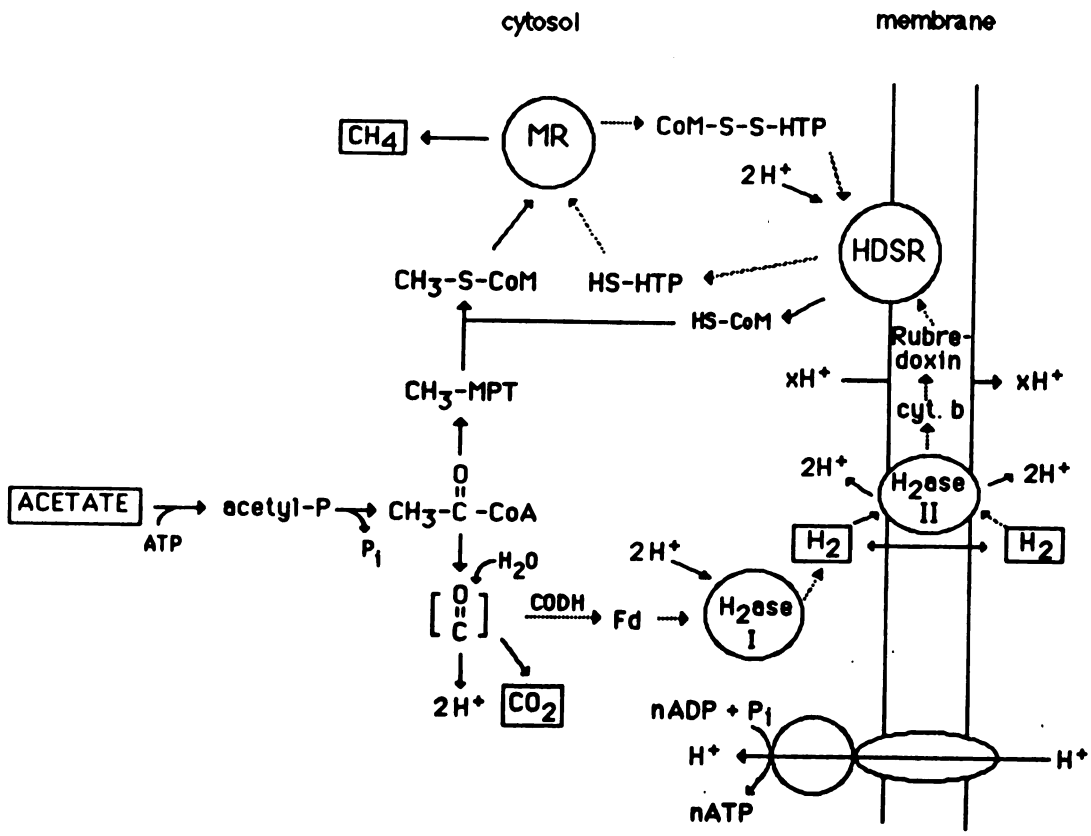


Fig 8. Model for function of hydrogenases during acetate catabolism by *Ms. barkeri* MS. HDSR, heterodisulfide reductase; MR, methylreductase; CoM, coenzyme M; HTP, 7-mercaptoheptanoylthreonine phosphate; MPT, tetrahydromethanopterin. Dashed lines indicate electron flow.

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CHAPTER 5

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

These studies on acetate degradation by *Methanosarcina barkeri* MS have made an important contribution to the understanding of acetotrophic methanogenesis. *Methanosarcina barkeri* is one of the most extensively studied methanogens, and this research adds to the current knowledge of methanogenic catabolism.

During acetate catabolism by methanogens, acetate is cleaved prior to reduction of the methyl group to methane using reducing equivalents generated by oxidation of the carbonyl group. The mechanism of electron transfer from the carbonyl to the methyl group of acetate was studied in relationship to hydrogen metabolism and energy conservation. A hypothesis was developed that electron-transport driven phosphorylation functions during acetate catabolism to couple electron transfer to chemiosmotic energy conservation. EPR spectroscopic analysis of the membrane of acetate-grown cells showed the presence of multiple iron-sulfur centers and a rubredoxin-like center, which were reducible by CO and H_2. The membrane-bound cytochrome *b* was shown by optical spectroscopy to be reduced by H_2 and oxidized by methyl-CoM. This suggested that these membrane-bound electron carriers may be part of an electron transfer chain.

Hydrogenase activity was found to be associated with the membrane of acetate-grown cells. This enzyme was of interest since it represents a membrane-bound oxidoreductase which may donate electrons to membrane-bound carriers. The purified enzyme did not couple to F_{420} or ferredoxin, but was found to link to cytochrome *b* in a detergent-solubilized system.

