A BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF COENZYME F_{420}-REDUCING HYDROGENASE FROM *METHANOBACTERIUM FORMICICUM*

by

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(ABSTRACT)

The coenzyme F_{420}-reducing hydrogenase of Methanobacterium formicicum was purified 87-fold to electrophoretic homogeneity. The enzyme formed aggregates (1,000 kd) of a coenzyme F_{420}-active monomer (109 kd) composed of 1 each of α, β, and γ subunits (43.6, 36.7, and 28.8 kd, respectively). It contained 1 mol of FAD, 1 mol of nickel, 12-14 mols of iron, and 11 mols of acid-labile sulfide per mol of the 109 kd species, but no selenium. The amino acid sequence I---P--R-EGH-----EV was conserved in the N-terminus of α subunit of the enzyme and the largest subunits of nickel-containing hydrogenases from Methanobacterium thermoautotrophicum, Desulfovibrio baculatus, and Desulfovibrio gigas. FAD dissociated from the coenzyme F_{420}-reducing hydrogenase during reactivation with H_2 and coenzyme F_{420}, unless KCl was present, yielding coenzyme F_{420}-inactive apoenzyme. The hydrogenase catalyzed H_2 production at a rate 3-fold less than that for

H_2 uptake. Specific antiserum inhibited the coenzyme F_{420}-dependent activity but not the methyl viologen-dependent activity of the purified enzyme.

Cell extract of *M. formicicum* contained a coenzyme F_{420}-mediated formate hydrogenlyase system. Formate hydrogenlyase activity was reconstituted with coenzyme F_{420}-reducing hydrogenase, coenzyme F_{420}-reducing formate dehydrogenase, and coenzyme F_{420}, all purified from *M. formicicum*. The reconstituted system required FAD for maximal activity (kinetic K_d= 4 μM). Without FAD, the formate dehydrogenase and hydrogenase rapidly lost coenzyme F_{420}-dependent activity relative to methyl viologen-dependent activity. Immunoadsorption of the formate dehydrogenase or hydrogenase from cell extract greatly reduced formate hydrogenlyase activity; addition of the purified enzymes restored activity. Formate hydrogenlyase activity of cell extract and the reconstituted system was reversible.

The coenzyme F_{420}-reducing hydrogenase and formate dehydrogenase of *M. formicicum* were shown to be located at the cytoplasmic membrane using immunogold labeling of thin-sectioned, Lowicryl-embedded cells. Neither enzyme was released from whole cells by osmotic shock treatment.

FOREWORD

This dissertation consists of nine sections. Section I is a brief introduction. Section II is a review of the literature that pertains to my research. Sections III, IV, V, and VI contain the results of the dissertation and are written in publication form. Section VII is a general summary of the results. Literature cited in Sections I, II, and VII is included in Section VIII; all other cited literature is listed at the end of the respective sections. Section IX is my *curriculum vitae*.

The following are the titles of the manuscripts that have been accepted or submitted for publication:

Section III: Baron, S. F., and J. G. Ferry. 1988. Purification and properties of the membrane-associated coenzyme F_{420}-reducing hydrogenase from *Methanobacterium formicicum*. J. Bacteriol. (submitted).

Section IV: Baron, S. F., and J. G. Ferry. 1988. Reconstitution and properties of coenzyme F_{420}-mediated formate hydrogenlyase in *Methanobacterium formicicum*. J. Bacteriol. (submitted).

Section V: Baron, S. F., and J. G. Ferry. 1987. Locations of the hydrogenases of Methanobacterium formicicum after subcellular fractionation of cell extract. J. Bacteriol. **169:**3823-3825.

Section VI: Baron, S. F., D. S. Williams, H. D. May, P. S. Patel, H. C. Aldrich, and J. G. Ferry. 1988. Immunogold localization of coenzyme F_{420}-reducing hydrogenase in Methanobacterium formicicum. Arch. Microbiol. (in press).

In addition to the above journal articles, I have contributed to the following publications while a student in Dr. Ferry's laboratory:

Abbanat, D. R., D. J. Aceti, S. F. Baron, L. L. Lundie, Jr., K. C. Terlesky, and J. G. Ferry. 1989. Microbiology and biochemistry of the methanogenic archaeobacteria, **In:** Advances in space research. Proceedings of the XXVIII plenary meetings of the committee on space research. Pergamon Press, New York. (in press).

Baron, S. F., H. D. May, K. C. Terlesky, and J. G. Ferry. 1987. Metabolic regulation in methanogenic bacteria

with potential influence on the performance of biomass digestors. Poultry Science. **66**:922-926.

Sowers, K. R., S. F. Baron, and J. G. Ferry. 1984. Methanosarcina acetivorans sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. Appl. Environ. Microbiol. **47**:971-978.

DEDICATION

To my wife, , who has filled the past four years of my life with happiness and peace.

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SECTION I. GENERAL INTRODUCTION

Methanobacterium formicicum is one of several methanogenic bacteria which obtain energy for growth by reducing CO_2 to methane with electrons derived from H_2 or formate. The oxidation of formate in this organism is catalyzed by formate dehydrogenase, which reduces the physiological electron acceptor, coenzyme F_{420} (F_{420}). The oxidation of H_2 is catalyzed by hydrogenase, two types of which have been identified in H_2-oxidizing methanogenic bacteria. One (F_{420}-hydrogenase) reduces F_{420}. The other (MV-hydrogenase) reduces the artificial electron acceptor, methyl viologen but not F_{420}, and its physiological electron acceptor is unknown. The F_{420}-hydrogenase may function in the methylreductase reaction, which is the last and energy-yielding step in methanogenesis, but this is still unclear.

Cell extracts of M. formicicum and several other formate-utilizing methanogenic bacteria have an electron transport system called formate hydrogenlyase which converts formate to H_2 and CO_2. Formate hydrogenlyase in coliform bacteria consists of a formate dehydrogenase, a hydrogenase, and one or more intermediate electron carriers. F_{420} is required for activity of the formate hydrogenlyase in methanogenic bacteria, but the other

components are unknown.

The goals of this research were i) to purify the components of the formate hydrogenlyase system of *M. formicicum* and reconstitute the system; ii) to determine the cellular locations of its enzymatic components; and iii) to characterize the hydrogenase component.

SECTION II. LITERATURE REVIEW

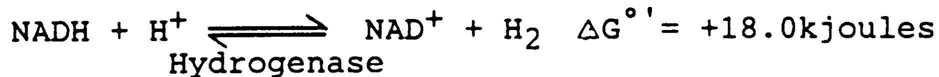
Ecology and Microbiology of Methanogenesis

The formation of methane by methanogenic bacteria (methanogenesis) occurs in anaerobic environments which have no terminal electron acceptors other than CO_2 and which receive large amounts of organic matter (8). These environments include the rumen and large intestines of animals, aquatic sediments, sewage digestors, and flooded soils, tundra, and bogs (87). The complete conversion of complex organic matter to methane and CO_2 is carried out by a microbial consortium consisting of three basic groups (87). First, fermentative microorganisms degrade complex organic polymers to smaller molecules, which are then fermented to H_2, CO_2, alcohols, fatty acids (formate, acetate, propionate, butyrate, etc.) and other organic acids (e.g., succinate and lactate). Second, H_2-producing acetogenic bacteria oxidize alcohols and higher fatty acids and produce acetate, H_2, and CO_2. Finally, methanogenic bacteria reduce CO_2 to methane with H_2 as electron donor and convert substrates including formate, acetate, methanol, and methylamines to methane and CO_2.

Acetate is the major substrate for methanogenesis in most anaerobic environments, accounting for 60-90% of the methane produced (87). In the rumen however, acetate and

other fatty acids are absorbed into the bloodstream and used as an energy source for the ruminant (18). Therefore, H_2/CO_2 is the major methane precursor in the rumen (61), and formate accounts for about 18% of the methane produced (62). Formate and H_2/CO_2 are equivalent substrates for methanogenesis, yielding about the same standard free energy change per mole of methane produced (87). About one half of all H_2-utilizing methanogenic bacteria isolated can also use formate as sole carbon and energy source (71), including Methanobacterium formicicum, the organism used for this dissertation work.

The methanogenic bacteria influence growth and end product formation by the fermentative microorganisms and H_2-producing acetogenic bacteria through a process termed 'interspecies H_2 transfer'. For example, the degradation of propionate to acetate and H_2 by the H_2-producing acetogenic bacteria is endergonic but is exergonic when coupled to methanogenesis from H_2 plus CO_2 (18). Therefore, growth of these bacteria depends entirely upon the rapid removal of H_2 by methanogenic bacteria, and the two are isolated and cultured in symbiotic association (17,19,88,89,108). In the fermentative microorganisms, NADH generated during glycolysis can be reoxidized through H_2 production via hydrogenase, which is thermodynamically unfavorable under standard conditions (18):



$$NADH + H^+ \underset{\text{Hydrogenase}}{\rightleftharpoons} NAD^+ + H_2 \quad \Delta G^{\circ\prime} = +18.0\text{kjoules}$$

In pure cultures of fermentative microorganisms, H_2 accumulates, and the organisms must therefore reoxidize NADH by reducing the glycolytic intermediate, pyruvate, to products such as alcohols and higher fatty acids. However, in cocultures with methanogenic bacteria, H_2 is maintained at low levels, and proportionally more of the oxidized products, CO_2 and acetate are formed (26,27,64,76,145).

Phylogeny of Methanogenic Bacteria

The methanogenic bacteria, along with extreme halophilic bacteria and extreme thermophilic bacteria, comprise a unique kingdom of prokaryotic organisms called archaeobacteria (71). This kingdom has been shown to be distinct from eukaryotes and typical prokaryotes (eubacteria) by comparative cataloguing of 16S rRNA (48,146,147) and by differences in cell wall structure (73), cytoplasmic membrane lipid composition (84,136), antibiotic sensitivity (60), and RNA polymerase structure (152). In addition, as illustrated in Fig. 1, the methanogenic archaeobacteria have several novel coenzymes including: coenzyme F_{420} (38), methanofuran (70), methanopterin (138), coenzyme M (128), factor F_{430} (32), and 7-mercaptoheptanoylthreonine phosphate (100).

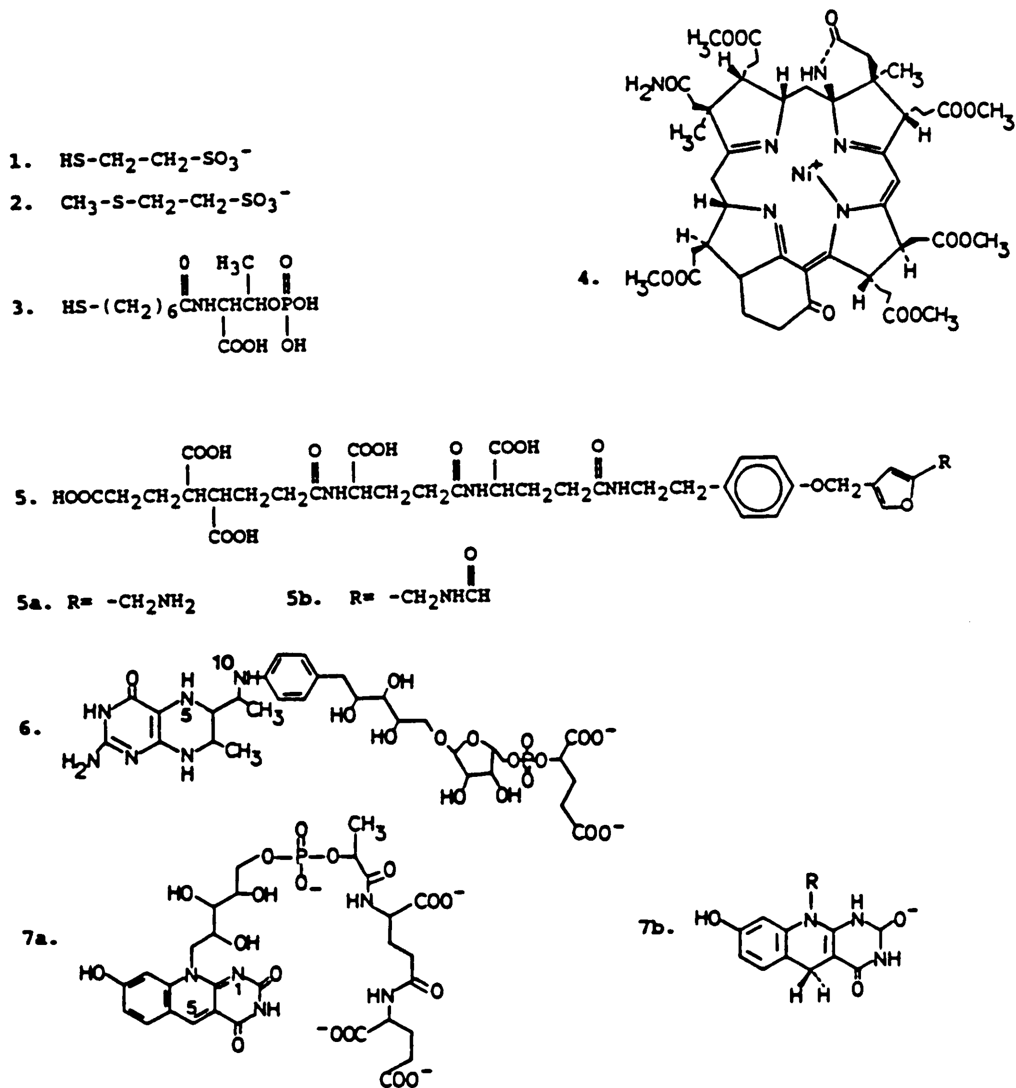


Figure 1. Structures of the unique coenzymes involved in methanogenesis. Taken from reference 71. 1. coenzyme M; 2. methyl coenzyme M; 3. 7-mercaptoheptanoylthreonine phosphate; 4. factor F_{430}; 5a,b. methanofuran and formylmethanofuran, respectively; 6. tetrahydromethanopterin; 7a,b. oxidized and reduced coenzyme F_{420}, respectively.

Biochemistry of Methanogenesis

In 1940, Barker (11) observed that methanogenic bacteria could reduce CO_2 to methane with H_2 as electron donor and proposed a pathway in which CO_2 is sequentially reduced to methane. Sparling and Daniels (125) demonstrated that formate is not reduced directly to methane but is first oxidized to CO_2, suggesting that the same CO_2 reduction pathway is involved. This pathway has largely been elucidated (110) and is illustrated in Fig. 2. Structures of the unique coenzymes involved are shown in Fig. 1. Electrons for the reductive steps are ultimately obtained from the oxidation of H_2, catalyzed by hydrogenase, or of formate, catalyzed by formate dehydrogenase. CO_2 is initially bound to methanofuran (MFR) and is reduced to the formyl level, yielding formyl-MFR (78). The formyl moiety is transferred to tetrahydromethanopterin (H_4MPT), yielding 5-formyl-H_4MPT (35), which is then dehydrated to 5,10-methenyl-H_4MPT (34). This product is reduced to methylene-H_4MPT with reduced coenzyme F_{420} (F_{420}) as the electron donor (57). Methylene-H_4MPT is reduced to methyl-H_4MPT and the methyl moiety then transferred to coenzyme M (HS-CoM) (110).

The last step of the CO_2 reduction pathway (Fig. 2) is the reduction of methyl-S-CoM to methane, catalyzed by the methyl-S-CoM methylreductase complex (methylreductase)

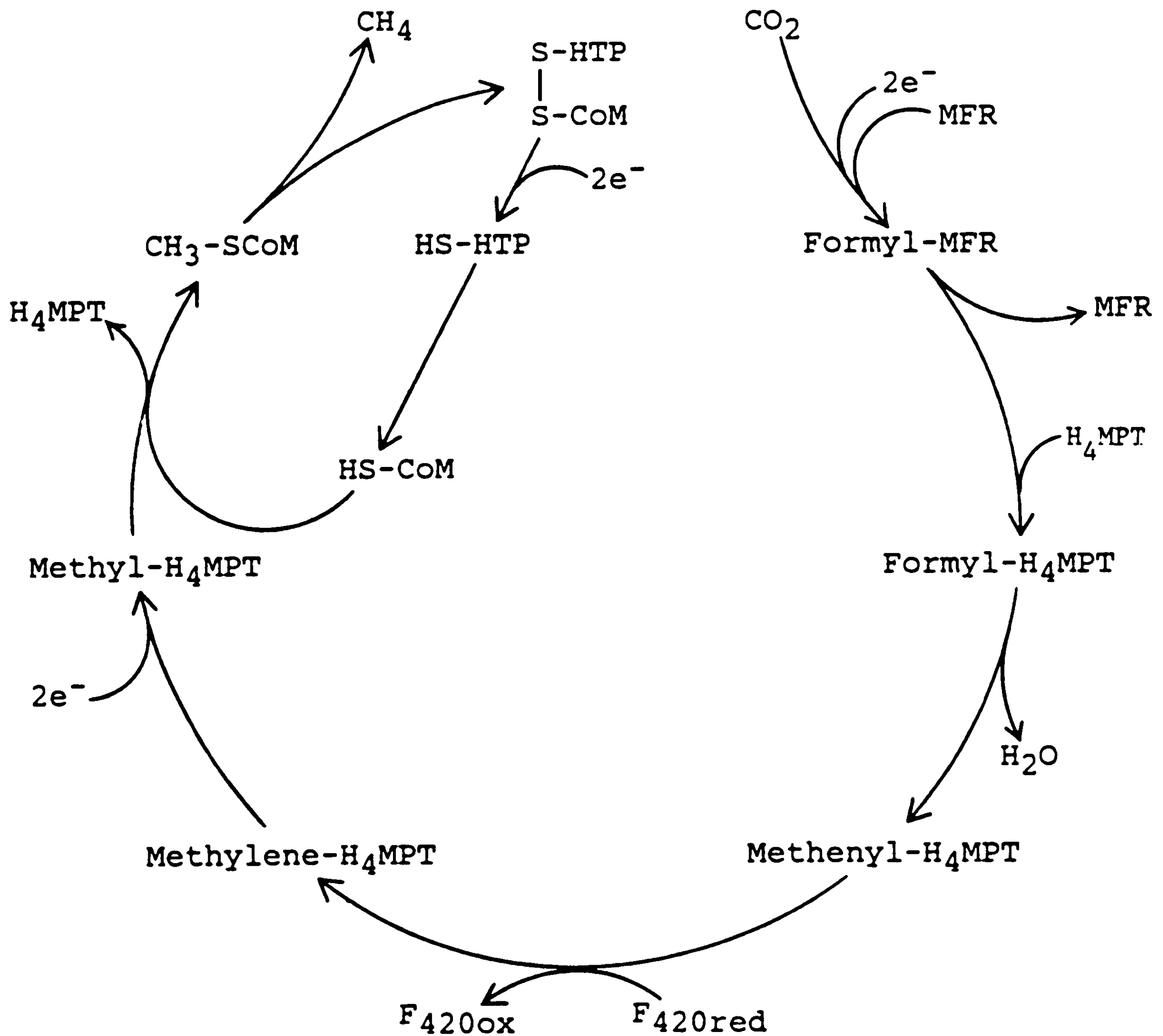


Figure 2. Proposed pathway for the reduction of CO_2 to methane. Taken from reference 110. MFR, methanofuran; H_4MPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-HTP, 7-mercaptoheptanoyl-threonine phosphate; F_{430}, factor F_{430}; F_{420ox}, F_{420red}, oxidized and reduced coenzyme F_{420}; e^-, electron.

(54,96). The H_2-coupled methylreductase system of *Methanobacterium thermoautotrophicum* has been resolved into four protein components: A1, A2, A3, and C, and four cofactors: ATP, Mg^{+2}, FAD, and component B (54,96). Component C contains two molecules of the nickel tetrapyrrole, factor F_{430} (39) and is thought to be the site where methyl-S-CoM is reduced (96). Component A1 has F_{420}-dependent hydrogenase activity (96), but an absolute requirement for this activity in the methylreductase system has not been demonstrated. The functions of A2 and A3 are unknown. The immediate electron donor for the reduction of methyl-S-CoM is component B (101), recently identified as 7-mercaptoheptanoyl-threonine phosphate (HS-HTP, see Fig. 1) (100), and the oxidized product of this reaction is a mixed disulfide of HS-CoM and HS-HTP (CoM-S-S-HTP) (16,41). The disulfide can be reductively cleaved back to HS-CoM and HS-HTP by an H_2-coupled enzyme system (59).

ATP Synthesis in Methanogenic Bacteria

Recent evidence suggests that ATP is synthesized by a chemiosmotic mechanism in methanogenic bacteria. A plausible source of a protonmotive force is the oxidation of H_2 by a membrane-associated hydrogenase (135) coupled to the methylreductase reaction (31). H_2-driven ATP synthesis has been demonstrated in membrane vesicles (33) and

protoplasts (91) of *M. thermoautotrophicum*. Blaut and Gottschalk (15) showed that methanogenesis from methanol plus H_2 drives ATP synthesis in *Methanosarcina barkeri* and *Methanosphaera stadtmaniae*. The F_{420}-dependent hydrogenase of *Methanococcus vannielii* (94) is membrane-associated. Component C of the methylreductase system is membrane-associated in *M. thermoautotrophicum* (7) and *M. vannielii* (103), and is located in a large, membrane-bound sacculus (methanoreductosome) in methanogenic bacterium strain Göl (86). Mayer *et al*. (85) also identified a membrane-bound ATPase in strain Göl. Ellerman *et al*. (41) suggested that the H_2-driven reduction of CoM-S-S-HTP could generate a protonmotive force, but the enzyme system catalyzing this reaction is soluble (59).

Growth and methanogenesis from certain substrates in methanogenic bacteria requires Na^+ (105), and a Na^+/H^+ antiporter is present in *M. thermoautotrophicum* (121), suggesting a role for Na^+ in ATP synthesis. Daniels *et al*. (31) proposed that respiratory extrusion of Na^+ and subsequent H^+ extrusion by a Na^+/H^+ antiporter could generate a protonmotive force. Blaut and Gottschalk (15) suggested that Na^+ gradients may be involved in coupling the endergonic and exergonic reactions of methanogenesis.

Coenzyme F_{420}

Coenzyme F_{420} (F_{420}) has been found in all methanogenic bacteria examined (38). Structurally, F_{420} is similar to the flavin, FAD; it consists of an 8-hydroxy-5-deazaflavin chromophore with a highly anionic lactyl-glutamyl-glutamate side chain (Fig. 1) (37). F_{420} is electronically similar to pyridine nucleotides, since it is reduced in a direct hydride ion (i. e., two electron) transfer at the number 5 position of the deazaflavin moiety (47). The midpoint redox potential of F_{420} is -340 to -350 mV (66), indicating that it functions as a low potential electron carrier in methanogenic bacteria. The coenzyme is an electron acceptor or donor for hydrogenase (49,97,95, 126,150), formate dehydrogenase (68,115,137), carbon monoxide dehydrogenase (30), F_{420}:NADP oxidoreductase (68,137), pyruvate synthase, α-ketoglutarate synthase (50,151), and methylene-H_4MPT dehydrogenase (57).

Hydrogenase

A. Definition and occurrence in nature.

The activation of H_2 is carried out by the enzyme hydrogenase, which catalyzes the reversible reaction: $H_2 \rightleftharpoons 2H^+ + 2e^-$ using suitable electron donors or acceptors. Hydrogenase was first discovered by Stephenson and Stickland (127) in enrichment cultures from polluted

river sediments and has since been detected in anaerobic bacteria, aerobic bacteria, oxygenic and anoxygenic photosynthetic bacteria, eukaryotic algae, and protozoa (4). Some hydrogenases preferentially catalyze the oxidation of H_2 (H_2 uptake) _in vitro_ and are therefore termed 'unidirectional' or 'uptake' hydrogenases. Others catalyze H_2 uptake and H_2 evolution at considerable rates and are therefore termed 'bidirectional'.

B. Physiological functions.

Uptake hydrogenases are found in aerobic and anaerobic bacteria which use H_2 as an energy source for growth (4), a list of which is shown in Table 1. In all of these organisms, a hydrogenase catalyzes the oxidation of H_2, and the electrons are passed down a membrane-bound electron transport chain to a terminal electron acceptor, thus generating a protonmotive force (4). For example, _Escherichia coli_ can grow anaerobically by reducing fumarate to succinate, using H_2 as the electron donor (14). This is mediated by a membrane-bound electron transport system consisting of an uptake hydrogenase (isoenzyme 2) (111), cytochrome b, menaquinone, and fumarate reductase (14).

Bidirectional hydrogenases are found in anaerobic bacteria and mediate the production of H_2 from electron

Table 1. Bacteria which use H_2 as an energy source.[a]

Representative genus	Reaction	Reference
Desulfovibrio[b]	$4H_2 + SO_4^{-2} \longrightarrow S^{-2} + 4H_2O$	10
Methanobacterium[b]	$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$	71
Acetobacterium[b]	$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O$	120
Escherichia[c]	H_2 + Fumarate $\longrightarrow$ Succinate	14
Escherichia[c]	$H_2 + (CH_3)_3NO \longrightarrow (CH_3)_3N + H_2O$	80
Escherichia[c]	$H_2 + (CH_3)_2SO \longrightarrow (CH_3)_2S + H_2O$	80
Campylobacter[c]	$H_2 + S^0 \longrightarrow H_2S$	148
Alcaligenes[d]	$H_2 + \frac{1}{2}O_2 \longrightarrow H_2O$	9
Paracoccus[d]	$5H_2 + 2NO_3^- + 2H^+ \longrightarrow N_2 + 6H_2O$	117

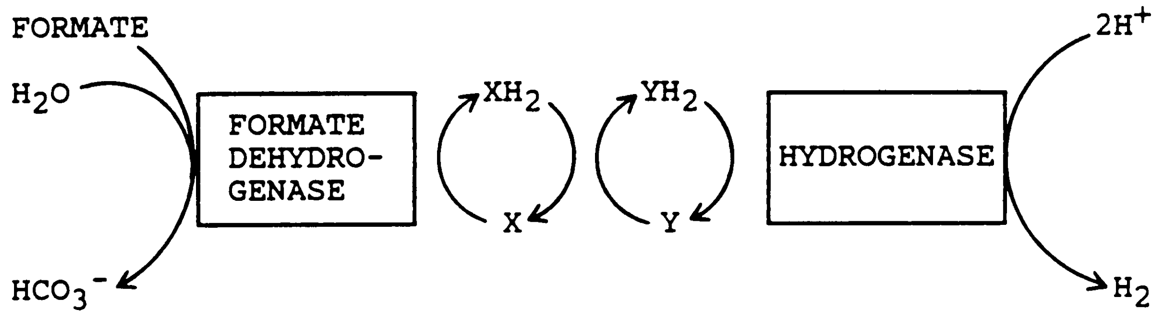
[a]Modified from reference 117.

[b]Obligately anaerobic

[c]Facultatively anaerobic or microaerophilic

[d]Aerobic or obligately respiring

carriers reduced during fermentations (4). For example, a bidirectional hydrogenase in *Clostridium pasteurianum* (25) oxidizes ferredoxin reduced during the oxidation of pyruvate. H_2 production allows anaerobic bacteria to regulate end product formation and ATP synthesis by providing an alternate route for disposal of reducing potential. *Ruminococcus albus* ferments glucose to ethanol, acetate, CO_2, and H_2 (64). NADH generated during glycolysis can be reoxidized either through H_2 production (catalyzed by hydrogenase) or through reduction of acetyl-CoA to ethanol. ATP is synthesized by the phosphoroclastic reaction, in which acetyl-CoA is converted to acetate. Therefore, production of H_2 allows more acetyl-CoA to be used for ATP synthesis. This mechanism is most effective when H_2 is rapidly removed by interspecies H_2 transfer (64). *Escherichia coli* and other anaerobic bacteria have an anaerobic electron transport system called formate hydrogenlyase (Fig. 3) which cleaves metabolically produced formate to H_2 and CO_2 (53). The formate hydrogenlyase system of *E. coli* is induced under anaerobic conditions (80) and consists of a soluble, benzyl viologen-linked formate dehydrogenase (28), a membrane-bound hydrogenase (isoenzyme 3) (111), and one or more as yet unidentified electron carriers (80). Cell extracts of methanogenic bacteria have a formate hydrogenlyase system



$\Delta G°' = +1.3$ kjoules

Figure 3. Schematic diagram of formate hydrogenlyase. X and Y represent intermediate electron carriers.

mediated by F_{420} (46,99,137), but the other components of the system have not been purified and recombined to reconstitute activity.

C. Cellular location.

Molecular H_2 can freely permeate biological membranes, while its oxidation product, protons, cannot. Therefore, hydrogenase itself could theoretically generate a proton gradient across the cytoplasmic membrane (10,77,102,135). Many hydrogenases are membrane-associated, such as the uptake enzymes from *Rhodopseudomonas capsulata* (124) and *Alcaligenes eutrophus* (116). In contrast, some hydrogenases are cytoplasmic, such as the NAD-reducing uptake enzyme of *A. eutrophus* (118) and the ferredoxin-dependent bidirectional enzyme of *C. pasteurianum* (25).

Many bacteria have multiple hydrogenases. *D. vulgaris* strain Hildenborough has a periplasmic, bidirectional hydrogenase (104) and a membrane bound, bidirectional hydrogenase (51) which may function in a hydrogen cycling mechanism (102), as illustrated in Fig. 4. Electrons from the oxidation of lactate are passed to an internal hydrogenase, which catalyzes the production of H_2. H_2 permeates the membrane and is reoxidized to protons *via* the periplasmic hydrogenase. The electrons are transferred

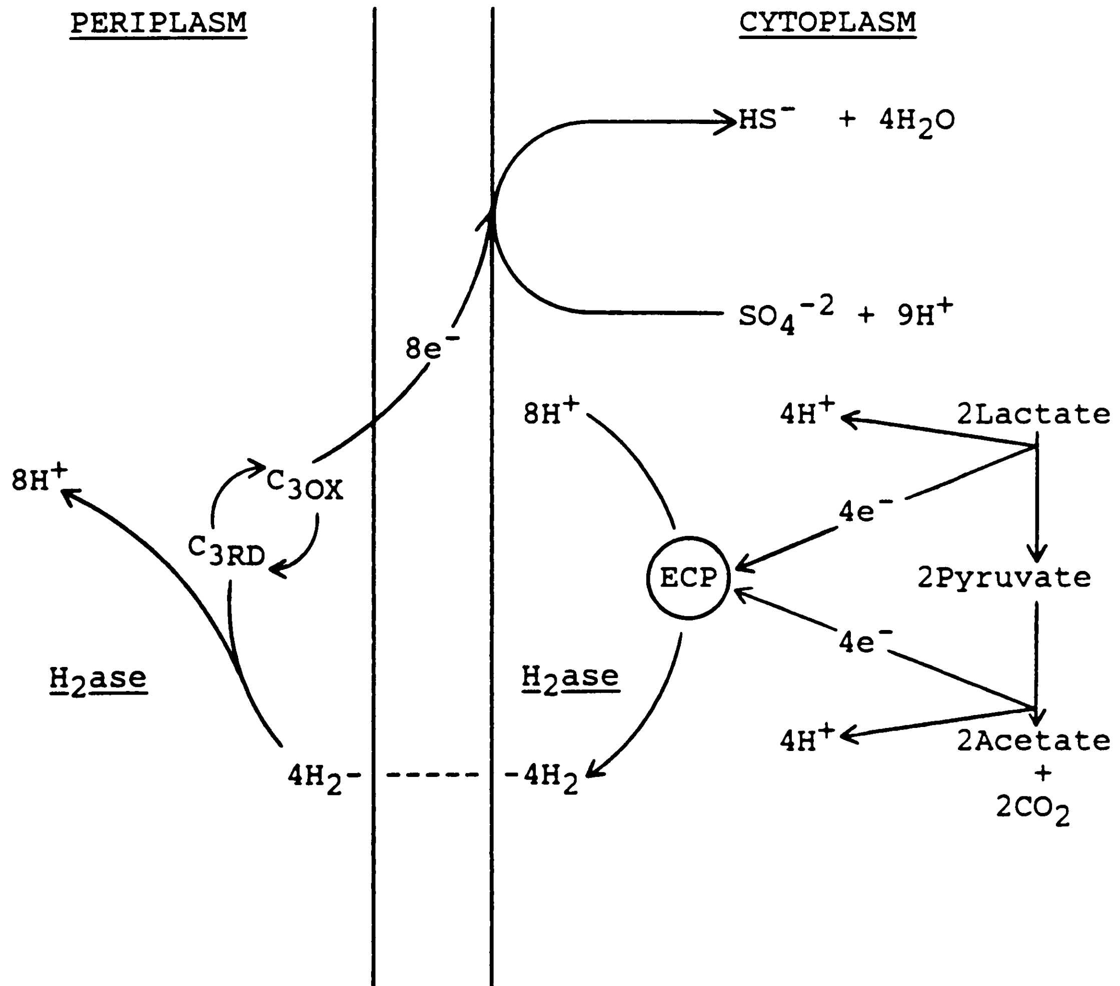


Figure 4. Proposed mechanism of H_2 cycling by Desulfovibrio during growth on lactate and sulfate. ECP, electron carrier proteins; $c_{3ox,rd}$, oxidized and reduced forms of cytochrome c_3; e^-, electron; H_2ase, hydrogenase. Taken from reference 102.

back across the membrane by an electron transport chain and used to reduce sulfate to sulfide. The uptake of protons in the cytoplasm and release in the periplasmic space thus generates a protonmotive force.

D. Physical and biochemical properties.

The properties of hydrogenases are highly variable, reflecting the diversity of organisms in which they are found. Yet, there are some conserved features. For example, all hydrogenases examined mediate an exchange between the protons in H_2 and the protons in water; when hydrogenases are incubated with deuterium gas (D_2), HD and H_2 are formed in various ratios, depending on the pH and the particular hydrogenase used (79).

All hydrogenases examined contain non-heme iron and acid-labile sulfide (4) present in [2Fe-2S], [3Fe-XS], or [4Fe-4S] clusters (4,77,117,130). Other metals detected in stoichiometric amounts include nickel (58,143), selenium (12,95,109,129,130,150), copper, and zinc (1). Based on their metal contents, hydrogenases can be classified into three broad groups: i) those which contain nickel and iron-sulfur centers (NiFe hydrogenases) (5,6,12,22,29,42, 75,92,130,131,132); ii) those which contain nickel, iron-sulfur centers, and selenium (NiFeSe hydrogenases) (12,95,109,129,130); and iii) those which contain only

iron-sulfur clusters (Fe hydrogenases) (2,63,139,144,149). These classes differ in several respects: i) The nickel-containing enzymes appear to function in H_2 uptake, since all but two uptake hydrogenases examined (3,106) contain nickel (21,58). In contrast, of the five Fe hydrogenases which have been identified (3,24,52,106,140), three are bidirectional (2). ii) The NiFe and NiFeSe hydrogenases are reversibly inactivated by oxygen (21) while the Fe hydrogenases are irreversibly inactivated (2). iii) The Fe and NiFeSe enzymes are inhibited by carbon monoxide, nitrate, and nitric oxide more than are the NiFe enzymes (13). iv) The three classes differ in pH optima for H_2 uptake, H_2 evolution, and proton-deuterium exchange activities (79).

The H_2-binding site in nickel-containing hydrogenases is thought to be a nickel atom bound by four sulfur ligands (81,122). The most accepted mechanism for H_2 oxidation involves a heterolytic cleavage: nickel binds H_2 and deprotonates it, yielding a nickel-hydride which is further deprotonated (143). The iron-sulfur centers interact with the nickel site (6,23) and probably oxidize the nickel center one electron at a time as it is reduced (143). Selenium may substitute for one of the sulfur ligands to nickel and help to destabilize the H_2 molecule during deprotonation (129). The oxidation of H_2 in Fe

hydrogenases may involve a novel iron-sulfur cluster (2,63,55,56).

Flavins are prosthetic groups of hydrogenases which reduce hydride ion acceptors. For example, the NAD-reducing uptake hydrogenase of A. eutrophus (118) contains FMN, and the F_{420}-reducing hydrogenases of three methanogenic bacteria contain FAD (49,97,95).

Nickel-containing hydrogenases are stable in air but are reversibly inactivated (4,21). They can be reactivated by incubation under H_2 with or without an electron acceptor, by treatment with glucose plus glucose oxidase, or by incubation with thiols (4). Cammack et al. (20) and Fernandez et al. (43,45) proposed that the nickel-containing hydrogenases from Desulfovibrio and Alcaligenes can exist in three distinct states: unready, ready, and active. When these hydrogenases are purified aerobically they are inactive (unready) but are slowly reactivated with H_2 or other reductants to the fully active state. The active state is rapidly converted to a temporarily inactive (ready) state by anaerobic incubation with an electron acceptor. The ready and active states are both converted to the unready state by exposure to O_2. Based on electron paramagnetic resonance data obtained with the hydrogenase of Desulfovibrio gigas, Fernandez et al. (44) hypothesized that the unready state is an inactive

conformation of the enzyme containing Ni(III); the ready state, an active conformation containing Ni(III); and the active state, an active conformation containing Ni(I).

E. Hydrogenases of methanogenic bacteria.

Two types of hydrogenase have been identified in methanogenic bacteria (65,67,97). One (F_{420}-hydrogenase) reduces F_{420} and the artificial electron acceptor, methyl viologen. The other (F_{420}-non-reactive or MV-hydrogenase) reduces methyl viologen but not F_{420}, and its physiological electron acceptor is unknown. The two enzymes have different subunit composition, electrophoretic mobility, and chromatographic properties (65,67,97), but it is not known whether they are immunologically related.

F_{420}-hydrogenases from several methanogenic bacteria have been purified (42,49,95,126,150). All are large, particulate enzymes, ranging from 720,000-1,300,000 in molecular weight, and contain nickel, iron, and acid-labile sulfide, the latter two presumably in 4Fe-4S clusters (81,42). In addition, the enzymes of *Methanococcus voltae* (95) and *Methanococcus vannielii* contain selenium. FAD is present in the F_{420}-hydrogenases of *M. thermoautotrophicum* (49), *M. formicicum* (97, this dissertation), and *M. voltae* (95); the enzyme from *M. barkeri* contains FMN or riboflavin (42). FAD is required for F_{420}-reducing

activity of the F_{420}-hydrogenase from _M_. _formicicum_ (97).

Only one MV-hydrogenase has been purified and characterized, the enzyme from _M_. _formicicum_ (1). In contrast to the F_{420}-hydrogenase, this enzyme is soluble and relatively small (molecular weight 70,000). It contains nickel, iron-sulfur centers, zinc, and copper, but not selenium. The functions of copper and zinc in the enzyme are unclear, but copper gives an intense electron paramagnetic resonance signal in the reduced enzyme (1).

Formate dehydrogenase

Formate dehydrogenase catalyzes the reversible oxidation of formate to CO_2 according to the equation:

$$HCOO^- + H_2O \rightleftharpoons HCO_3^- + 2e^- + 2H^+$$

using suitable electron acceptors or donors. This enzyme has several physiological functions: i) oxidation of formate for use as an energy source by aerobic bacteria, methanogenic bacteria, and yeasts (134); ii) dissimilation of formate by the formate hydrogenlyase system of anaerobic bacteria (53); iii) reduction of CO_2 to acetate during fermentation of carbohydrates or purines by clostridia (83); and iv) reduction of CO_2 to formate for use in biosynthetic reactions of clostridia (72).

Formate dehydrogenase activity in methanogenic bacteria is linked to F_{420} (46,69,115,137). _M_. _vannielii_

has two distinct F_{420}-reducing formate dehydrogenases (69). One is a large complex which contains tungsten and selenium in the form of selenocysteine. The other is a smaller enzyme (molecular weight 105K) which contains molybdenum, iron, and acid-labile sulfide. The smaller enzyme, together with an F_{420}:$NADP^+$ oxidoreductase purified from *M. vannielii*, reconstitutes a formate-$NADP^+$ oxidoreductase system (68). *M. formicicum* has only one formate dehydrogenase: an enzyme of molecular weight 177K which has two subunits (85K and 53K) and contains molybdopterin, iron, acid-labile sulfide, zinc, and FAD (115). The flavin is required for F_{420}-reducing activity of the enzyme (114,115). This enzyme is operationally soluble, since the majority of formate dehydrogenase activity is present in the supernatant fraction after ultracentrifugation of cell extract (114).

SECTION III. PURIFICATION AND PROPERTIES OF

COENZYME F_{420}-REDUCING HYDROGENASE

FROM *METHANOBACTERIUM FORMICICUM*

SUMMARY

The membrane-associated coenzyme F_{420}-reducing hydrogenase of *Methanobacterium formicicum* was purified 87-fold to electrophoretic homogeneity. The enzyme contained α, β, and γ subunits (molecular weights 43,600, 36,700, and 28,800, respectively) and formed aggregates (molecular weight 1,020,000) of a coenzyme F_{420}-active $\alpha_1\beta_1\gamma_1$ trimer (molecular weight 109,000). The hydrogenase contained 1 mol of FAD, 1 mol of nickel, 12-14 mols of iron, and 11 mols of acid-labile sulfide per mol of the molecular weight 109,000 species, but no selenium. The isoelectric point was 5.6. The amino acid sequence I---P--R-EGH------V was conserved in the N-termini of the α subunits of the F_{420}-hydrogenases from *M. formicicum* and *Methanobacterium thermoautotrophicum* and of the largest subunits of nickel-containing hydrogenases from *Desulfovibrio baculatus*, *Desulfovibrio gigas*, and *Rhodobacter capsulatus*. The hydrogenase was reversibly inactivated by exposure to O_2 and could be reductively reactivated. FAD dissociated from the enzyme during reactivation, unless potassium salts were present, yielding apoenzyme unable to reduce coenzyme F_{420}.

The enzyme catalyzed H_2 production at a rate 3-fold less than that for H_2 uptake and reduced coenzyme F_{420}, methyl viologen, flavins, and 7,8-didemethyl-8-hydroxy-5-deazariboflavin. Maximal coenzyme F_{420}-reducing activity was obtained at 55°C and pH 7.0-7.5, and with 0.2-0.8 M KCl in the reaction mixture. Specific antiserum inhibited the coenzyme F_{420}-dependent but not the methyl viologen-dependent activity of the purified enzyme.

INTRODUCTION

The methanogenic bacteria that oxidize H_2 and reduce carbon dioxide ($4H_2 + CO_2 \rightleftharpoons CH_4 + 2H_2O$) obtain energy for growth by a chemiosmotic mechanism that couples ATP synthesis to electron transport (8). At least one step in the CO_2 reduction pathway (16) is driven by the reduced form of the physiological electron acceptor, coenzyme F_{420} (F_{420}). Many H_2-oxidizing methanogens studied have two distinct hydrogenases (19,20,29). One (F_{420}-hydrogenase) reduces F_{420}, and also the artificial electron acceptor, methyl viologen (MV). The other (F_{420}-non-reactive hydrogenase or MV-hydrogenase) reduces MV but not F_{420}. F_{420}-hydrogenases from several H_2-oxidizing methanogens have been purified and characterized (12,15,28,38,42). All of these enzymes form large aggregates of molecular weight 720,000-1,300,000 (720-1,300K), and contain nickel and

iron-sulfur centers. The enzymes from Methanobacterium thermoautotrophicum (15), Methanobacterium formicicum (29) and Methanococcus voltae (28) also contain FAD, which is required for reduction of F_{420} (29).

The location of F_{420}-hydrogenases on the cytoplasmic membrane (4,6,27) is consistent with an involvement in H_2-driven electron transport coupled to ATP synthesis. The F_{420}-hydrogenase of M. formicicum is membrane-associated (4,6) and is a component of the formate hydrogenlyase system in this organism (5). Here we describe the purification of this hydrogenase and compare its properties to F_{420}-hydrogenases from other carbon dioxide-reducing methanogenic bacteria.