The function of hydrogenase(s) in production and consumption was studied further. Acetate-grown cells were found to contain levels of hydrogenase comparable to hydrogen-grown cells, and could consume hydrogen at comparable rates. Acetate-grown cells contained two types of hydrogenase activity, both F_{420}-reactive (hydrogenase I) and F_{420}-nonreactive (hydrogenase

II). CO-dependent hydrogen formation was reconstituted in vitro with purified CO dehydrogenase, ferredoxin, and a hydrogenase I fraction. A putative hydrogen production route linked to acetate catabolism was thereby described: reduced ferredoxin generated by oxidation of the carbonyl group of acetate by CO dehydrogenase could be used by hydrogenase I to form hydrogen.

Membrane-bound hydrogenase (hydrogenase II) was shown to couple the consumption of hydrogen and CoM-S-S-HTP reduction to ATP synthesis. These findings suggest that electron transfer from hydrogen to CoM-S-S-HTP is part of a proton-translocating electron transfer chain. The function of hydrogen-producing and consuming activities in acetate catabolism may be one of several possibilities. Hydrogen production and consumption may proceed in a stoichiometric fashion, with hydrogen as an obligate intermediate. Alternatively, hydrogen production and consumption may serve as a redox-regulated system and allow the methanogen to regulate the oxidation state of its catabolic machinery. In either case, the clumping morphology of this organism would aid in the recapture of hydrogen produced from neighboring cells.

The role of cytochromes in methanogenic catabolism is currently a matter of discussion. We have demonstrated that oxidation and reduction of the membrane-bound cytochrome *b* is associated with electron transfer to methyl-CoM. These data are consistent with a function of the cytochrome in acetate catabolism. An alternative hypothesis is that cytochromes participate in oxidation of methyl groups (4). However, this theory was advanced on the basis of the nature of catabolic substrates used by cytochrome-containing methanogens and not on direct biochemical evidence. It is possible that hydrogen consumption linked to a membrane-bound electron transfer chain containing cytochromes is a general feature of methyl-CoM reduction and energy

conservation by *Methanosarcina*, whether hydrogen, acetate, or methanol is the substrate.

In addition to H_2-dependent reduction of CoM-S-S-HTP, there may be other sites involved in energy conservation during methanogenesis from acetate. Methyl-H_4MPT:CoM transferase has been shown to be a primary sodium pump in *Methanosarcina* strain Gö1 (1, 2). Also, oxidation of CO to CO_2 and H_2 by whole cells blocked in methane formation is coupled to the chemiosmotic phosphorylation of 0.1 mol ADP/mol CO (3). In this reaction, electrons from CO oxidation may be transferred directly to membrane-bound carriers and subsequently to hydrogenase, perhaps by a membrane-associated reduced ferredoxin:acceptor oxidoreductase activity.

The results described in this thesis lead to several avenues of future research. Purification to homogeneity of hydrogenase I, which participates in CO-dependent hydrogen formation, would determine if this enzyme is one of the previously reported F_{420}-reactive hydrogenases or an as yet undescribed enzyme. Immunocytochemical localization of the hydrogenase II would provide more information on the cellular compartment of this enzyme in situ. Also, since hydrogenase I and II are expressed simultaneously in the same organism, it would be of interest to study the genetic organization of the genes which encode these enzymes. The molecular biology of methanogenic archaea is a rapidly expanding field (5), and may lead to interesting findings about gene expression in Archaea.

The large ferredoxin (13kDa) isolated from acetate-grown cells merits further study. Isolation of ferredoxin from methanol-adapted cells and comparison to the ferredoxin from acetate-grown cells would show if this protein is specifically expressed during acetate catabolism.

Finally, the membrane-bound electron carriers are of particular interest. Redox titration has indicated the presence of at least three different *b* cytochromes in *Ms. barkeri* (4); these may represent several different membrane-bound proteins. Purification of the cytochromes may show what oxidoreductase(s) they are associated with, and allow measurement of kinetics of oxidoreduction. The detergent-solubilized system of hydrogen-dependent cytochrome *b* reduction is a starting point for the dissection of this reaction. Chromatographic separation and reconstitution of fractions will offer a means of studying the enzyme(s) and cofactor(s) involved in cytochrome oxidoreduction. In addition, cytochromes are potential 'molecular chronometers' for phylogenetic analysis (6). Amino acid sequence information may provide further information on the interrelatedness of methanogenic archaea which possess this electron carrier.

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