MATERIALS AND METHODS

Purification. Methanobacterium formicicum JF-1 (DSM 2639) was cultured with 89 mM formate and H_2:CO_2 (4:1) harvested, and stored as described (34). Anaerobic procedures were as described (31), except that sodium dithionite was omitted from buffers. The term 'vacuum degassed' refers to solutions made anaerobic by 8 cycles of alternate evacuation and pressurization with O_2-free N_2 [82 kPa]. Cell paste was flushed with N_2 for 30 min and resuspended in twice its weight of vacuum degassed breakage buffer (75 mM potassium TES, pH 7.5; 15mM $MgCl_2$; 3 mM

2-mercaptoethanol; 10 µg/ml DNAase I). The resuspended cells were broken anaerobically by one passage through a French pressure cell (Aminco model FA073) at 138 MPa (20,000 lb/in^2). The cell lysate was collected into serum bottles under N_2 and stored as frozen pellets in liquid N_2. All subsequent steps were done aerobically at 4°C. Cell lysate was made to 2% (v/v) in Triton X-100, mixed gently for 1 h on a rocking platform, and centrifuged (20 min, 30,000 x *g*, 5°C). The supernatant solution was loaded onto a column (5 x 12 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with buffer A (50 mM potassium TES pH 7.5, 10 mM $MgCl_2$, 5% (v/v) glycerol, 2 mM 2-mercaptoethanol). The column was then washed with 1.5 bed volumes of buffer A, and hydrogenase was eluted (4 ml/min) with a linear gradient of KCl (0-0.5 M, 5 bed volumes). F_{420}-hydrogenase eluted in 0.18-0.28 M KCl, while MV-hydrogenase (29) eluted in 0.28-0.41 M KCl. Fractions with a high ratio of F_{420}-dependent to MV-dependent activity were pooled and made to 40% saturation (0°C) with $(NH_4)_2SO_4$, followed by centrifugation (19,000 x *g*, 30 min, 4°C). The supernatant solution was made to 70% saturation (final concentration) with $(NH_4)_2SO_4$ and centrifuged (19,000 x *g*, 30 min, 4°C). The precipitated protein pellet was resuspended in 60 ml of buffer A containing 1 M KCl and loaded onto a column (2.6 x 10 cm) of Phenyl Sepharose CL-4B (Pharmacia, Inc.)

equilibrated with the same buffer. The column was washed (2 ml/min) with 4 bed volumes of this buffer, and hydrogenase activity was eluted with 3 bed volumes of a linear gradient decreasing from 1 to 0 M KCl and simultaneously increasing from 0 to 1.5% (v/v) Triton X-100. Active fractions were pooled and applied to a Mono Q HR 10/10 anion exchange column (Fast Protein Liquid Chromatography system, Pharmacia, Inc.) equilibrated with buffer A. The column was washed (2 ml/min) with 30 ml of buffer A containing 0.25 M KCl, and hydrogenase was eluted with a linear gradient of KCl (0.25-0.60 M, 140 ml). Active fractions were pooled and dialyzed overnight against 1 liter of 34 mM asparagine-tris (pH 7.3) (10) containing 2.5 uM FAD and 2.5% (v/v) glycerol. Samples (3 mg protein) were applied to 3 mm thick, 4% polyacrylamide slab gels and electrophoresed at 30 mA constant current. The brown hydrogenase band (R_f=0.40) was excised from the gels and minced into small pieces. The protein was eluted into 1 mM tris-Cl (pH 7.5) using an electrophoretic concentrator (Model 1750, ISCO, Inc., Omaha, Nebr.), made to 5% (v/v) in glycerol, and frozen in liquid N_2. The purified enzyme could also be stored in air at -20°C for at least 1 month without loss of activity. Anti-F_{420}-hydrogenase antiserum was prepared as described (6).

Hydrogenase assay. Samples were made to 48 µM in F_{420} and 30 µM in FAD, vacuum degassed with N_2, flushed with H_2 for 1 min, and then incubated for 30-60 min at 35°C. H_2 uptake was assayed spectrophotometrically at 35°C as described (29). The standard assay mixture (0.5 ml) contained: 50 mM potassium phosphate buffer pH 7.5, 20 mM 2-mercaptoethanol, and 48 µM F_{420} or 20 mM MV, under 82 KPa H_2. The standard assay mixture without F_{420} or MV was also used to assay for reduction of alternative electron acceptors by the F_{420}-hydrogenase. The following extinction coefficients were used (all in $mM^{-1}cm^{-1}$ at pH 7.5): FAD, ε_{450}=11.3; FMN or riboflavin, ε_{450}=12.2; benzyl viologen, ε_{550}=8.1; NAD or NADP, ε_{339}=6.22; and 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (2), ε_{420}=33.8. A unit of activity was the reduction of 1 µmol of acceptor per min; initial velocities were used for calculations. Protein was determined by the dye binding assay (micro-method) of Bradford (9), with bovine serum albumin as the standard. As a control, a standard curve was also prepared with F_{420}-hydrogenase from the DEAE-cellulose purification step which had been extensively dialyzed against distilled water and lyophilized; this standard curve was similar to that obtained with bovine serum albumin.

Electrophoresis. Native polyacrylamide gel

electrophoresis was done using the asparagine-tris buffer system (10). Hydrogenase activity was located by incubating the gel in standard F_{420} or MV assay mixture under H_2. Activity patterns were preserved by adding 5 mM 2,3,5-triphenyltetrazolium chloride (final concentration). SDS polyacrylamide gel electrophoresis was carried out using 12% gels according to the method of Laemmli (21). Native and SDS gels were stained for protein with Coomassie Brilliant Blue R-250. A Zeinek Soft LASER scanning densitometer (LKB Instruments, Inc., Rockville, MD) was used for densitometric scanning of the gels.

Isoelectric focusing. Samples (5-10 µg protein) were electrophoresed on chilled (10°C) LKB ampholine Pageplates (pH 3.5-10.0) using an LKB 2117 Multiphor II (Pharmacia LKB Biotechnology). The standards (Pharmacia LKB Biotechnology) included (pI in parentheses): human carbonic anhydrase (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), glucose oxidase (4.15), and amyloglucosidase (3.50).

Molecular weight determination. Native molecular weights were estimated by gel filtration on a column of Sephacryl S-300 (Pharmacia, Inc.) (0.9 x 53 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.5)containing 0.1 M KCl, 2 mM 2-mercaptoethanol, and 0.01%

(w/v) NaN_3. Samples (0.2 ml) in equilibration buffer containing 10% (v/v) glycerol were applied at a flow rate of 0.04 ml/min. Fractions were monitored for A_{280} and hydrogenase activity. The standards (Pharmacia, Inc.) included (molecular weight in parentheses): ferritin dimer (880K) and monomer (440K); thyroglobulin (669K); catalase (232K); aldolase (158K); bovine serum albumin (67K); ovalbumin (43K); chymotrypsinogen (25K); and ribonuclease (13.7K).

Subunit molecular weights were determined from SDS polyacrylamide gels calibrated with protein standards (Bio-Rad Laboratories, Inc.). Subunit stoichiometries were determined by integrating peaks from densitometric scans of the gels and normalizing the peak areas on the basis of the subunit molecular weights.

Component analysis. Enzyme preparations used for the cofactor analyses were washed three times with distilled-deionized water using a Centricon-30 ultrafiltration unit (Amicon Inc.; molecular weight cutoff 30K). Iron, nickel, and zinc were determined by atomic absorption spectrophotometry (Savannah River Ecology Laboratory, Aiken, S.C.) on Hitachi Models 180-70 and 180-80 spectrometers operated in the Zeeman background correction mode and using pyrolytically coated graphite furnace tubes. Enzyme samples were digested with concentrated ultrapure

nitric acid (J.T. Baker) at 160°C for 1 h and brought to 0.5 ml with 10 mM nitric acid prior to analysis. Iron was also quantitated by a colorimetric assay (13). Acid-labile sulfide was determined by the method of Beinert (7) in culture tubes (6 x 50 mm) fitted with serum stoppers. Selenium was determined fluorimetrically by the method of Spallholz _et al_. (36), except that reagent and sample volumes were decreased 10-fold. FAD was determined by reconstitution of apo-D-amino acid oxidase, which is specific for FAD (14). Apo-D-amino acid oxidase was prepared by the method of Massey and Curti (25). FAD standards were purified by thin layer chromatography on Kodak 13181 silica gel G plates using 50 mM potassium phosphate buffer (pH 7.0) as the solvent system. Bound flavin was released by boiling enzyme solutions for 15 min, followed by centrifugation to remove precipitated protein (34). An attempt was made to reconstitute any deflavo-enzyme present in the purified F_{420}-hydrogenase preparation. A sample (128 µl) in buffer A containing 0.5 M KCl, 48 µM F_{420}, and 30 µM FAD, was vacuum degassed with N_2, flushed with H_2 for 1 min, and incubated for 30 min at 35°C. After removal of H_2 by vacuum degassing with N_2, the sample was dialyzed aerobically in a Centricon-30 ultrafiltration unit (Amicon, Inc.; molecular weight cutoff 30K) with three changes (1.9 ml) of buffer A containing 0.5

M KCl and then assayed for FAD. The F_{420}-hydrogenase subunits were obtained by electroelution from preparative SDS gels (18) and their N-terminal amino acids sequenced with a gas phase microsequenator as described (17).

Chemicals. F_{420} was purified from cell extract of *M. formicicum* by DEAE-cellulose chromatography, followed by gel filtration as described (33), except that Sephadex G-10 was used for gel filtration. FO was a gift from W. T. Ashton, Merck Sharp & Dohme, West Point, PA. $FADH_2$ was prepared by reducing FAD with zinc dust under N_2. All other chemicals were obtained from commercial sources.

RESULTS

Purification, molecular weight, and subunit composition. A representative purification of the F_{420}-hydrogenase from *M. formicicum* is shown in Table 1. Chromatography on DEAE-cellulose and Phenyl Sepharose CL-4B resolved the F_{420}-hydrogenase from the MV-hydrogenase, as previously reported (29). H_2-producing activity copurified with H_2 uptake activity.

SDS gel electrophoresis revealed that the electroeluted enzyme contained three subunits (α, β, and γ) with molecular weights of 43.6K, 36.7K, and 28.8K, respectively (Fig. 1). Native gradient gel electrophoresis of the electroeluted enzyme revealed two protein bands

TABLE 1. Purification of F_{420}-hydrogenase from *M. formicicum*.

Step	Protein (mg)	Units/ mg protein[a]	Fold purification	Yield[b] (%)	Activity ratios	
					F_{420}/MV[c]	H_2production/ H_2 uptake[d]
Cell lysate	2609	0.605	1.0	100	0.011	0.124
Triton X-100 treated cell lysate	1940	0.834	1.4	102	0.011	0.183
DEAE-cellulose	1005	0.742	1.2	47.3	0.424	0.156
Ammonium sulfate precipitation	686	0.736	1.2	32.0	0.288	ND[e]
Phenyl-Sepharose CL-4B	215	2.55	4.2	34.8	0.385	0.151
Mono Q HR 10/10	6.2	34.6	57.2	13.6	0.266	0.215
Preparative electrophoresis	0.53	52.7	87.1	1.8	0.266	0.224

[a]F_{420}-dependent H_2 uptake activity determined with the standard assay. Samples were reactivated before assay as described in Materials and Methods.

[b]Based on F_{420}-dependent H_2 uptake activity.

[c]Ratio of F_{420}-dependent to MV-dependent H_2 uptake.

[d]Ratio of F_{420}-dependent H_2 production to F_{420}-dependent H_2 uptake (standard assay). Activities are reported as μmol H_2 produced or oxidized/min/mg protein. H_2-producing activity was determined with the reconstituted formate hydrogenlyase as described (5), except that the hydrogenase sample to be assayed was substituted for purified F_{420}-hydrogenase. Each assay contained 114 μg of purified formate dehydrogenase and ≈0.06 units of F_{420}-dependent H_2 uptake activity from the hydrogenase sample to be assayed.

[e]Not determined.

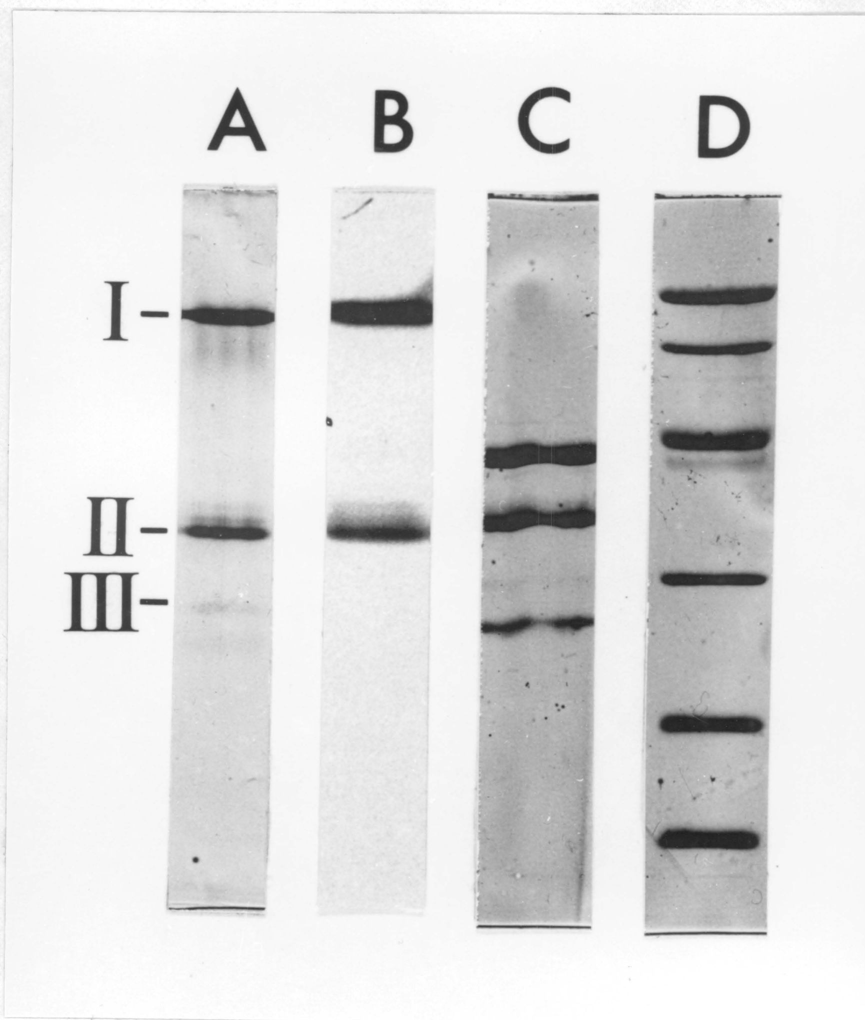


Figure 1. Native and SDS polyacrylamide gel electrophoresis of purified F_{420}-hydrogenase from M. formicicum. 25 µg of purified F_{420}-hydrogenase was electrophoresed on a native polyacrylamide gradient gel (4-15%, 0.75 mm slab) and stained for (A) protein with Coomassie R-250 or (B) for F_{420}-dependent H_2 uptake activity; Roman numerals indicate protein bands discussed in the text. (C) 11 µg of purified F_{420}-hydrogenase was electrophoresed on an SDS polyacrylamide gel (12%, 0.75 mm slab) and stained for protein with Coomassie R-250. (D) SDS gel molecular weight markers: 92.5K, 66.2K, 45K, 31K, 21.5K, and 14.4K.

which stained for F_{420}-dependent activity (Fig. 1, bands Iand II). Band I contained about 4-fold more protein than band II as estimated by densitometric scans. Two dimensional native-SDS polyacrylamide gel electrophoresis indicated the same $\alpha_1\beta_1\gamma_1$ subunit composition for both species (Fig. 2). Two F_{420}-active hydrogenase species were also resolved by gel filtration chromatography, with molecular weights of 1,020K ± 100 (n=5) and 109K ± 16 (n=5). The smaller species was probably an $\alpha_1\beta_1\gamma_1$ trimer, since its native molecular weight obtained by gel filtration agreed with the sum of the subunit molecular weights (109K). Electrophoresis of the enzyme electroeluted from the preparative gel also revealed a third, faint protein band void of F_{420}-dependent activity (Fig. 1, band III) but which stained for MV-dependent H_2 uptake activity. This minor species contained predominantly the α subunit, less of the γ subunit, and none of the β subunit (Fig. 2). The results suggest that the F_{420}-hydrogenase was purified to electrophoretic homogenity primarily as an aggregate of a 109K molecular weight F_{420}-reducing species. These results are similar to those reported for the F_{420}-reducing hydrogenase purified from _M. thermoautotrophicum_ (15).

The N-terminal amino acid sequences of the α, β, and γ subunits of the F_{420}-hydrogenase of _M. formicicum_ are

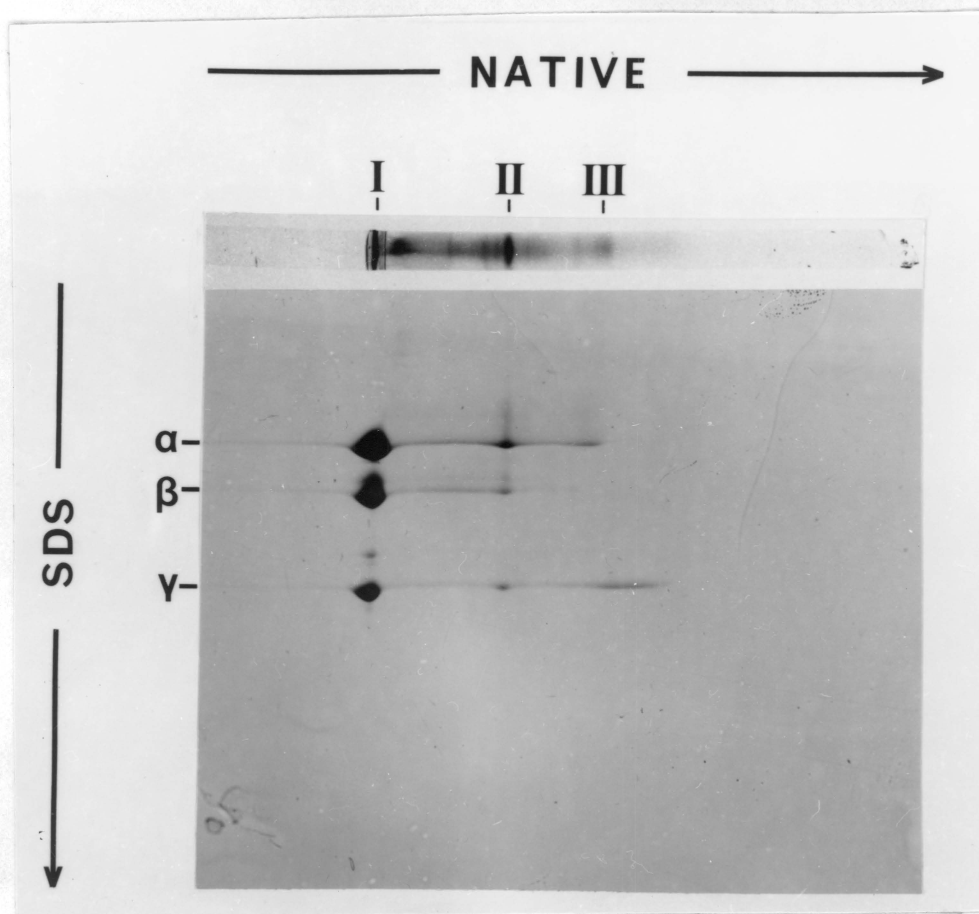


Figure 2. Two dimensional native-SDS polyacrylamide gel electrophoresis of purified F_{420}-hydrogenase from M. formicicum. Samples (71 µg) of purified F_{420}-hydrogenase were electrophoresed on native polyacrylamide gradient (4-15%) tube gels (5 mm inside diameter) using the asparagine-tris buffer system (10). One of the gels was incubated in SDS sample buffer (21) for 90 min at 25°C and then for 5 min at 100°C, placed lengthwise onto the top of an SDS polyacrylamide gel (12%), and subjected to electrophoresis essentially as described by O'Farrell (30). A duplicate tube gel and the SDS gel were stained for protein using Coomassie Brilliant Blue R-250.

compared to the published sequences (15) from the F_{420}-hydrogenase of M. thermoautotrophicum in Fig. 3. The α subunits shared 92% identity (24/26 residues); the β subunits, 86% (24/28 residues); and the γ subunits, 86% (12/14 residues). Although the F_{420}-hydrogenase of M. formicicum is membrane-associated and strongly hydrophobic (4,6), inspection of the N-terminal amino acid sequences revealed no predominantly hydrophobic segments. The N-terminal amino acid sequence of the α subunit from the M. formicicum F_{420}-hydrogenase contained 48%, 44%, and 46% identity with those of the large subunits from the NiFeSe-hydrogenase of Desulfovibrio baculatus (25) and the NiFe-hydrogenases from Desulfovibrio gigas (22) and Rhodobacter capsulatus (22), respectively (Fig. 2), but no homology with that from the Desulfovibrio vulgaris Fe-hydrogenase (39). The sequence I---P--R-EGH------V was conserved in the N-termini of the largest subunits from the nickel-containing hydrogenases of M. formicicum, M. thermoautotrophicum, D. baculatus, D. gigas, and R. capsulatus. Considerable functional homology was observed in the other parts of the N-terminal sequences.

Components. Based on the results in Table 2, the purified F_{420}-hydrogenase contained 1 mol of nickel, 12-14 mols of iron, 11 mols of acid-labile sulfide, 1 mol of FAD, and 1 mol of zinc per mol of the molecular weight 109K

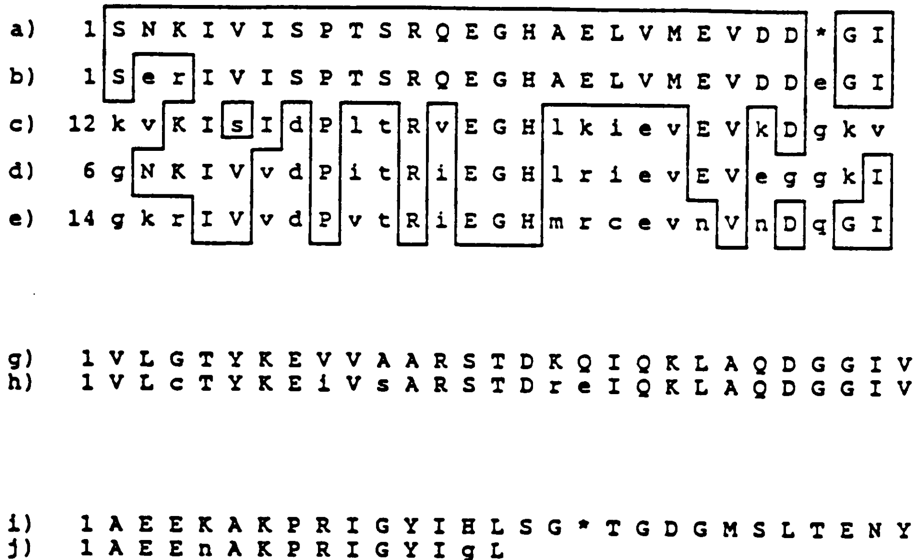


Figure 3. Comparison of the N-terminal amino acid sequences of the subunits of F_{420}-hydrogenase from M. formicicum to those of other nickel-containing hydrogenases. The sequences are presented using the single letter amino acid code and are aligned for maximum identity. Numbering is relative to the N-terminus. Upper case letters represent identity with the sequences from M. formicicum, and lower case letters, differences. Boxes indicate conserved amino acids, and asterisks, uncertainty in the sequences. a), g), and i), from the α, β, and γ subunits of the M. formicicum enzyme, respectively; b), h), and j), from the α, β, and γ subunits of the F_{420}-hydrogenase of Methanobacterium thermoautotrophicum (15), respectively; c), from the 57K subunit of the Desulfovibrio baculatus periplasmic NiFeSe hydrogenase (26); d), from the 62K subunit of the Desulfovibrio gigas periplasmic NiFe hydrogenase (23); e), from the 66K subunit of the NiFe hydrogenase of Rhodobacter capsulatus (22).

TABLE 2. Metal and cofactor composition of F_{420}-hydrogenase from *M. formicicum*.

Component	Amount (mol/mol of enzyme)[a]
FAD (as purified enzyme)	0.73 ± 0.08 (3)[b]
FAD (reconstituted enzyme)	0.83 ± 0.06 (3)[b]
Ni	0.65 ± 0.03 (2)[c]
Fe	11.9 ± 0.3 (4)[d]
	13.7 ± 0.9 (2)[c]
S^{-2}	10.9 ± 2.3 (5)[d]
Zn	0.55 ± 0.06 (2)[c]
Se	<0.015[e]

[a]Values were calculated on the basis of a molecular weight of 109K and are reported as mean ± standard deviation from the number of determinations indicated in parentheses.

[b]Determined by reconstitution of apo-D-amino acid oxidase. As purified F_{420}-hydrogenase was reconstituted with FAD as described in Materials and Methods.

[c]Determined by atomic absorption spectroscopy.

[d]Determined by colorimetric assays.

[e]Determined by fluorimetric assay. Detection limit, 0.015 nmol Se/nmol of enzyme (up to 0.41 nmol of enzyme used in assay).

species. Selenium was not present in significant amounts. The FAD content was determined by reconstitution of apo-D-amino acid oxidase, which is specific for FAD (14).

Properties. The enzyme was reversibly inactivated by exposure to O_2 and required reactivation before assay. Untreated samples of F_{420}-hydrogenase (as purified) reduced F_{420} or MV in the standard anaerobic assay only after a lag period (Fig. 4). Preincubation of the samples with glucose plus glucose oxidase to scavenge residual O_2 greatly reduced the lag period, as did extensive vacuum degassing of the enzyme solution with N_2. These results imply that residual O_2 present in the untreated samples may have been consumed in the assay during the lag period. Preincubation of vacuum degassed samples for 2 h under H_2 increased maximal activities about 2-fold. A 30 min preincubation of vacuum degassed samples under H_2 in the presence of F_{420} and FAD eliminated the lag period and increased the maximal activities by about 10-fold; therefore, this method was routinely used to reactivate hydrogenase samples before assay.

Reactivation in the absence of FAD resulted in a loss of F_{420}-dependent activity relative to MV-dependent activity unless KCl was present (Table 3). $FADH_2$, FMN, and riboflavin could not substitute for FAD (data not shown). When KCl was replaced with K_2SO_4, KCH_3COO, $(NH_4)_2SO_4$,

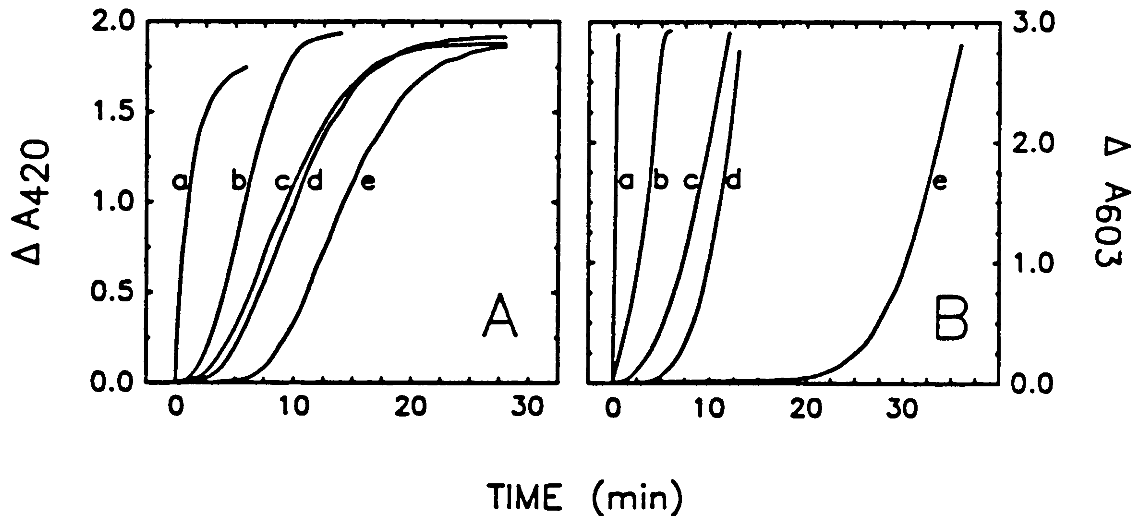


Figure 4. Reactivation of F_{420}-hydrogenase from M. formicicum. Aliquots (5 ul, 2.3 μg protein) from samples of purified F_{420}-hydrogenase (0.47 mg/ml) were assayed for (A) F_{420}-dependent or (B) MV-dependent H_2 uptake using the standard assays. (a) as purified enzyme vacuum degassed 8 times with O_2-free N_2, flushed for 1 min with H_2, and preincubated for 30 min at 35°C with 48 μM F_{420} and 30 μM FAD; (b) same as (a), except preincubated under H_2 for 2 h at 25°C without F_{420} and FAD; (c) as purified enzyme vacuum degassed 8 times with O_2-free N_2; (d) as purified enzyme and the assay mixtures both preincubated with 10 mM glucose and 54 units/ml glucose oxidase for 10 min at 35°C; (e) as purified enzyme assayed without treatment.

TABLE 3. Effect of exogenous FAD and KCl on reactivation of F_{420}-hydrogenase from *M. formicicum*.

Addition to reactivation mixture[a]	Activity[b] (units/ml) with:		
	F_{420}	MV	Ratio[c]
None	0.067 ± 0.014	5.00 ± 0.40	0.013
0.8 M KCl	0.970 ± 0.103	4.31 ± 0.29	0.225
30 μM FAD	1.58 ± 0.23	4.96 ± 0.76	0.319
30 μM FAD, 0.8 M KCl	1.67 ± 0.05	5.25 ± 0.42	0.318

[a]Vacuum degassed samples of purified F_{420}-hydrogenase (15 μl, 1.7 μg protein) containing 48 μM F_{420} and the indicated additions (final concentrations) were incubated under H_2 for 1 h at 35°C and then assayed immediately for F_{420}-dependent and MV-dependent H_2 uptake using the standard assays.

[b]Mean ± standard deviation from 4 replicates.

[c]Ratio of F_{420}-dependent to MV-dependent activity.

NH_4Cl, or NaCl (all at 0.8N) the F_{420}-dependent activity obtained after reactivation was 102, 128, 65, 30, and 20%, respectively, of that in the presence of KCl. Therefore, salts other than KCl were less effective in protecting F_{420}-dependent activity during reactivation. Previous results have shown that the F_{420}-hydrogenase (29) and formate dehydrogenase of *M. formicicum* (33,34) require FAD to reduce F_{420} but not MV. The results in Table 4 show that FAD dissociated from the F_{420}-hydrogenase under conditions of reactivation (F_{420} plus H_2); KCl prevented the dissociation. This dissociation was apparently complete, since boiling the enzyme released nearly the same amount of FAD as reduction with H_2 in the presence of F_{420} (Table 4).

The optimum temperature for F_{420}-dependent activity of the F_{420}-hydrogenase was 55°C, and the activation energy was calculated to be 10.0 Kcal/mol from an Arrhenius plot. F_{420}-dependent activity was increased by increasing concentrations of KCl up to 200 mM; however, MV-dependent activity slightly decreased as the KCl concentration was increased (Fig. 5). An absolute requirement for K^+ was not established, since the assay mixture contained 50 mM K^+. Incorporation of 400 mM NaCl or 10 mM $MgCl_2$ into the assay mixture stimulated F_{420}-dependent activity by about 2-fold. The effect of pH on F_{420}-dependent H_2 uptake and H_2

TABLE 4. Dissociation of FAD from F_{420}-hydrogenase of *M. formicicum*.

Treatment[a]	FAD released[b] (μM)
48 μM F_{420}, N_2 atm.	<0.03[c]
48 μM F_{420}, 0.8 M KCl, H_2 atm.	<0.03
H_2 atm.	0.050 ± 0.050
48 μM F_{420}, H_2 atm.	0.564 ± 0.151
100°C, 15 min	0.534 ± 0.046

[a]Samples (0.2 ml, 0.5 mg protein) of F_{420}-hydrogenase from the Mono Q HR 10/10 step (Table 1) which had been dialyzed for 12 h (4°C) against 1 liter of Buffer A were vacuum degassed with N_2, flushed with N_2 or H_2 for 1 min, and incubated for 45 min at 35°C with the indicated additions (final concentrations). Another sample was simply heated at 100°C for 15 min. The samples were ultrafiltered anaerobically using Centricon-30 units (Amicon, Inc., molecular weight cutoff 30K) and the ultrafiltrates assayed for FAD using the reconstitution of apo-D-amino acid oxidase.

[b]Mean ± standard deviation of 6 replicates from two experiments.

[c]Detection limit, 0.03 μM.

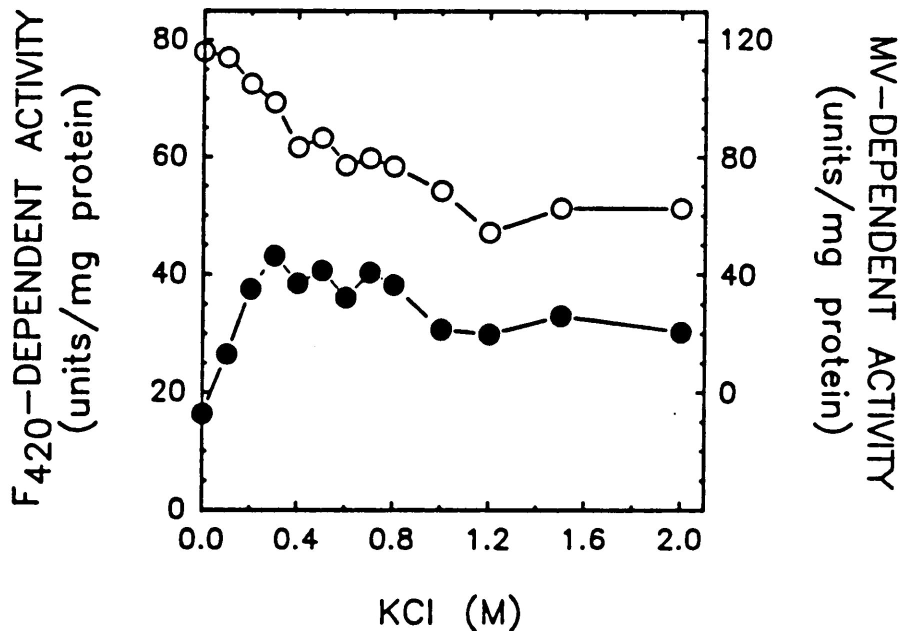


Figure 5. Effect of KCl concentration on activity of F_{420}-hydrogenase from M. formicicum. Each assay contained 1.4 µg of purified, reactivated F_{420}-hydrogenase. The standard H_2 uptake assays were used, except that the indicated concentrations of KCl were incorporated into the reaction mixtures. Symbols: ●, F_{420}-dependent activity; ○, MV-dependent activity.

evolution activities is shown in Fig. 6. Both activities were optimal around pH 7.0 to 7.5, and the ratio of H_2 evolution to H_2 uptake was approximately 0.3 in this range, indicating that the enzyme is bidirectional. The isoelectric point of the hydrogenase was 5.6.

Kinetics. The purified F_{420}-hydrogenase reduced F_{420}, FO (the riboflavin analogue of F_{420}) (2), flavins, and MV (Table 5). The enzyme did not reduce NAD or NADP (data not shown). The apparent Km's for reduction of FO and flavins were approximately 2-fold greater than that for F_{420}, while that for MV was 42-fold greater.

Immunoinhibition. Anti-F_{420}-hydrogenase antiserum reacts specifically with the α, β, and γ subunits of the hydrogenase in cell extract, as previously shown by Western blot analysis (6). Incubation of purified F_{420}-hydrogenase with the antiserum inhibited F_{420}-dependent H_2 uptake but had little effect on the MV-dependent activity (Fig. 7A). Similar results were obtained with cell extract (Fig. 7B).

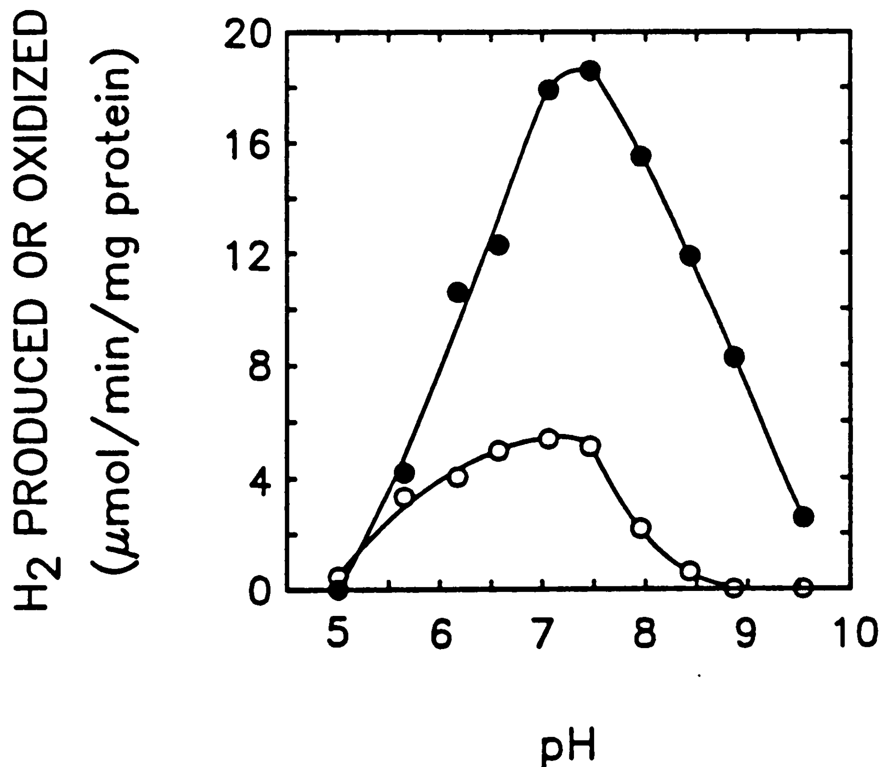


Figure 6. Effect of pH on F_{420}-dependent H_2-production and H_2 uptake by F_{420}-hydrogenase from M. formicicum. H_2 production (○) was assayed using the reconstituted formate hydrogenlyase as described (5), except that phosphate buffer was replaced with a mixture of potassium phosphate and bis-tris-propane (100 mM each, final concentration) titrated to the indicated pH with KOH or HCl. F_{420}-hydrogenase (2.6 ug) was not limiting in the assay. Activities are reported on the basis of F_{420}-hydrogenase protein. F_{420}-dependent H_2 uptake (●) was assayed spectrophotometrically as described in Materials and Methods, except that the standard reaction mixture was replaced with H_2-saturated formate hydrogenlyase reaction mixture described above, and the reduction of F_{420} was monitored at its isosbestic point, 401 nm (11); (ε_{401} = 26.9 $mM^{-1}cm^{-1}$). Each assay contained 2.6 μg purified F_{420}-hydrogenase.

TABLE 5. Kinetic properties of F_{420}-hydrogenase from *M. formicicum*.

Acceptor[a]	K_m (µM)	V_{max} (µmol/min/mg)
F_{420}	37	97.6
FO	80	98.6
FAD	87	53.5
FMN	61	121
Riboflavin	89	78.0
Methyl viologen	1560	172

[a]Each assay contained 0.9-1.4 ug of purified, reactivated F_{420}-hydrogenase. The standard H_2 uptake assay was used except that the following electron acceptors were substituted (concentration ranges used indicated in parentheses): F_{420} (5-20 µM); FO (5-48 µM); FAD (10-200 µM); FMN (5-200 µM); riboflavin (7.5-150 µM); or methyl viologen (0.1-5 mM). The data were determined by linear regression analysis of double-reciprocal plots of initial velocity versus substrate concentration (correlation coefficients were 0.98 or greater).

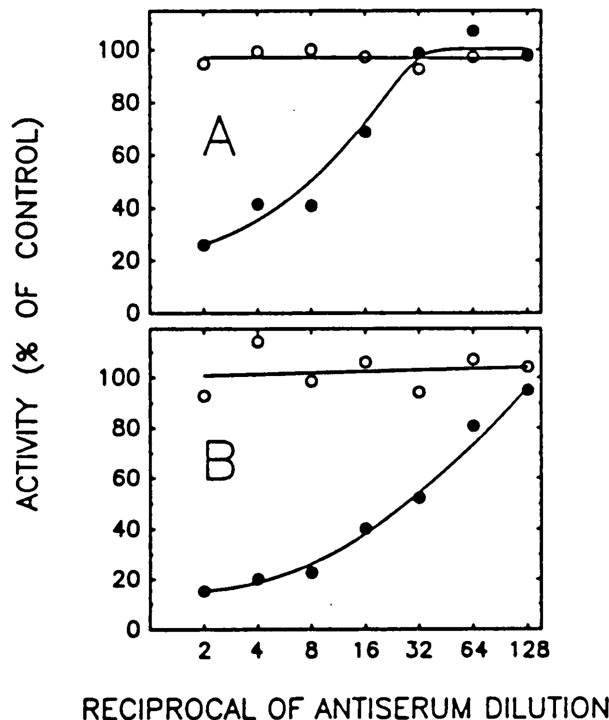


Figure 7. Immunoinhibition of F_{420}-hydrogenase from M. formicicum by specific antiserum. (A) Aliquots (10 µl, 1.7 µg protein) of purified, reactivated F_{420}-hydrogenase were mixed with aliquots (10 µl) of vacuum degassed anti-F_{420}-hydrogenase antiserum or control serum diluted in 50 mM potassium phosphate buffer (pH 7.5). The samples were incubated for 30 min at 35°, and immediately assayed for F_{420}-dependent (●) and MV-dependent (○) H_2 uptake using the standard assays. 100% = 1.54 units/ml F_{420}-dependent activity and 5.59 units/ml MV-dependent activity. (B) as in (A), except that cell extract containing 2% (v/v) Triton X-100 was substituted for the purified hydrogenase; cell extract refers to the supernatant solution from a centrifugation of cell lysate (30,000 x g, 20 min, 5°C). 100% = 1.40 units/ml F_{420}-dependent activity and 138 units/ml MV-dependent activity. Activities are expressed as percentages of those obtained with a 2-fold final dilution of control serum substituted for the antiserum.

DISCUSSION

The purified F_{420}-hydrogenase from *M. formicicum* resembles the enzymes from *M. thermoautotrophicum* (15) and *M. voltae* (28) in containing a minimal F_{420}-reducing unit of molecular weight approximately 110K comprised of α, β, and γ subunits. In contrast, the F_{420}-hydrogenase of *Methanospirillum hungatii* (38) has only two subunits (molecular weights 51K and 31K), in a ratio of 1:3, and that of *Methanosarcina barkeri* (12) has only one subunit (molecular weight 60K). Most F_{420}-hydrogenases studied are hydrophobic, judged from their behavior during hydrophobic interaction chromatography (4,15,28,29), and all form large aggregates ranging from molecular weight 720K to 1,300K (12,15,28,38,42, this study). The F_{420}-hydrogenases of *M. thermoautotrophicum* (15), *M. voltae* (28), and *M. hungatii* (38) are visible in electron micrographs as circular structures. Wackett *et al.* (40) proposed that these structures from the *M. thermoautotrophicum* enzyme represent the aggregated form assembled as two stacked rings, each containing four F_{420}-reducing $\alpha\beta\gamma$ trimers. Circular structures like those of the *M. thermoautotrophicum* enzyme were observed in electron micrographs of purified F_{420}-hydrogenase from *M. formicicum* (L. Wackett, personal communication) suggesting a similar arrangement of the aggregated form.

As previously suggested, all known hydrogenases contain iron-sulfur clusters, and many contain nickel (41). The F_{420}-hydrogenase of *M. formicicum* contained these metals in amounts similar to those for the *M. thermoautotrophicum* enzyme (15). The F_{420}-hydrogenase from *M. voltae* (28) also contains 1 mol of nickel, but only 4.5 mols of iron were detected. Nickel is also present in the enzymes from *M. hungatii* (38) and *Methanococcus vannielii* (S. Yamazaki, Fed. Proc., **42**:2977, 1983).

The conserved sequence I---P--R-EGH------V was present in the N-termini of the largest subunits of the F_{420}-hydrogenases of *M. formicicum* and *M. thermoautotrophicum* (15), the periplasmic hydrogenases of *D. baculatus* (26) and *D. gigas* (23), and the H_2 uptake hydrogenase of *Rhodobacter capsulatus* (22). These enzymes all contain nickel and iron. The conserved sequence was not present in the periplasmic hydrogenase of *Desulfovibrio vulgaris*, which contains iron but not nickel (23). These combined observations suggest that this sequence may function to position nickel at the active site, especially since it is conserved among phylogenetically distinct bacteria. However, this hypothesis requires further study.

The *M. formicicum* F_{420}-hydrogenase contained 0.6 mols of zinc. Zinc is often adventitiously bound during purification of proteins. However, zinc is present at high

concentrations (50-630 ppm) in methanogens (35) and has been found in formate dehydrogenase (34) and F_{420}-non-reactive hydrogenase (1) of *M. formicicum*. Selenium was not found in the enzyme from *M. formicicum* and has only been reported present in the F_{420}-hydrogenases from *M. vannielii* (42) and *M. voltae* (28).

FAD is present in the F_{420}-hydrogenases of *M. formicicum* (this report), *M. thermoautotrophicum* (15), and *M. voltae* (28), as well as the F_{420}-reducing formate dehydrogenase of *M. formicicum* (34). The flavin is thought to shuttle electrons between one-electron iron-sulfur clusters and the obligate two-electron acceptor F_{420} (19). The F_{420}-hydrogenase (29) and formate dehydrogenase (33) of *M. formicicum* lose bound FAD when purified under reduced conditions, yielding apoenzymes which reduce MV but not F_{420}; addition of exogenous FAD restores F_{420}-reducing activity. Bound FAD dissociated from the F_{420}-hydrogenase of *M. formicicum* when it was reductively reactivated, yielding F_{420}-inactive enzyme. Exogenous FAD, but not $FADH_2$, protected the F_{420}-dependent activity of the enzyme during reactivation; high concentrations of potassium salts (0.8 N) prevented the dissociation of FAD. These observations support the proposal by Fox *et al.* (15) that $FADH_2$ is less tightly bound than FAD in F_{420}-hydrogenase. High salt concentrations may prevent loss of

flavin through a conformational change, or by promoting aggregation. The stimulation of F_{420}-dependent H_2 uptake by KCl (Fig. 5) may partly be due to its effect in preventing dissociation of FAD from the reduced enzyme. Since the intracellular potassium concentration of Methanobacterium (0.8 M) (37) is the same as that required for retention of FAD by the reduced enzyme, dissociation in the intact cell is unlikely. The ability of anti-F_{420}-hydrogenase antiserum to inhibit F_{420}-dependent but not MV-dependent activity of purified F_{420}-hydrogenase (Fig. 7) suggests that the sites where these electron acceptors are reduced are spatially separated, as proposed for the M. thermoautotrophicum enzyme (24).

F_{420}-hydrogenases in H_2-utilizing CO_2-reducing methanogenic bacteria apparently function to oxidize H_2 to provide electrons for at least one reductive step in the pathway (16). However, H_2 is produced during the utilization of formate as reductant in M. formicicum (31) implying the presence of a bidirectional hydrogenase in this organism. The F_{420}-hydrogenase described here has been shown to function as a bidirectional enzyme and to participate in the formate hydrogenlyase system of this organism (5).

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REFERENCES

1. Adams, M. W. W., S. -L. C. Jin, J. -S. Chen, and L. E. Mortenson. 1986. The redox properties and activation of the F_{420}-non-reactive hydrogenase of Methanobacterium formicicum. Biochim. Biophys. Acta. **869**:37-47.

2. Ashton, W. T., R. D. Brown, F. Jacobson, and C. Walsh. 1979. Synthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin and confirmation of its identity with the deazaisoalloxazine chromophore of Methanobacterium redox coenzyme F_{420}. J. Am. Chem. Soc. **101**:4419-4420.

3. Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260-296.

4. Baron, S. F., D. P. Brown, and J. G. Ferry. 1987. Locations of the hydrogenases of Methanobacterium formicicum after subcellular fractionation of cell extract. J. Bacteriol. **169**:3823-3825.

5. Baron, S. F., and J. G. Ferry. 1988. Reconstitution and properties of a coenzyme F_{420}-mediated formate hydrogenlyase system in Methanobacterium formicicum. J. Bacteriol. (Submitted).

6. Baron, S. F., D. S. Williams, H. D. May, P. S. Patel, H. C. Aldrich, and J. G. Ferry. 1988. Immunogold localization of coenzyme F_{420}-reducing hydrogenase in Methanobacterium formicicum. Arch. Microbiol. (In press).

7. Beinert, H. 1983. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. Anal. Biochem.
131:373-378.

8. Blaut, M. and G. Gottschalk 1985. Evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria. T.I.B.S. 7:354-357.

9. Bradford, M. 1976. A rapid and sensitive method for the quantitation of protein using the principle of protein-dye binding. Anal. Biochem. 72:248-254.

10. Brewer, J. M., A. J. Pesce, and R. B. Ashworth. 1974. Electrophoresis, p. 351-354. In Experimental techniques in biochemistry. Prentice-Hall, Inc., Englewood Cliffs, N.J.

11. Eirich, L. D., G. D. Vogels, and R. S. Wolfe. 1979. Distribution of coenzyme F_{420} and properties of its hydrolytic fragments. J. Bacteriol. 140:20-27.

12. Fauque, G., M. Texeira, I. Moura, P. A. Lespinat, A. V. Xavier, D. V. derVartanian, H. D. Peck, Jr., J. LeGall, and J. G. Moura. 1984. Purification, characterization and redox properties of hydrogenase from Methanosarcina barkeri (DSM 800). Eur. J. Biochem. 142:21-28.

13. Fish, W. W. 1988. Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. Meth. Enzymol. 158:357-364.

14. Fonda, M. L., and B. M. Anderson. 1967. D-Amino acid oxidase. I. Spectrophotometric studies. J. Biol. Chem. 242:3957-3962.

15. Fox, J. A., D. J. Livingston, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 1. Purification and characterization. Biochemistry USA. 26:4219-4227.

16. Hartzell, P. L., G. Zvilius, J. C. Escalante-Semerena, and M. I. Donnelly. 1985. Coenzyme F_{420} dependence of the methylenetetrahydromethanopterin dehydrogenase of Methanobacterium thermoautotrophicum. Biochem. Biophys. Res. Commun. 133:884-890.

17. Hunkapiller, M. W., R. M. Hewick, W. J. Dreyer, and L. E. Hood. 1983. High-sensitivity sequencing with a gas-phase sequenator. Methods in Enzymology. **91**:399-413.

18. Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods in Enzymology. **91**:227-237.

19. Jacobson, F. S., L. Daniels, J. A. Fox, C. T. Walsh, and W. H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. **257**:3385-3388.

20. Jin, S.-L. C., D. K. Blanchard, and J. -S. Chen. 1983. Two hydrogenases with distinct electron-carrier specificity and subunit composition in Methanobacterium formicicum. Biochim. Biophys. Acta. **748**:8-20.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

22. Leclerc, M., A. Colbeau, B. Cauvin, and P. M. Vignais. 1988. Cloning and sequencing of the genes encoding the large and the small subunits of the H_2 uptake hydrogenase (hup) of Rhodobacter capsulatus. Mol. Gen. Genet. **214**:97-107.

23. Li, C., H. D. Peck, Jr., J. LeGall, and A. E. Przybyla. 1987. Cloning, characterization, and sequencing of the genes encoding the large and small subunits of the periplasmic [NiFe] hydrogenase of Desulfovibrio gigas. DNA. **6**:539-551.

24. Livingston, D. J., J. A. Fox, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 2. Kinetic and hydrogen-transfer studies. Biochemistry USA. **26**:4228-4236.

25. Massey, V. and B. Curti. 1966. New method of preparation of D-amino acid oxidase apoprotein and a conformational change after its combination with flavin adenine dinucleotide. J. Biol. Chem. **241**:3417-3423.

26. Menon, N. K., H. D. Peck, Jr., J. LeGall, and A. E. Przybyla. 1987. Cloning and sequencing of the genes encoding the large and small subunits of the periplasmic (NiFeSe) hydrogenase of Desulfovibrio baculatus. J. Bacteriol. **169**:5401-5407.

27. Muth, E. 1988. Localization of the F_{420}-reducing hydrogenase in Methanococcus vannielii by immuno-gold technique. Arch. Microbiol. **150**:205-207.

28. Muth, E., E. Mörschel, and A. Klein. 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium Methanococcus voltae. Eur. J. Biochem. **169**:571-577.

29. Nelson, M. J. K., D. P. Brown, and J. G. Ferry. 1984. FAD requirement for the reduction of coenzyme F_{420} by hydrogenase from Methanobacterium formicicum. Biochem. Biophys. Res. Commun. **120**:775-781.

30. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.

31. Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in Methanobacterium formicicum. J. Bacteriol. **142**:800-807.

32. Schauer, N. L., and J. G. Ferry. 1982. Properties of formate dehydrogenase in Methanobacterium formicicum. J. Bacteriol. **150**:1-7. Erratum: **151**:1642.

33. Schauer, N. L., and J. G. Ferry. 1983. FAD requirement for the reduction of coenzyme F_{420} by formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **155**:467-472.

34. Schauer, N. L., and J. G. Ferry. 1986. Composition of the coenzyme F_{420}-dependent formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **165**:405-411.

35. Scherer, P., H. Lippert, and G. Wolff. 1983. Composition of the major elements and trace elements of 10 methanogenic bacteria determined by inductively coupled plasma emission spectrometry. Biol. Trace Element Res. 5:149-163.

36. Spallholz, J. E., G. F. Collins, and K. Schwarz. 1978. A single-test-tube method for the fluorimetric microdetermination of selenium. Bioinorg. Chem. **9**:453-459.

37. Sprott, G. D., and K. F. Jarrell. 1981. K^+, Na^+, and Mg^{+2} content and permeability of Methanospirillum hungatei and Methanobacterium thermoautotrophicum. Can. J. Microbiol. **27**:444-451.

38. Sprott, G. D., K. M. Shaw, and T. J. Beveridge. 1987. Properties of the particulate enzyme F_{420}-reducing hydrogenase isolated from Methanospirillum hungatei. Can. J. Microbiol. **33**:896-904.

39. Voordouw, G., and S. Brenner. 1985. Nucleotide sequence of the gene encoding the hydrogenase from Desulfovibrio vulgaris (Hildenborough). Eur. J. Biochem. **148**:515-520

40. Wackett, L. P., E. A. Hartwieg, J. A. King, W. H. Orme-Johnson, and C. T. Walsh. 1987. Electron microscopy of nickel-containing methanogenic enzymes: methyl reductase and F_{420}-hydrogenase. J. Bacteriol. **169**:718-727.

41. Walsh, C. T., and W. H. Orme-Johnson. 1987. Nickel enzymes. Biochemistry USA. **26**:4901-4906.

42. Yamazaki, S. 1982. A selenium-containing hydrogenase from Methanococcus vannielii. J. Biol. Chem. **257**:7926-7929.

SECTION IV. RECONSTITUTION AND PROPERTIES OF A COENZYME F_{420}-MEDIATED FORMATE HYDROGENLYASE IN *METHANOBACTERIUM FORMICICUM*

SUMMARY

Formate hydrogenlyase activity in cell extract of *Methanobacterium formicicum* was abolished by removal of coenzyme F_{420}; addition of purified coenzyme F_{420} restored activity. Formate hydrogenlyase activity was reconstituted with three purified components from *M. formicicum*: coenzyme F_{420}-reducing hydrogenase, coenzyme F_{420}-reducing formate dehydrogenase, and coenzyme F_{420}. The reconstituted system required FAD for maximal activity (kinetic K_d= 4 µM). Without FAD, the formate dehydrogenase and hydrogenase rapidly lost coenzyme F_{420}-dependent activity relative to methyl viologen-dependent activity. Immunoadsorption of formate dehydrogenase or coenzyme F_{420}-reducing hydrogenase from cell extract greatly reduced formate hydrogenlyase activity; addition of the purified enzymes restored activity. The formate hydrogenlyase was reversible, since both cell extract and the reconstituted system produced formate from H_2 plus CO_2/HCO_3^-.

INTRODUCTION

Methanobacterium formicicum is one of several methanogenic bacteria which can use either formate or H_2/CO_2 for growth and methanogenesis (1). During growth on either substrate, this organism synthesizes a hydrogenase and formate dehydrogenase which both reduce the physiological electron acceptor, coenzyme F_{420} (F_{420}) (28). The F_{420}-reducing formate dehydrogenase of *M. formicicum* has been characterized (2,3,17,29,30,31,32), the genes encoding its two subunits (α and β) have been cloned and sequenced (33), and its regulation has been investigated (18,24). *M. formicicum* and other H_2-oxidizing methanogens have two distinct hydrogenases (12,13,21). One (F_{420}-hydrogenase) reduces F_{420} and methyl viologen (MV); the other (MV-hydrogenase) reduces MV but not F_{420}. Several F_{420}-hydrogenases have been purified and characterized (5,10,19,34,39). Component A1 of the H_2-coupled methyl coenzyme M methylreductase system of *Methanobacterium thermoautotrophicum* has F_{420}-dependent hydrogenase activity (20), suggesting a role for F_{420}-hydrogenase in H_2 uptake. A function for F_{420}-hydrogenase in H_2 production has not been investigated.

Cell extracts of methanogenic bacteria contain a formate hydrogenlyase system which requires F_{420} for activity (9,22,38). However, a requirement for additional

electron carriers has not been investigated, and the system has not been reconstituted *in vitro* with purified components. This system is reversible, since formate is synthesized from H_2 plus CO_2/HCO_3^- in cell extract (22) and whole cells (8).

We have previously described the purification and properties of the F_{420}-hydrogenase of *M. formicicum* (5). Here, we report that F_{420}-hydrogenase, formate dehydrogenase and F_{420} purified from *M. formicicum* together reconstitute formate hydrogenlyase activity. We examine some properties of the reconstituted system and address its physiological role in formate metabolism.

MATERIALS AND METHODS

Cell extract preparation. Cell extract of *M. formicicum* was prepared by centrifugation (30,000 x *g*, 20 min, 4°C) of French pressure cell lysate (5). Cell extract was depleted of F_{420} and other highly anionic materials by anaerobic DEAE-cellulose chromatography essentially as described by Tzeng *et al*. (38). However, bound protein was eluted with equilibration buffer containing 0.35 M KCl, and F_{420} and other highly anionic material was eluted with 2 M NaCl. Boiled cell extract was prepared as described (20).

Enzyme purification. The F_{420}-hydrogenase of *M. formicicum* was purified as described (5). The

MV-hydrogenase of M. formicicum was partially purified by chromatography on DEAE-cellulose (5), Phenyl Sepharose CL-4B (Pharmacia, Inc.), and Mono Q HR 10/10 ion exchange resin (Fast Protein Liquid Chromatography System, Pharmacia, Inc.), followed by preparative electrophoresis on 7.5% polyacrylamide gels. The formate dehydrogenase of M. formicicum was purified (31) and low molecular weight compounds removed by ultrafiltration as described (30).

Enzyme assays. Hydrogen uptake (21) and formate dehydrogenase (30) activities were assayed spectrophotometrically at 35°C and pH 7.5 as described. One unit was the reduction of 1 µmol of acceptor per min. Initial velocities were used for calculations. Hydrogenase samples for H_2-uptake assays were reductively reactivated as described (5). Protein was determined by the dye-binding assay (micromethod) of Bradford (7), with bovine serum albumin as the standard.

Formate hydrogenlyase activity in cell extracts was assayed at 35°C in 2 ml serum vials (No. 223713, Wheaton Scientific, Millville, N.J.) fitted with black butyl rubber bungs. The complete reaction mixture (0.1 ml) contained 48 µM F_{420}, 30 µM FAD, 50 µM sodium 2-bromoethane sulfonate (BES), 30 mM NaHCOO, and cell extract. All components except cell extract and formate were combined in the vials and taken to dryness under vacuum. The vials were then

flushed for 3 min with N_2, cell extract was added anaerobically, and flushing was continued for 3 min. The reaction was initiated with formate. Gas samples (50 µl) were removed at intervals using a Pressure-Lok syringe (Supelco, Inc., Bellefonte, Pa.) and assayed for H_2 using thermal conductivity gas chromatography as described (16). Activities were calculated from the linear portion of the time course obtained.

The reconstituted formate hydrogenlyase was assayed at 35°C in 5 ml serum vials (No. 223738, Wheaton Scientific) sealed with black butyl rubber bungs. The complete reaction mixture (0.5 mL) contained: 50 mM potassium phosphate buffer pH 7.5, 30 mM sodium formate, 30 µM FAD, 10 mM NaN_3, 20 mM 2-mercaptoethanol, and 48 µM F_{420}. Purified, vacuum degassed F_{420}-hydrogenase was added to vacuum degassed reaction mixture and incubated at 35°C for 30 sec, followed by the addition of formate dehydrogenase. Gas samples (0.2 ml) were withdrawn at intervals and assayed for H_2 as above.

Formate hydrogenlyase activity in the reverse direction was assayed at 35°C using 5 ml serum vials as described above. The complete reaction mixture (0.5 ml) contained 50 mM potassium phosphate buffer pH 7.5, 60 mM $NaHCO_3$, 30 µM FAD, 10 mM NaN_3, 20 mM 2-mercaptoethanol, and 48 µM F_{420} (final pH 7.5). 50 µM sodium 2-bromoethane

sulfonate was included when cell extract was assayed. The reaction mixture was vacuum degassed with N_2, and $NaHCO_3$ (1 M stock solution) was added anaerobically through the stopper. Just before assay, the vials were flushed for 3 min with $H_2:CO_2$ (4:1). For assay of cell extract, the reaction was initiated with DEAE-cellulose-treated cell extract. For assay of the reconstituted system, purified formate dehydrogenase was added to the reaction mixture and incubated for 30 sec at 35°C. The reaction was then initiated with purified F_{420}-hydrogenase which had previously been reactivated by incubation for 30 min at 35°C in the presence of 82 kPa H_2, 48 µM F_{420}, and 0.7 M KCl. Aliquots (40 µl) of the reaction mixture were removed at intervals, boiled for 10 min, and centrifuged to remove protein. Formate was measured by ion exclusion HPLC as described (16), except that the flow rate was 0.5 ml/min. The detection limit was about 50 µM.

Enzyme stability in the reconstituted formate hydrogenlyase. The formate hydrogenlyase system was reconstituted with or without 30 µM FAD as described above. Samples (20 µl) were withdrawn from the reaction mixture at intervals; 10 µl was used to assay for F_{420}-dependent activity and the other 10 µl for MV-dependent activity of the formate dehydrogenase or (in separate experiments) the hydrogenase. The standard formate dehydrogenase and H_2

uptake assays were used. However, the formate dehydrogenase assays were initiated with enzyme sample rather than formate, and endogenous activity was not determined. Because 0.6 mM formate was carried over from the formate hydrogenlyase assay to the hydrogenase assay, 10 mM NaN_3 was included in the hydrogenase assay mixtures to inhibit residual formate dehydrogenase activity (29). Hydrogenase activity was not affected by azide at this concentration.

Immunoadsorption. Anti-F_{420}-hydrogenase antiserum (6) and anti-formate dehydrogenase antiserum (33) were prepared as described. All of the following steps were performed in an anaerobic glove bag. Columns (2 ml bed volume) of Protein A-Sepharose CL-4B (Pharmacia, Inc.) contained in 3 ml plastic disposable syringes (Becton Dickinson, Inc.) were equilibrated with vacuum degassed 50 mM potassium phosphate buffer (pH 7.0). Samples (4 ml) of vacuum degassed buffer, antiserum, or control serum were passed over the columns in 0.5 ml aliquots. Each aliquot was allowed to drain completely into the bed and incubate at 25°C for 5-10 min before the next was loaded. Unbound protein was removed by washing with 10 bed volumes of buffer. Most of the excess buffer was removed by centrifugation. Samples (2 ml) of cell extract containing 5 µM FAD were loaded onto separate columns in successive

0.5 ml aliquots as above, and the eluates were collected in chilled 5 ml amber serum vials. Residual extract was removed from the columns by centrifugation and pooled with the rest of the eluate. The treated extracts were stored in liquid N_2.

Chemicals. Coenzyme F_{420} was purified from cell extract of *M. formicicum* by DEAE-cellulose chromatography, followed by gel filtration as described (30), except that Sephadex G-10 was used for gel filtration. All other chemicals were obtained commercially.

RESULTS

Reconstitution of formate hydrogenlyase. Formate hydrogenlyase activity in cell extract of *M. formicicum* was abolished when highly anionic material was removed by DEAE-cellulose chromatography (Fig. 1). However, addition of purified F_{420} restored activity (Fig. 1) to a level (47 nmoles H_2 produced/min/mg protein) similar to that of untreated cell extract. FAD stimulated formate hydrogenlyase activity when present with F_{420} but did not restore activity by itself (Fig. 1). FMN, NAD, or NADP (all at 30 µM) did not replace F_{420}, either alone or in combination with FAD. These results are similar to those obtained with F_{420}-depleted cell extracts from other methanogenic bacteria (9,22,38).

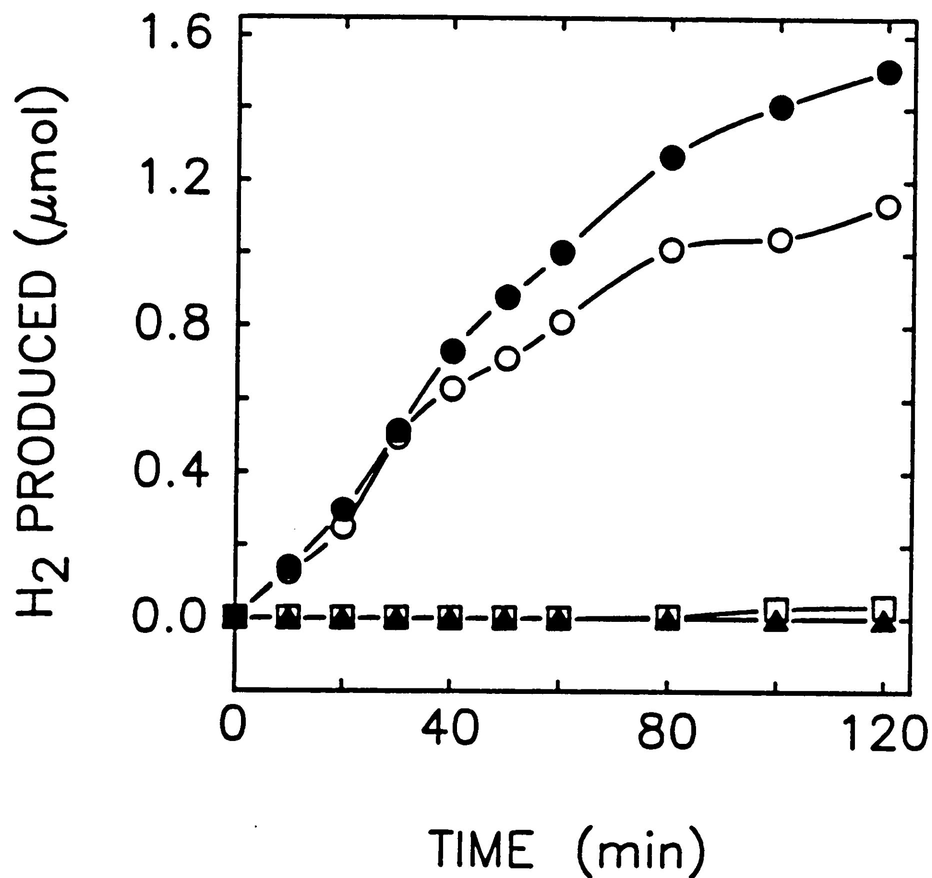

Figure. 1. Coenzyme F_{420}-dependence of formate hydrogenlyase activity in cell extract of M. formicicum. Each assay contained 0.46 mg protein (in 0.1 ml) from DEAE-cellulose treated cell extract. Symbols: ●, complete reaction mixture; ○, FAD omitted; □, F_{420} omitted, with or without FAD; △, formate omitted.

F_{420}-hydrogenase, formate dehydrogenase, and F_{420}, all purified from <u>M</u>. <u>formicicum</u>, together reconstituted formate hydrogenlyase activity (3.26 μmoles H_2/min/mg F_{420}-hydrogenase) (Fig. 2). No activity was observed if any of these components or formate was omitted (data not shown). The K_m for F_{420} in the reconstituted system was 12 μM. Optimal activity was obtained between pH 6.5 and 7.5, and no H_2 was produced below pH 5.0 (5). Activity was dependent upon the amounts of purified hydrogenase or formate dehydrogenase added (Fig. 3). The system was routinely reconstituted with the formate dehydrogenase activity in excess, and under these conditions, F_{420} remained fully reduced for at least 60 min.

<u>FAD requirement for formate hydrogenlyase activity</u>. FAD is a component of the formate dehydrogenase (31) and F_{420}-hydrogenase (5) of <u>M</u>. <u>formicicum</u>. Both enzymes lose bound FAD under reduced conditions (5,21,30,31), yielding apoenzymes which can reduce MV but not F_{420}. FAD was also required for maximal activity of the reconstituted formate hydrogenlyase system; the rate of H_2 production decreased after 25 min in its absence (Fig. 2). Boiled cell extract substituted for FAD, but $FADH_2$, FMN, riboflavin, or NAD(P) did not (data not shown). As illustrated in Fig. 4, after 25 min into the formate hydrogenlyase reaction, the F_{420}-dependent and MV-dependent activities of the formate

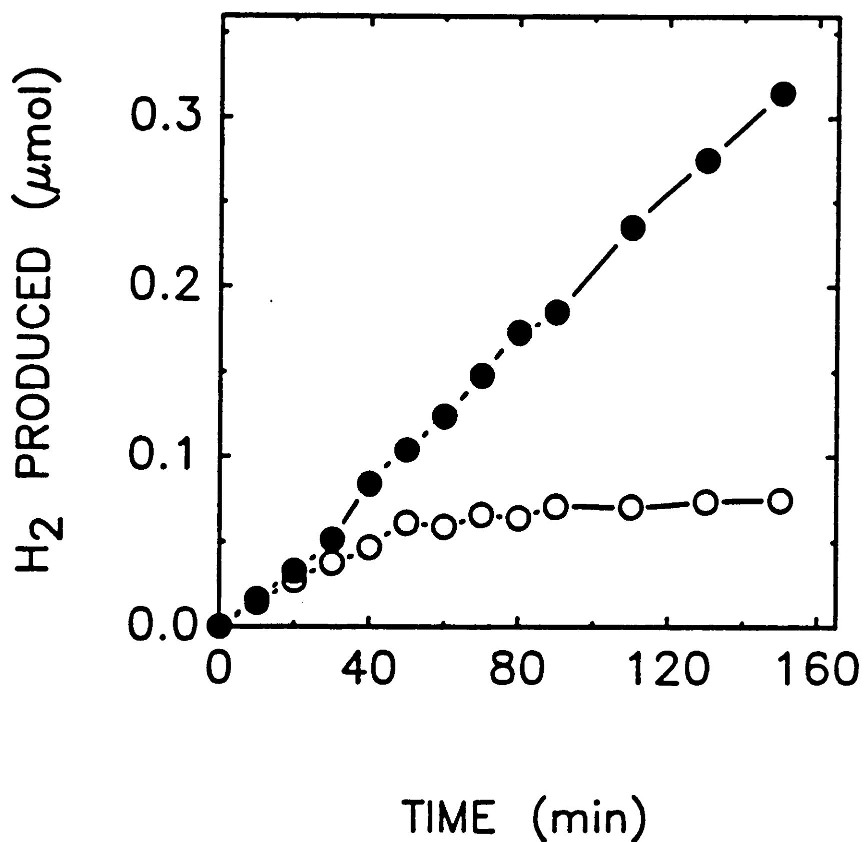

Figure 2. Reconstitution of the formate hydrogenlyase of *M. formicicum* with purified components. Purified formate dehydrogenase (14 μg protein) was added to the reaction mixture first and allowed to incubate for 5 min. The reaction was then initiated with purified F_{420}-hydrogenase (0.7 μg protein). Symbols: ●, complete reaction mixture; ○, FAD omitted.

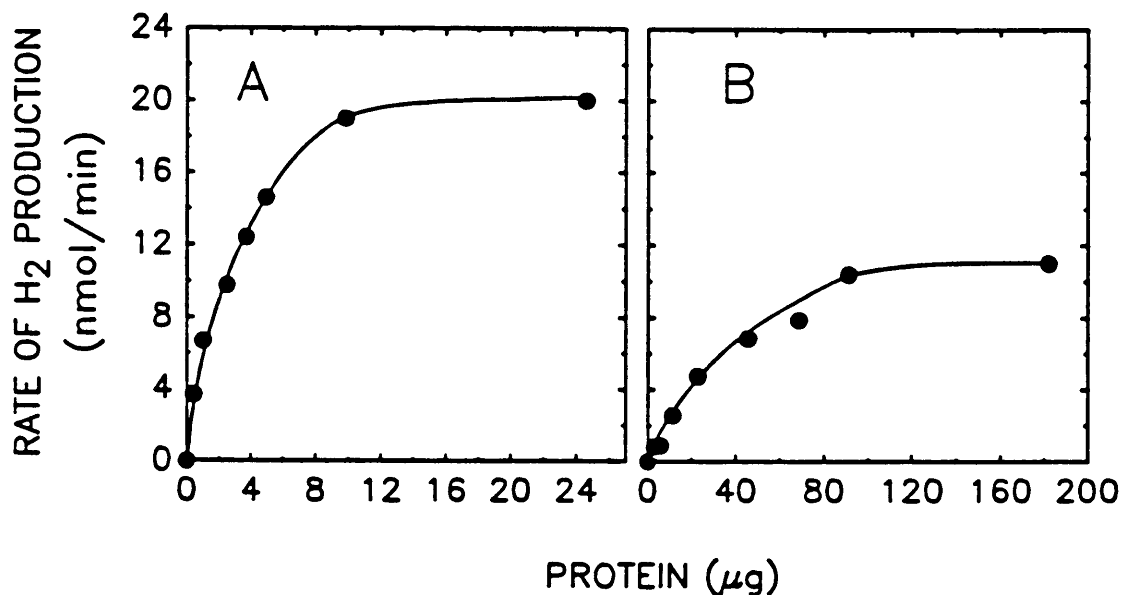

Figure 3. Dependence of the reconstituted formate hydrogenlyase activity on the amounts of formate dehydrogenase and F_{420}-hydrogenase added. (A) F_{420}-hydrogenase varied, formate dehydrogenase held constant (114 μg protein); (B) Formate dehydrogenase varied, F_{420}-hydrogenase held constant (2 μg protein).

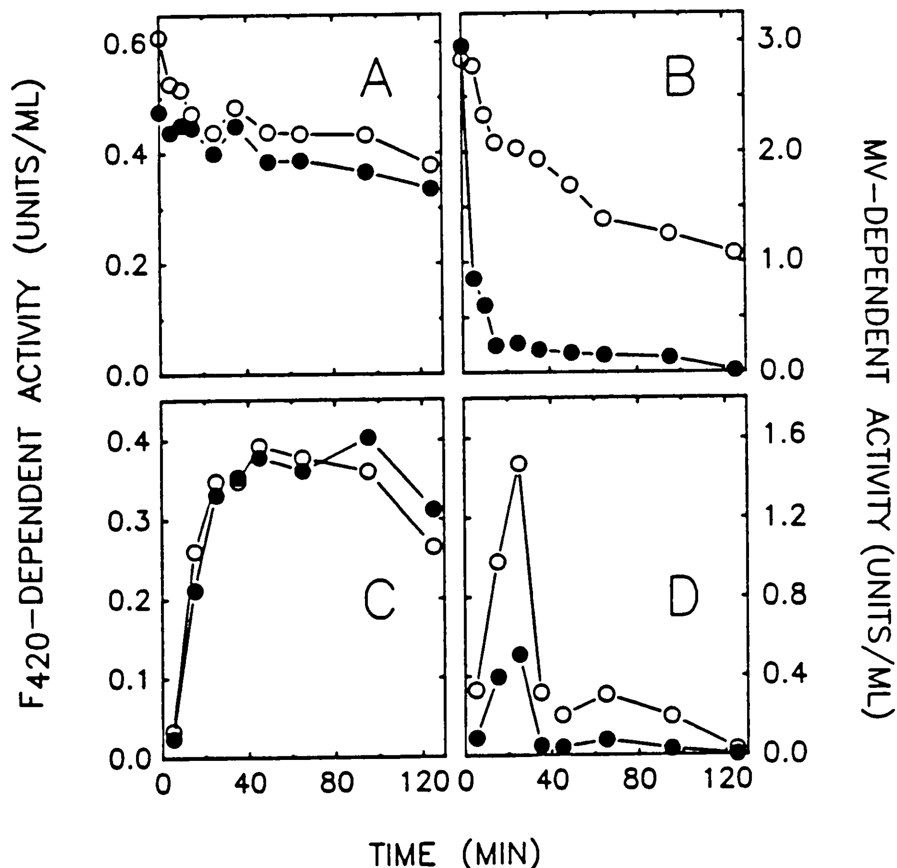

Figure 4. Effect of FAD on stability of the formate dehydrogenase and F_{420}-hydrogenase during the formate hydrogenlyase reaction. The formate hydrogenlyase system was reconstituted with 40 µg formate dehydrogenase and 4 µg F_{420}-hydrogenase in the presence (A,C) or absence (B,D) of FAD. F_{420}-dependent (●) and MV-dependent (○) formate dehydrogenase (A,B) or H_2 uptake (C,D) activities were assayed at intervals as described in Materials and Methods.

dehydrogenase were 0.40 units/ml and 2.2 units/ml in the presence of FAD; in the absence of FAD, they were 0.05 units/ml and 2.0 units/ml. Similarly, after 25 min, the F_{420}-dependent and MV-dependent activities of the F_{420}-hydrogenase were 0.34 units/ml and 1.4 units/ml in the presence of FAD, while in the absence of FAD, they were 0.07 units/ml and 0.90 units/ml. This loss of F_{420}-dependent activity did not occur when formate was omitted from the formate hydrogenlyase assay mixture (data not shown). These results suggest that FAD dissociated from both enzymes during turnover and that exogenously added FAD reconstituted the deflavoenzymes and restored F_{420}-dependent activity. The kinetic K_d for FAD in the reconstituted formate hydrogenlyase system was estimated to be 4 µM (Fig. 5).

Effect of immunoadsorption of F_{420}-hydrogenase or formate dehydrogenase from cell extract on formate hydrogenlyase activity. Western blot analysis of cell extract reveals that anti-F_{420}-hydrogenase and anti-formate dehydrogenase antisera react specifically with the respective enzymes (6). Immunoadsorption of F_{420}-hydrogenase from cell extract decreased F_{420}-dependent H_2 uptake activity but did not affect the MV-dependent activity (Table 1). This result indicates that the F_{420}-hydrogenase was removed from cell extract, while the

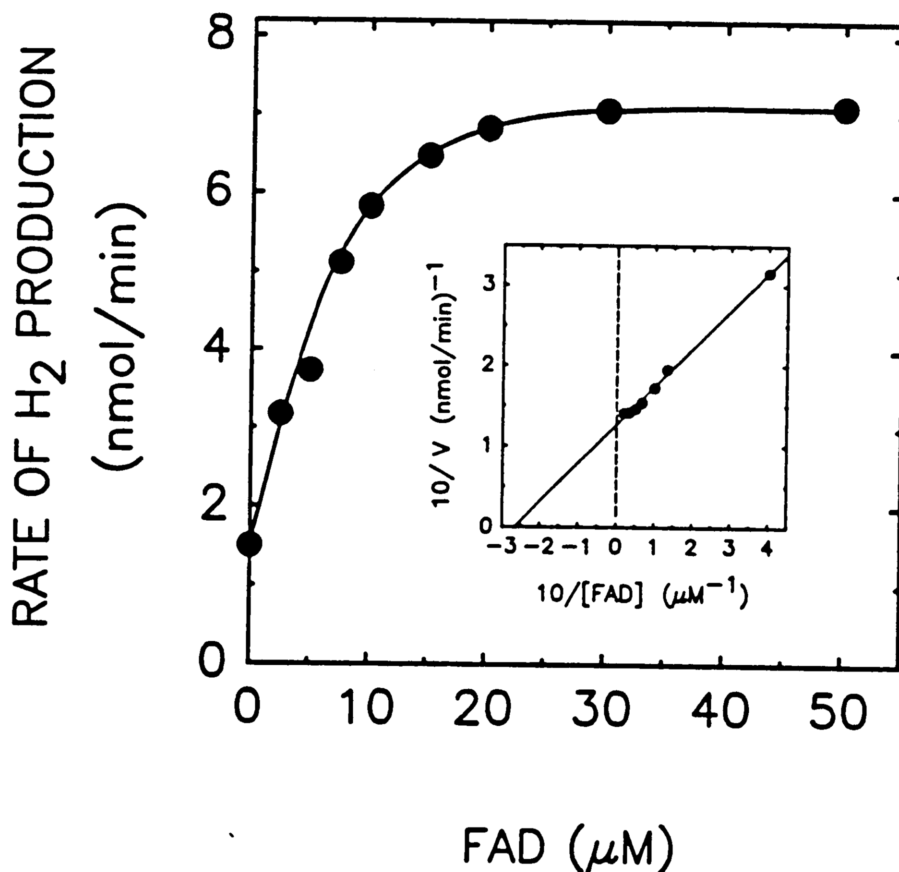

Figure 5. FAD dependence of the reconstituted formate hydrogenlyase from M. formicicum. Each assay contained 114 μg of purified formate dehydrogenase, 2.6 μg of F_{420}-hydrogenase, and the indicated concentration of FAD. H_2 production rates were calculated from the slope of the time courses at 45 min. **Inset:** a Lineweaver-Burk plot of the data.

TABLE 1. Effect of immunoadsorption of formate dehydrogenase or F_{420}-hydrogenase from cell extract of M. *formicicum* on formate-dependent and H_2-dependent activities

Enzyme assayed[a]	Activity (units/mg protein) after treatment of cell extract with:[b]		
	Control serum	Anti-formate dehydrogenase antiserum	Anti-F_{420}-hydrogenase antiserum
Hydrogenase:			
F_{420}-dependent	0.394	0.347 (88.1)	0.090 (22.8)
Methyl viologen-dependent	44.0	44.3 (101)	44.3 (101)
Formate dehydrogenase:			
F_{420}-dependent	0.659	0.087 (13.2)	0.642 (97.4)
Methyl viologen-dependent	4.36	0.44 (10.0)	4.47 (103)
Formate hydrogenlyase	35.6	4.3 (11.1)	7.9 (22.3)

[a]Hydrogenase and formate dehydrogenase were assayed spectrophotometrically; one unit is the reduction of 1 µmole of acceptor per min. Formate hydrogenlyase was assayed using gas chromatography; one unit is the production of 1 nmole of H_2 per min. Cell extract was treated with the indicated antiserum adsorbed to Protein A-Sepharose CL-4B. Activities reported are means from at least triplicate assays. Parentheses indicate the percentage of activity remaining after immunoadsorption (control serum-treated=100%).

MV-hydrogenase, which contributes about 98% of the MV-dependent H_2 uptake activity in cell extract (5), was not removed. The immunoadsorption of F_{420}-hydrogenase did not significantly affect formate dehydrogenase activity (Table 1). Immunoadsorption of formate dehydrogenase from cell extract substantially decreased F_{420}-dependent and MV-dependent formate dehydrogenase activity (Table 1), indicating a removal of the formate dehydrogenase. This treatment slightly decreased F_{420}-dependent but not MV-dependent H_2 uptake activity. Immunoadsorption of F_{420}-hydrogenase or formate dehydrogenase from cell extract substantially reduced formate hydrogenlyase activity (Table 1), and addition of purified F_{420}-hydrogenase or purified formate dehydrogenase fully restored activity (Fig. 6). Addition of partially purified MV-hydrogenase to F_{420}-hydrogenase-depleted extract did not restore formate hydrogenlyase activity (Fig. 6). These results indicate that the F_{420}-hydrogenase is the only hydrogenase in cell extract capable of supporting formate hydrogenlyase activity. The results also confirm the requirement for the formate dehydrogenase in this system; moreover, only one formate dehydrogenase is known to be present in *M. formicicum* (31).

Reversibility of the formate hydrogenlyase. F_{420}-depleted cell extract produced formate from H_2 plus

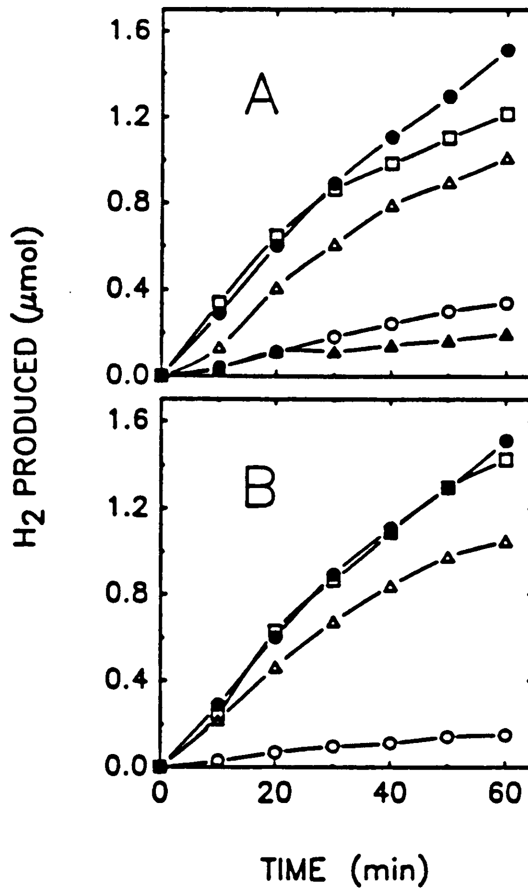

Figure 6. Restoration of formate hydrogenlyase activity in formate dehydrogenase-depleted or F_{420}-hydrogenase-depleted cell extract of M. formicicum by addition of the purified enzymes. F_{420}-hydrogenase or formate dehydrogenase was removed from cell extract by immunoadsorption. Vacuum degassed solutions of F_{420}-hydrogenase, partially purified MV-hydrogenase, or formate dehydrogenase were added to the assay vials just prior to initiation of the assays. (A) F_{420}-hydrogenase-depleted extract with: ○, no additions; 3.6 (△) or 9.6 (□) μg F_{420}-hydrogenase added; ▲, 101 μg partially purified MV-hydrogenase added; ●, control serum-treated extract with no additions. (B) Formate dehydrogenase-depleted extract with: ○, no additions; 11 (△) or 41 (□) μg formate dehydrogenase added; ●, control serum-treated extract with no additions.

CO_2/HCO_3^- when purified F_{420} was added (Fig. 7), indicating that F_{420}-mediated formate hydrogenlyase activity was reversible. No formate was detected when H_2, CO_2/HCO_3^-, or F_{420} was omitted (data not shown). FAD was stimulatory when present with F_{420} (Fig. 7) but did not support activity without F_{420} (data not shown). The ratio of formate hydrogenlyase activity in the forward direction (H_2 production) to that in the reverse direction (formate production) by the F_{420}-depleted extract was 0.44.

The reconstituted formate hydrogenlyase also produced formate from H_2 plus CO_2/HCO_3^- (Fig. 7). In these assays, hydrogenase activity was kept in 5.5 fold excess of the formate dehydrogenase activity so that F_{420} would remain reduced during the reaction. No formate was produced when F_{420}-hydrogenase, formate dehydrogenase, F_{420}, H_2, or CO_2/HCO_3^- was omitted (data not shown). FAD stimulated activity in the presence of F_{420} (Fig. 7) but could not replace F_{420} (data not shown).

DISCUSSION

Formate hydrogenlyase systems function in a variety of anaerobic bacteria (11); however, none of these systems have completely been defined. The formate hydrogenlyase system of _M. formicicum_ consisted of only two protein components (F_{420}-hydrogenase and formate dehydrogenase), a

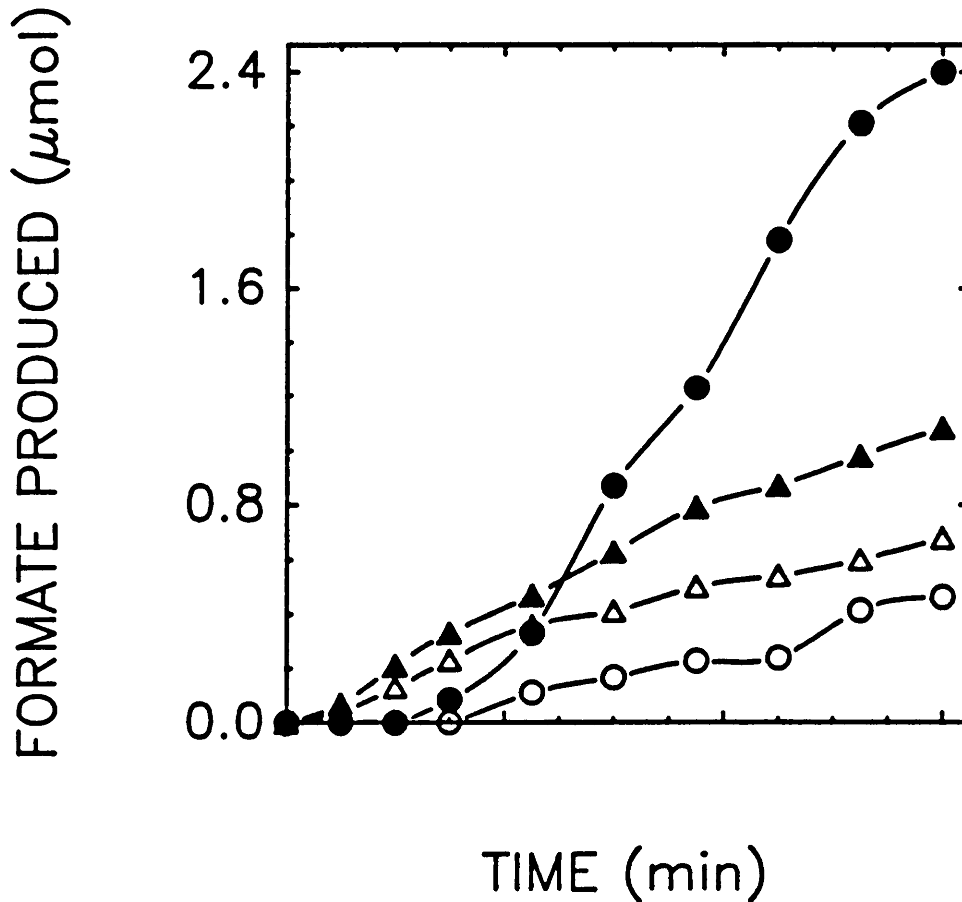

Figure 7. Reversibility of the formate hydrogenlyase of _M. formicicum_. Formate production from H_2 plus CO_2/HCO_3^- was assayed as described in MATERIALS AND METHODS. (●,○), DEAE-cellulose-treated extract, 0.46 mg protein (in 0.1 ml) per assay. (▲,△), reconstituted formate hydrogenlyase; each assay contained 5.4 μg purified formate dehydrogenase and 15.5 μg of purified, reactivated F_{420}-hydrogenase. Symbols: ●, ▲, complete reaction mixture; ○, △, FAD omitted.

soluble intermediate electron carrier (F_{420}), and FAD. The function of FAD in the M. formicicum formate hydrogenlyase was not as a free electron carrier between formate dehydrogenase and F_{420}-hydrogenase, since no activity was obtained in the presence of FAD and absence of F_{420}; rather, FAD appears to stabilize F_{420}-dependent activity of the component enzymes. An F_{420}:NADP oxidoreductase is present in several methanogenic bacteria (14,29,38); however, NADP was not required for formate hydrogenlyase activity, nor were any other intermediate electron carriers besides F_{420}. Although M. formicicum has both F_{420}-hydrogenase and MV-hydrogenase (12,13,21), the latter could not support formate hydrogenlyase activity since addition of the partially purified enzyme did not restore formate hydrogenlyase activity of F_{420}-hydrogenase-depleted cell extract.

The formate hydrogenlyase systems of Escherichia coli (25) and Rhodopseudomonas palustris (26) consist of a soluble formate dehydrogenase, a particulate hydrogenase, and one or more unidentified intermediate electron carriers. Cytochrome C3 has been identified as an intermediate electron carrier for the formate hydrogenlyase system in cell extracts of Desulfovibrio desulfuricans (J.P. Williams, J.T. Davidson, and H.D. Peck, Jr., Bacteriol. Proc., p. 110, 1964). A formate hydrogenlyase

has been studied in cell extracts of the acetogenic 'S organism' and shown to consist of at least an NAD-dependent formate dehydrogenase, an NADH:ferredoxin oxidoreductase, a ferredoxin, and a ferredoxin dependent hydrogenase (27).

The formate dehydrogenase (6) and F_{420}-hydrogenase (4,6) of M. formicicum are both membrane associated, suggesting a role in electron transport. However, the physiological function of formate hydrogenlyase in methanogenic bacteria is not known. Cultures of Methanococcus vannielii produce increasing amounts of H_2 as the pH rises from 7.6 to 8.8 during growth on formate (35). Cultures of M. formicicum growing on formate produce only small amounts of H_2 relative to CH_4 at pH 7.6 and 37°C (28). However, when the growth temperature is raised to 63°C, more H_2 is produced than CH_4 (28). These observations are consistent with a role for the formate hydrogenlyase system in maintaining the redox balance in formate-grown cells, especially when methanogenesis is impaired. The ability to interconvert formate and H_2/CO_2 may also allow the cell to dispose of excess reducing potential during growth on either substrate.

However, the involvement of H_2 as an obligatory intermediate in one or more reductive steps during formate-dependent CO_2 reduction to methane can not be ruled out. Likewise, H_2 produced from the formate hydrogenlyase

reaction may be required for reductive biosynthesis. Because the reaction has a standard free energy change of +1.3 kjoules (37) the cell may not be able to derive additional energy unless the H_2 partial pressure were decreased by a membrane-dependent H_2-cycling mechanism similar to that proposed for the sulfate-reducing bacteria (23). Interestingly, molar growth yields of *M. formicicum* grown with formate are about 1.4-fold greater than those for cells grown on H_2/CO_2 (28), even though these two substrates yield virtually the same free energy change per mole of CH_4 produced.

The reversibility of the formate hydrogenlyase suggests that this system may allow the cell to fix CO_2 as formate for biosynthesis. In fact, formate dehydrogenase is synthesized at high levels in *M. formicicum* cells grown on H_2/CO_2 alone (28). Recently, mutants of *Methanobacterium thermoautotrophicum* have been isolated which require formate for growth on H_2/CO_2 (R. S. Tanner and D. P. Nagle, Abstr. Ann. Meet. Am. Soc. Microbiol., I-10, p. 182, 1988). Formate is a precursor of one carbon units for the biosynthesis of purines, thymidine, and methionine in clostridia (36).

Reconstitution of the *M. formicicum* formate hydrogenlyase offered a convenient assay for the H_2-producing activity of the F_{420}-hydrogenase; because formate

and formate dehydrogenase activity were in excess during the reaction, a constant supply of reduced F_{420} was available for H_2 production by the hydrogenase. The ratio of H_2-evolution to H_2-uptake for the F_{420}-hydrogenase was 0.30 (5), indicating that the enzyme is bidirectional. The optimal pH for H_2 production by hydrogenases is often more acidic than that for H_2 oxidation (15). However, the optimal pH for both H_2 production and H_2 oxidation by the *M. formicicum* F_{420}-hydrogenase was near pH 7.5 (5).

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LITERATURE CITED

1. Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260-296.

2. Barber, M. J., H. D. May, and J. G. Ferry. 1986. Inactivation of formate dehydrogenase from *Methanobacterium formicicum* by cyanide. Biochemistry. **25**:8150-8155.

3. Barber, M. J., L. M. Siegel, N. L. Schauer, H. D. May, and J. G. Ferry. 1983. Formate dehydrogenase from Methanobacterium formicicum. Electron paramagnetic resonance spectroscopy of the molybdenum and iron-sulfur centers. J. Biol. Chem. **258**:10839-10845.

4. Baron, S. F., D. P. Brown, and J. G. Ferry. 1987. Locations of the hydrogenases of Methanobacterium formicicum after subcellular fractionation of cell extract. J. Bacteriol. **169**:3823-3825.

5. Baron, S. F., and J. G. Ferry. 1988. Purification and properties of coenzyme F_{420}-reducing hydrogenase of Methanobacterium formicicum. J. Bacteriol. (Submitted).

6. Baron, S. F., D. S. Williams, H. D. May, P. S. Patel, H. C. Aldrich, and J. G. Ferry. 1988. Immunogold localization of coenzyme F_{420}-reducing formate dehydrogenase and coenzyme F_{420}-reducing hydrogenase in Methanobacterium formicicum. Arch. Microbiol. (In press).

7. Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

8. Eguchi, S. Y., N. Nishio, and S. Nagai. 1985. Formic acid production from H_2 and bicarbonate by a formate-utilizing methanogen. Appl. Microbiol. Biotechnol. **22**:148-151.

9. Ferry, J. G., and R. S. Wolfe. 1977. Nutritional and biochemical characterization of Methanospirillum hungatei. Appl. Environ. Microbiol. **34**:371-376.

10. Fox, J. A., D. J. Livingston, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 1. purification and characterization. Biochemistry. **26**:4219-4227.

11. Gray, C. T., and H. Gest. 1965. Biological formation of molecular hydrogen. Science. **148**:186-192.

12. Jacobson, F. S., L. Daniels, J. A. Fox, C. T. Walsh, and W. H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazariboflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. **257**:3385-3388.

13. Jin, S. -L. C., D. K. Blanchard, and J. -S. Chen. 1983. Two hydrogenases with distinct electron-carrier specificity and subunit composition in Methanobacterium formicicum. Biochim. Biophys. Acta. **748**:8-20.

14. Jones, J. B., and T. C. Stadtman. 1980. Reconstitution of a formate-$NADP^+$ oxidoreductase from formate dehydrogenase and a 5-deazaflavin-linked $NADP^+$ reductase isolated from Methanococcus vannielii. J. Biol. Chem. **255**:1049-1053.

15. Lespinat, P. A., Y. Berlier, G. Fauque, M. Czechowski, B. Dimon, and J. LeGall. 1986. The pH dependence of proton-deuterium exchange, hydrogen production and uptake catalyzed by hydrogenases from sulfate-reducing bacteria. Biochimie. **68**:55-61.

16. Lovley, D. R., R. C. Greening, and J. G. Ferry. 1984. Rapidly growing rumen methanogenic organism that synthesizes coenzyme M and has a high affinity for formate. Appl. Environ. Microbiol. **48**:81-87.

17. May, H. D., N. L. Schauer, and J. G. Ferry. 1986. Molybdopterin cofactor from Methanobacterium formicicum formate dehydrogenase. J. Bacteriol. **166**:500-504.

18. May, H. D., P. S. Patel, and J. G. Ferry. 1988. Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in Methanobacterium formicicum. J. Bacteriol. **170**:3384-3389.

19. Muth, E., E. Mörschel, and A. Klein. 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium Methanococcus voltae. Eur. J. Biochem. **169**:571-577.

20. Nagle Jr., D. P., and R. S. Wolfe. 1983. Component A of the methyl coenzyme M methylreductase system of Methanobacterium: resolution into four components. Proc. Natl. Acad. Sci. U.S.A. **80**:2151-2155.

21. Nelson, M. J. K., D. P. Brown, and J. G. Ferry. 1984. FAD requirement for the reduction of coenzyme F_{420} by hydrogenase from Methanobacterium formicicum. Biochem. Biophys. Res. Commun. **120**:775-781.

22. Nishio, N., S. Y. Eguchi, H. Kawashima, and S. Nagai. 1983. Mutual conversion between H_2 plus CO_2 and formate by a formate-utilizing methanogen. J. Ferment. Technol. **61**:557-561.

23. Odom, J. M., and H. D. Peck, Jr. 1981. Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, Desulfovibrio spp. FEMS Microbiol. Lett. **12**:47-50.

24. Patel, P. S., and J. G. Ferry. 1988. Characterization of the upstream region of the formate dehydrogenase operon of Methanobacterium formicicum. J. Bacteriol. **170**:3390-3395.

25. Peck, Jr., H. D., and H. Gest. 1957. Formic dehydrogenase and the hydrogenlyase enzyme complex in coli-aerogenes bacteria. J. Bacteriol. **73**:706-721.

26. Qadri, S. M. H., and D. S. Hoare. 1968. Formic hydrogenlyase and the photoassimilation of formate by a strain of Rhodopseudomonas palustris. J. Bacteriol. **95**:2344-2357.

27. Reddy, C. A., M. P. Bryant, and M. J. Wolin. 1972. Ferredoxin and nicotinamide adenine dinucleotide-dependent H_2 production from ethanol and formate in extracts of S organism isolated from "Methanobacillus omelianskii". J. Bacteriol. **110**:126-132.

28. Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in Methanobacterium formicicum. J. Bacteriol. **142**:800-807.

29. Schauer, N. L., and J. G. Ferry. 1982. Properties of formate dehydrogenase in Methanobacterium formicicum. J. Bacteriol. **150**:1-7. (Erratum **151**:1642).

30. Schauer, N. L., and J. G. Ferry. 1983. FAD requirement for the reduction of coenzyme F_{420} by formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **155**:467-472.

31. Schauer, N. L., and J. G. Ferry. 1986. Composition of the coenzyme F_{420}-reducing formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **165**:405-411.

32. Schauer, N. L., J. G. Ferry, J. F. Honek, W. H. Orme-Johnson, and C. T. Walsh. 1986. Mechanistic studies of the coenzyme F_{420}-reducing formate dehydrogenase from Methanobacterium formicicum. Biochemistry. **25**:7163-7168.

33. Schuber, A. P., E. C. Orr, M. A. Recny, P. F. Schendel, H. D. May, N. L. Schauer, and J. G. Ferry. 1986. Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from Methanobacterium formicicum. J. Biol. Chem. **261**:12942-12947.

34. Sprott, G. D., K. M. Shaw, and T. J. Beveridge. 1987. Properties of the particulate enzyme F_{420}-reducing hydrogenase isolated from Methanospirillum hungatei. Can. J. Microbiol. **33**:896-904.

35. Stadtman, T. C., and H. A. Barker. 1951. Studies on the methane fermentation. X. A new formate-decomposing bacterium, Methanococcus vannielii. J. Bacteriol. **62**:269-280.

36. Thauer, R. K., G. Fuchs, and K. Jungermann. 1977. Role of iron-sulfur proteins in formate metabolism, p. 121-156. In W. Lovenberg (ed.), Iron-sulfur proteins, Vol. III. Academic Press, Inc., N. Y.

37. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.

38. Tzeng, S. -F., M. P. Bryant, and R. S. Wolfe. 1975. Factor F_{420}-dependent pyridine nucleotide-linked formate metabolism of Methanobacterium ruminantium. J. Bacteriol. **121**:192-196.

39. Yamazaki, S. 1982. A selenium-containing hydrogenase from Methanococcus vannielii. J. Biol. Chem. **257**:7926-7929.

SECTION V. LOCATIONS OF THE HYDROGENASES OF *METHANOBACTERIUM FORMICICUM* AFTER SUBCELLULAR FRACTIONATION OF CELL EXTRACT

SUMMARY

The F_{420}-hydrogenase of *Methanobacterium formicicum* was associated with membranes isolated by sucrose density gradient ultracentrifugation of cell extract. The MV-hydrogenase was present in the soluble fractions. Column chromatography with Phenyl Sepharose CL-4B revealed that the F_{420}-hydrogenase was strongly hydrophobic, suggesting that it associates with isolated membranes through hydrophobic interactions.

INTRODUCTION

Two hydrogenases have been reported in H_2-oxidizing methanogens (6,7,9,15). One enzyme (F_{420}-hydrogenase) reduces coenzyme F_{420} (F_{420}) and methyl viologen (MV). The other enzyme (MV-hydrogenase) reduces MV but not F_{420}. Jin *et al*. (7) showed that these two hydrogenases in *M. formicicum* are distinct enzymes on the basis of subunit composition and peptide mapping. The cellular locations of these enzymes are unclear. *Methanobacterium formicicum* strain G2R (phenotypically similar to *M. formicicum*) contains a membrane-associated hydrogenase which reduces

viologen dyes but not F_{420} (8). The F_{420}-hydrogenase is unstable in this organism under the conditions used, sinceno F_{420}-dependent activity is detected in either the soluble or membrane fraction of cell extract; thus the identity of this membrane-associated hydrogenase could not be determined (8). We have developed methods to stabilize the F_{420}-hydrogenase of *M. formicicum* and were thus able to study the location of this enzyme.

MATERIALS AND METHODS

Methanobacterium formicicum JF-1 (DSM 2639) was cultured with H_2:CO_2 (4:1) and harvested anaerobically as described (11). All subsequent procedures were performed under an atmosphere of O_2-free N_2 as described (10,11), except that sodium dithionite was not included in buffers. Basal buffer contained 50 mM potassium N-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonate pH 7.5, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol.

Although anaerobic procedures were used throughout, both hydrogenases were reversibly inactivated and required reductive reactivation prior to assay. The samples were degassed by 8 cycles of alternate evacuation and pressurization with N_2, and flushed with H_2 for 1 min. They were then made to 48 µM in F_{420} and 30 µM in FAD andincubated in the dark for 1 h at 35°C. Under these

conditions, the F_{420}-hydrogenase is stabilized toward F_{420}-dependent activity (to be published).

Hydrogenase was assayed spectrophotometrically at 35°C and pH 7.5 as previously described (10). One unit of activity is the reduction of 1 µmole of F_{420} or MV per min. F_{420} was purified as described (12). Protein was determined by the dye-binding assay (micro-method) of Bradford (3) with bovine serum albumin as the standard.

Sucrose density gradient ultracentrifugation was employed to separate membranes from soluble proteins in cell extracts of *M. formicicum*. Sucrose solutions with or without Triton X-100 (1% [v/v] final concentration) were prepared in basal buffer and layered in polycarbonate centrifuge tubes (16 x 78 mm) fitted with gas-tight screw caps. The gradients consisted of 5.5 mL of 20% (w/v) sucrose and 2.0 mL of 30% (w/v) sucrose over a 70% (w/v) sucrose shelf (1.5 mL). Samples (1.0 mL, 20 mg protein) of cell extract with or without 1.5% (v/v) Triton X-100 were layered onto the gradients and centrifuged in a Beckman 50Ti fixed angle rotor (90 min; 226,000 x g; 5°C).

DEAE-cellulose chromatography was used to remove unbound Triton X-100 and sucrose from hydrogenase samples prior to hydrophobic interaction chromatography. Fractions from the sucrose gradients were loaded onto a column (1 x2cm) of DEAE-cellulose (Whatman DE-52) equilibrated with

basal buffer containing 5% (v/v) glycerol (buffer A). Unadsorbed material was washed from the column with 11 mL of buffer A. Adsorbed protein was batch eluted with the same buffer (1 mL/min) containing 1 M KCl (buffer B). Recovery of F_{420}-dependent and MV-dependent activity was greater than 70% and 80%, respectively. Hydrophobic interaction chromatography was done as follows. Samples were loaded onto a column (0.9 x 5 cm) of Phenyl Sepharose CL-4B (Pharmacia, Inc.) equilibrated with buffer B. The column was washed with 3.2 mL of buffer B, then with 9.6 mL of buffer A, and finally with a linear gradient of Triton X-100 (0-1.5% [v/v], 9.6 mL) in buffer A (0.4 mL/min). It was then washed with an additional 9.6 mL of buffer A containing 1.5% (v/v) Triton X-100. Fractions of 0.4 mL were collected.

RESULTS

After ultracentrifugation of the sucrose gradients, a membrane fraction was present in a narrow, light-brown, translucent band at the top of the 70% sucrose shelf. Electron micrographs (not shown) of this material confirmed the presence of membrane vesicles. This band was absent in the sucrose gradients loaded with cell extract which had been treated with Triton X-100 to solubilize membranes.

After ultracentrifugation of untreated extract, 58% of the F_{420}-dependent activity was detected in the membrane fraction (I, Fig. 1A), and the remaining 42% was distributed between the soluble proteins (III) and a broad band (II) situated between the membrane and soluble fractions.

After ultracentrifugation of Triton X-100-treated extract, no F_{420}-dependent activity was present at the top of the 70% sucrose shelf (Fig. 1B); instead, the activity increased near the middle of the gradient (IV) and in the soluble fractions (III). The total F_{420}-dependent activity recovered throughout the sucrose gradient without Triton X-100 (4.6 units) was nearly the same as that with Triton X-100 (4.8 units), and in both cases was greater than 85% of that loaded. Thus, all of the membrane-associated F_{420}-dependent activity (I, Fig. 1A) could be accounted for in III and IV (Fig. 1B) after solubilization of membranes.

These results show that the F_{420}-hydrogenase of _M. formicicum_ is associated with isolated membranes and suggest that the enzyme may be located on membranes in the intact cell. The F_{420}-hydrogenases studied to date are monomers which form aggregates of up to M_r 800,000 (5,6,7,15), including the enzyme from _M. formicicum_ for which aggregates never exceed M_r 790,000 (unpublished

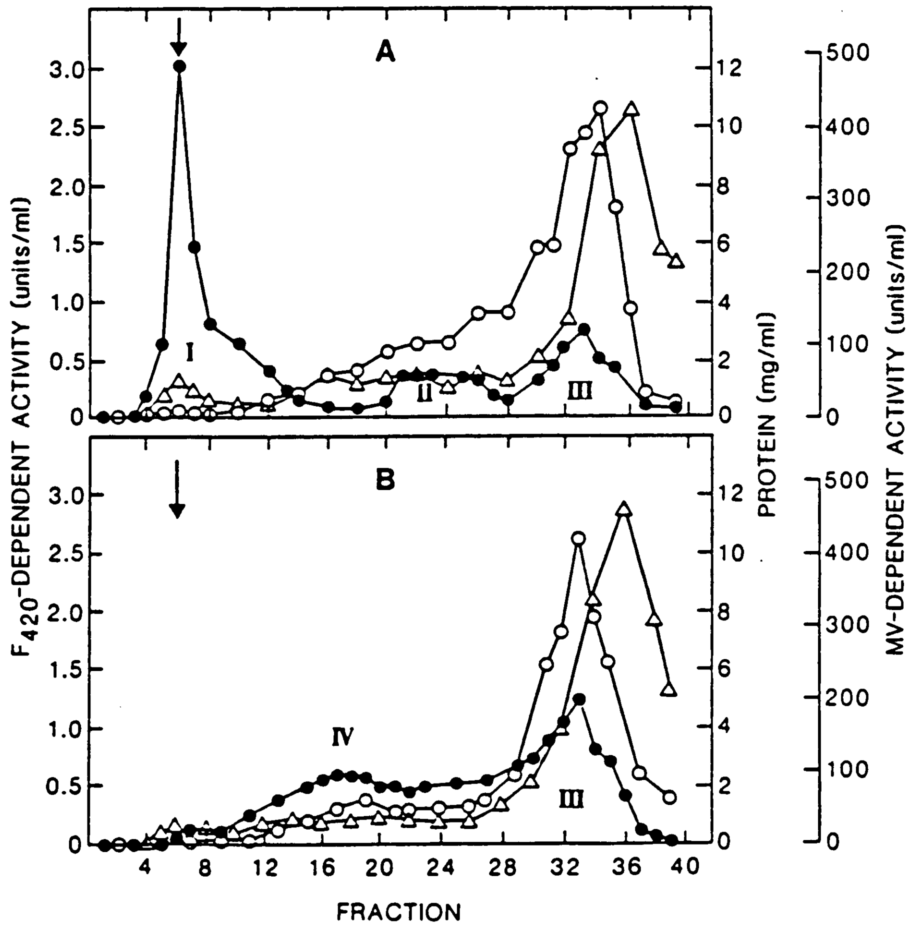

Figure 1. Separation of the F_{420}-hydrogenase and MV-hydrogenase of M. formicicum by sucrose density gradient ultracentrifugation. (A) Cell extract was centrifuged through sucrose gradients containing no Triton X-100. (B) Cell extract containing 1.5% (v/v) Triton X-100 was centrifuged through sucrose gradients containing 1% (v/v) Triton X-100. Symbols: ●, F_{420}-dependent activity; ○, MV-dependent activity; △, protein. Arrow denotes the top of the 70% sucrose shelf. Roman numerals denote peaks of hydrogenase activity described in the text. Fractions (0.25 mL) are numbered from the bottom of the tube.

results). Therefore, II and III (Fig. 1A) may represent aggregates and monomers, respectively, of the F_{420}-hydrogenase which may have dissociated from the membranes. The carbon monoxide dehydrogenase complex of *Methanosarcina thermophila* (M_r 1,000,000) (13) sediments only to the top of the 30% sucrose layer when ultracentrifuged under the same conditions used in this study (K. Terlesky, personal communication). Therefore, the membrane-associated F_{420}-hydrogenase (I, Fig. 1A) does not represent free aggregates large enough to sediment to the same position as membranes in sucrose gradients.

When hydrogenase from solubilized membranes was chromatographed on the hydrophobic interaction column, the F_{420}-dependent activity eluted as a single peak at the end of a linear gradient of Triton X-100 (Fig. 2A). This peak coincided with a peak of MV-dependent activity, but no other peaks of activity were observed. Similarly, when the soluble fractions were chromatographed over the same column, the F_{420}-dependent activity eluted in a single peak at the end of the Triton X-100 gradient, and was accompanied by a peak of MV-dependent activity (Fig. 2B). However, the majority of the MV-dependent activity (79%) eluted in two peaks well resolved from the F_{420}-dependent activity, indicating that they represented the MV-hydrogenase. These results show that the F_{420}-hydrogenase

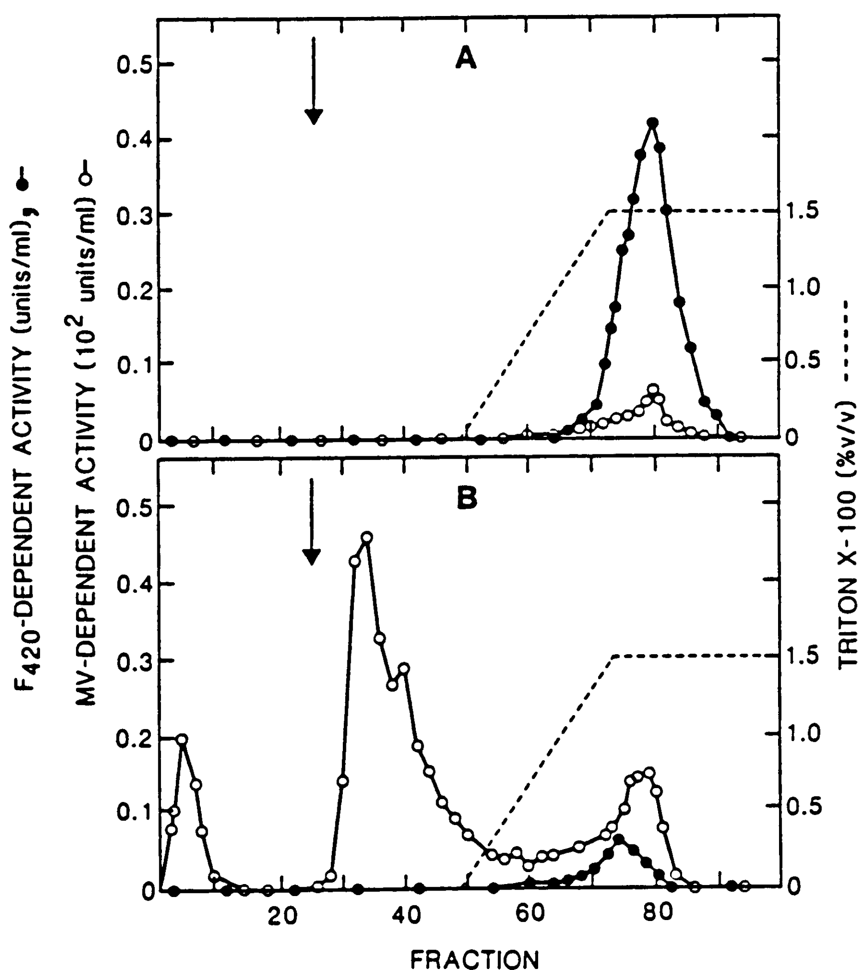

Figure 2. Hydrophobic interaction chromatography of the F_{420}-hydrogenase and MV-hydrogenase of M. formicicum. (A) Triton X-100-solubilized membranes (fractions 3-14 from the sucrose gradient in Fig. 1A) were passed over a DEAE-cellulose column and then chromatographed on a column of Phenyl Sepharose CL-4B as described in the text. (B) As in (A) except fractions 28-39 from the sucrose gradient in Fig. 1A were used and were not treated with Triton X-100 prior to DEAE-cellulose chromatography. Symbols: ●, F_{420}-dependent activity; ○, MV-dependent activity; ----, Triton X-100 gradient. Arrow denotes the start of washing with 9.6 mL of buffer A (see text).

is more hydrophobic than the MV-hydrogenase and suggests that the F_{420}-hydrogenase associates with isolated membranes through hydrophobic interactions.

After ultracentrifugation of either untreated or Triton X-100-treated cell extract, the soluble fractions (III, Figs. 1A,B) contained most of the MV-dependent activity, suggesting that the MV-hydrogenase was present in these fractions. Three observations support this conclusion: i) The ratio of F_{420}-dependent to MV-dependent activity in the most active fraction of I (Fig. 1A) was 385-fold greater than that in the most active fraction of III (Fig. 1A). ii) The total MV-dependent activity (about 600 units) in III was similar regardless of the presence of Triton X-100. iii) Hydrophobic interaction chromatography of the soluble fractions resolved distinct hydrogenases, one capable of reducing F_{420} and MV, and the other capable of reducing MV but not F_{420} (Fig. 2B). These results do not necessarily suggest that the MV-hydrogenase is a soluble enzyme _in vivo_, since it could be loosely bound to the membrane and become dislodged during preparation of cell extract or sucrose gradient fractionation.

DISCUSSION

The synthesis of ATP in *Methanosarcina barkeri* and *Methanosphaera stadtmanae* is driven by a protonmotive force generated across the membrane by methanogenesis from methanol and H_2 (1,2), and membrane preparations from the H_2-oxidizing methanogen, *Methanobacterium thermoautotrophicum*, carry out H_2-driven ATP synthesis (4). Our results indicate that the F_{420}-hydrogenase of *M. formicicum* is associated with membranes, and therefore may be involved in ATP synthesis in this and other H_2-oxidizing methanogens.

The F_{420}-hydrogenases of *Methanobacterium* spp. are reported to be unstable (8,9). The F_{420}-hydrogenase from *M. formicicum* JF-1 contains bound FAD required for reduction of F_{420} (9). Hydrophobic interaction chromatography converts the enzyme to a deflavo-species which reduces MV but not F_{420}; preincubation of the enzyme with FAD restores the F_{420}-dependent activity (9). Similarly, when the enzyme is reactivated by incubation under H_2 with F_{420}, it loses all F_{420}-dependent activity, unless FAD is present, and retains most of the MV-dependent activity (to be published). In this study, the reactivation mixture contained 30 µM FAD which stabilized reactivated F_{420}-hydrogenase and reconstituted any deflavo-enzyme present. The ratio of F_{420}-dependent to

MV-dependent activity of the peak in Fig. 2A was 21-fold greater than that of the peak of F_{420}-dependent activity in Fig. 2B. The reason for this is unknown; perhaps the dissociated form of the F_{420}-hydrogenase is irreversibly converted to deflavo-enzyme, while the conversion of the membrane-associated form is reversible.

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LITERATURE CITED

1. Blaut, M. and G. Gottschalk. 1984. Protonmotive force-driven synthesis of ATP during methane formation from molecular hydrogen and formaldehyde or carbon dioxide in Methanosarcina barkeri. FEMS Microbiol. Lett. **24**:103-107.

2. Blaut, M. and G. Gottschalk. 1984. Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in Methanosarcina barkeri. Eur. J. Biochem. **141**:217-222.

3. Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

4. Doddema, H. J., C. van der Drift, G. D. Vogels, and M. Veenhuis. 1979. Chemiosmotic coupling in Methanobacterium thermoautotrophicum: hydrogen-dependent adenosine 5'-triphosphate synthesis by subcellular particles. J. Bacteriol. **140**:1081-1089.

5. Fuchs, G., J. Moll, P. Scherer, and R. K. Thauer. 1979. Activity, acceptor specificity and function of hydrogenase in Methanobacterium thermoautotrophicum, pp. 83-92. **In** H. G. Schlegel and K. Schneider (eds.), Hydrogenases: their catalytic activity, structure and function. E. Goltz KG, Gottingen.

6. Jacobson, F. S., L. Daniels, J. A. Fox, C. T. Walsh, and W. H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. **257**:3385-3388.

7. Jin, S.-L. C., D. K. Blanchard, and J.-S. Chen. 1983. Two hydrogenases with distinct electron-carrier specificity and subunit composition in Methanobacterium formicicum. Biochim. Biophys. Acta. **748**:8-20.

8. McKellar, R. C. and G. D. Sprott. 1979. Solubilization and properties of a particulate hydrogenase from Methanobacterium strain G2R. J. Bacteriol. **139**:231-238.

9. Nelson, M. J. K., D. P. Brown, and J. G. Ferry. 1984. FAD requirement for the reduction of coenzyme F420 by hydrogenase from Methanobacterium formicicum. Biochem. Biophys. Res. Commun. **120**:775-781.

10. Nelson, M. J. K. and J. G. Ferry. 1984. Carbon monoxide-dependent methyl coenzyme M methylreductase in acetotrophic Methanosarcina spp. J. Bacteriol. **160**:526-532.

11. Schauer, N. L. and J. G. Ferry. 1982. Properties of formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **151**:1642.

12. Schauer, N. L. and J. G. Ferry. 1983. FAD requirement for the reduction of coenzyme F420 by formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **155**:467-472.

13. Terlesky, K. C., M. J. K. Nelson and J. G. Ferry. 1986. Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate-grown *Methanosarcina thermophila*. J. Bacteriol. **168**:1053-1058.

14. Wackett, L. P., E. A. Hartwieg, J. A. King, W. H. Orme-Johnson and C. T. Walsh. 1987. Electron microscopy of nickel-containing methanogenic enzymes: methyl reductase and F_{420}-reducing hydrogenase. J. Bacteriol. **169**:718-727.

15. Yamazaki, S. 1982. A selenium-containing hydrogenase from *Methanococcus vannielii*. J. Biol. Chem. **257**:7926-7929.

SECTION VI. IMMUNOGOLD LOCALIZATION OF COENZYME F_{420}-REDUCING FORMATE DEHYDROGENASE AND COENZYME F_{420}-REDUCING HYDROGENASE IN *METHANOBACTERIUM FORMICICUM*

SUMMARY

The ultrastructural locations of the coenzyme F_{420}-reducing formate dehydrogenase and coenzyme F_{420}-reducing hydrogenase of *Methanobacterium formicicum* were determined using immunogold labeling of thin-sectioned, Lowicryl-embedded cells. Both enzymes were located predominantly at the cell membrane. Whole cells displayed minimal F_{420}-dependent formate dehydrogenase activity or F_{420}-dependent hydrogenase activity, and little activity was released upon osmotic shock treatment, suggesting that these enzymes are not soluble periplasmic proteins. Analysis of the deduced amino acid sequences of the formate dehydrogenase subunits revealed no hydrophobic regions that could qualify as putative membrane-spanning domains.

INTRODUCTION

The methanogenic bacteria obtain energy for growth by reducing CO_2 to CH_4 with electrons derived from the oxidation of H_2 and other substrates. At least one step in the CO_2 reduction pathway is driven by the dihydro form of coenzyme F_{420} (F_{420}) (13), an 8-hydroxy-5-deazaflavin, low

potential electron carrier present in all known methanogens. Methanobacterium formicicum can use formate or H_2 as the electron donor for CO_2 reduction (28). The oxidation of these substrates is catalyzed by formate dehydrogenase and hydrogenase, respectively, both of which are synthesized during growth on either substrate (28).

The F_{420}-reducing formate dehydrogenase of M. formicicum has been characterized (4,5,20,29,30,31,32), the genes encoding its two subunits (α and β) have been cloned and sequenced (33), and its regulation has been investigated (19,27). This enzyme is operationally soluble, since the majority of formate dehydrogenase activity is present in the supernatant fraction after ultracentrifugation of cell extract prepared by French pressure cell lysis (28). However, it can not be ruled out that the enzyme is detached from the cytoplasmic membrane during cell rupture.

M. formicicum and other H_2-oxidizing methanogens have two distinct hydrogenases (14,15,25,36). One (F_{420}-hydrogenase) reduces F_{420} and methyl viologen (MV); the other (MV-hydrogenase) reduces MV but not F_{420}. Several F_{420}-hydrogenases have been purified and characterized (11,23,34,36, S. F. Baron and J. G. Ferry, manuscript in preparation). The F_{420}-hydrogenase of M. formicicum is a hydrophobic enzyme that loosely associates with membranes

isolated by sucrose gradient ultracentrifugation of cell extract prepared by French pressure cell lysis (6). This result suggests, but does not prove, that the hydrogenase is associated with the membrane in intact cells.

Results of recent immunogold labeling experiments indicate that methyl coenzyme M reductase, which catalyzes the last step in methanogenesis, is located at the cell membrane in *Methanococcus voltae* (26) and *Methanobacterium thermoautotrophicum* (1). Here we use immunogold labeling and biochemical techniques to examine the cellular locations of the formate dehydrogenase and F_{420}-hydrogenase of *M. formicicum* and report that both enzymes are associated with the cytoplasmic membrane of intact cells.

MATERIALS AND METHODS

Organism and growth conditions. *Methanobacterium formicicum* JF-1 (DSM 2639) was grown in a basal salts-yeast extract-tryptone medium with 89 mM formate and H_2:CO_2 (4:1) as described (31).

Preparation of cell extract. Cell paste was resuspended in twice its weight of breakage buffer (75 mM potassium TES [pH 7.5], 1.5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 10 µg/ml DNAase I) and anaerobically passed through a French pressure cell at 138 MPa. The cell lysate was collected anaerobically and frozen in liquid N_2.

When required, the lysate was centrifuged (20 min, 30,000 x *g*, 5°C) in pressurized (82 KPa N_2) polycarbonate centrifuge tubes, and the supernatant solution (cell extract) was removed and stored in liquid N_2.

Preparation and testing of antisera. The formate dehydrogenase of *M. formicicum* was purified (31) and anti-formate dehydrogenase antiserum prepared (33) as described. The F_{420}-hydrogenase of *M. formicicum* was purified to homogeneity from cell lysate by chromatography on DEAE-cellulose, Phenyl Sepharose CL-4B (Pharmacia, Inc.), and Mono Q HR 10/10 ion exchange resin (Fast Protein Liquid Chromatography System, Pharmacia, Inc.), followed by preparative native polyacrylamide gel electrophoresis (S.F. Baron and J.G. Ferry, manuscript in preparation). The brown band of hydrogenase (R_m=0.4) from the preparative gel was excised, macerated by passage through a 23 gauge needle, and emulsified with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant. Samples (about 25 µg protein) were injected subcutaneously into 4 kg New Zealand White rabbits every 2 weeks for 8 weeks. Blood was collected 2 weeks after the last injection, chilled to 4°C, centrifuged at 5,000 x *g* for 15 min, filtered through a 0.2 µm Acrodisc filter (Gelman Instrument Co.), and stored at -20°C. Control serum was obtained from an unimmunized rabbit.

Western blot analysis was used to determine the specificity of the anti-formate dehydrogenase and anti-F_{420}-hydrogenase antisera. Samples were electrophoresed on SDS polyacrylamide gels according to the method of Laemmli (18). The proteins were electrophoretically transferred to nitrocellulose paper (Bio-Rad Laboratories, Richmond, CA) as described (35). The blot was incubated with 0.5% casein and 0.5% gelatin prepared in PBST (10 mM potassium phosphate [pH 7.0], 100 mM NaCl, 0.1% [v/v] Triton X-100). It was then incubated with antiserum or control serum appropriately diluted in PBST containing 0.1% casein and 0.1% gelatin, washed with PBST, incubated with ^{125}I-labeled goat-anti-rabbit IgG conjugate (0.6 µCi) (New England Nuclear), washed with PBST, and autoradiographed on Kodak SB 5 diagnostic film.

Immunogold labeling. The procedure used was a modification of the method of Bendayan (8). Bacteria from mid-log phase tube cultures (A_{550}=0.15) were centrifuged, and the culture medium was replaced with a mixture of 0.1 M cacodylate buffer (pH 7.2), 0.5% glutaraldehyde, and 3% formaldehyde, the latter freshly prepared from paraformaldehyde powder. Osmium tetroxide was not used as a secondary fixative for these cells; this somewhat compromises ultrastructural preservation, particularly of membranes, but enhances the likelihood of immunoreactivity

(3). The samples were then dehydrated in a series of methanols, embedded in Lowicryl K4M, and polymerized under indirect ultraviolet radiation at -40° C for 24 h and under direct ultraviolet light at room temperature for 24 h, as described (3). Ultrathin sections were picked up on Formvar-coated nickel grids. Grids containing sections were preadsorbed on 1% bovine serum albumin, incubated for one h on the primary antiserum or control serum diluted 1:100 in phosphate-buffered saline (PBS, pH 7.36: 15 mM sodium phosphate and 135 mM sodium chloride) containing 0.1% Tween 20. They were then washed in PBS + Tween 20, incubated for 1 h on protein A-gold (15 nm average diameter) diluted 1:10 in PBS, washed in PBS, and finally washed in deionized water. As controls, the thin sections were incubated with protein A-gold alone or control serum instead of antiserum. After poststaining with 5% uranyl acetate and lead citrate, the grids were viewed and photographed on a Philips EM-301 electron microscope at 60 KV.

Gold particles appearing in the electron micrographs were counted to determine the percentage located at the cell membrane. Particles located within 15 nm (the average diameter of the gold particles) of the general outline of the membrane were considered to be located at the membrane, while those more than 15 nm away were considered to be

located in the cytoplasm.

Osmotic shock experiments. The procedure used was a modification of the method of Bell et al. (7). Cells were centrifuged in a stoppered, pressurized (82 KPa N_2) polycarbonate screw cap centrifuge tube, which was kept anaerobic during transfers by flushing with a stream of N_2. Cell pellets were resuspended by vortexing. Cell paste (2.0 g) from a late log phase culture (A_{550}=0.9) was resuspended to a final volume of 8 ml with buffer (50 mM tris-HCl, pH 7.5; 0.5 M sucrose; 10 mM NaN_3; 2 mM 2-mercaptoethanol) and centrifuged (10,000 x *g*, 20 min, 4°C). The cells were washed two more times in this manner, and the cell pellet from the last wash was resuspended to 8 ml with buffer ('washed cell suspension'). A 0.8 ml sample of the washed cell suspension was combined with 7.2 ml of buffer containing 0.1 mM EDTA and then centrifuged (10,000 x *g*, 20 min, 4°C). The supernatant solution was removed, and the cell pellet was rapidly resuspended in 8 ml of ice cold 0.5 mM $MgCl_2$ containing 10 mM NaN_3 and 2 mM 2-mercaptoethanol. The suspension was centrifuged (10,000 x *g*, 20 min, 4°C), and the supernatant solution ('shock fluid') was removed and immediately assayed. The cell pellet was resuspended in 8 ml of buffer without sucrose and anaerobically passed through a French pressure cell (138 MPa), yielding a 'shocked cell lysate'. For

comparison, a lysate of the washed cell suspension was also prepared ('untreated cell lysate'). A sample of the washed cell suspension was diluted 1:10 with buffer and assayed directly for formate dehydrogenase activities to determine activities in whole cells. The sample was then flushed with H_2, incubated at 35°C for 15 min, and assayed for hydrogenase activity.

Enzyme assays. Formate dehydrogenase (29) and hydrogenase (25) were assayed spectrophotometrically as described. Hydrogenase samples were reductively reactivated before assay as described (6). A unit of activity was the reduction of 1 μmol of F_{420} or MV per min at 35°C and pH 7.5. Protein was estimated using the dye-binding assay of Bradford (10) with bovine serum albumin as the standard.

Hydropathy analysis. The amino acid sequences of the α and β subunits of the formate dehydrogenase of *M. formicicum* were deduced from the DNA sequences of the *fdhA* and *fdhB* genes (33). Hydropathy plots were generated from the amino acid sequences using the method of Kyte and Doolittle (17) and the Pustell DNA/protein sequence analysis program (International Biotechnologies, Inc.).

RESULTS

Specificity of the antisera. The formate dehydrogenase of *Methanobacterium formicicum* has two subunits: α and β, of M_r 85,000 and 53,000, respectively (31). Anti-formate dehydrogenase antiserum reacted specifically with the two subunits of the enzyme in samples of cell extract (Fig. 1). The F_{420}-hydrogenase purified from *M. formicicum* contains three subunits: α, β, and γ, of M_r 44,000, 37,000, and 29,000, respectively (S.F. Baron and J.G. Ferry, manuscript in preparation), similar to the enzyme purified from *Methanobacterium thermoautotrophicum* (11). Western blot analysis revealed that anti-F_{420}-hydrogenase antiserum was specific for the three subunits of the enzyme in samples of cell lysate (Fig. 1). Control serum contained no antibodies against *M. formicicum* cell extract proteins (Fig. 1).

Immunogold labeling. Thin sections of *M. formicicum* whole cells incubated with anti-formate dehydrogenase antiserum were marked with gold particles in the vicinity of the cell membrane (Fig. 2). A survey of 38 cells from several electron micrographs showed that an average 78% of the gold label was located in the immediate vicinity of the cell membrane and only 22% in the cytoplasm. Thin sections incubated with control serum (Fig. 2) or protein A-gold alone (data not shown) exhibited little or no labeling.

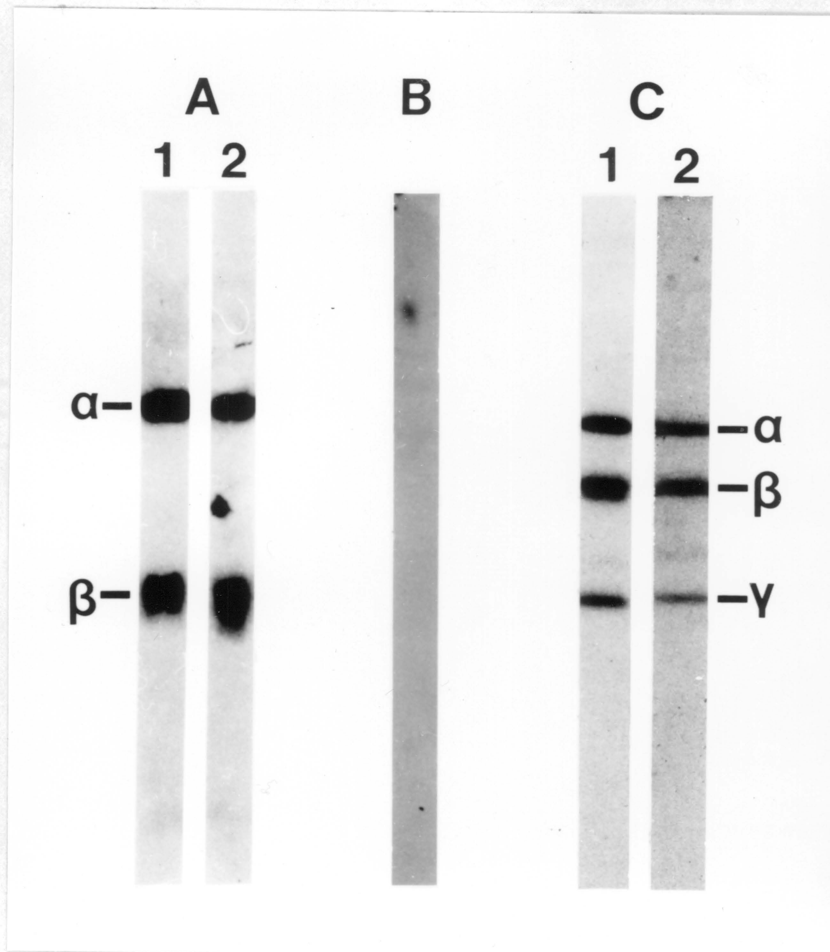

Figure 1. Western blot analysis of the formate dehydrogenase and F_{420}-hydrogenase of Methanobacterium formicicum. (**A**) Purified formate dehydrogenase, 1 µg (**1**) or cell extract, 10 µg protein (**2**) treated with anti-formate dehydrogenase antiserum (1:7500 dilution). A 10% gel was used. α and β refer to the M_r=83,000 and 53,000 subunits, respectively. (**B**) Cell extract (100 µg protein) treated with control serum (1:200 dilution). A 12% gel was used. (**C**) Purified F_{420}-hydrogenase, 1 µg (**1**) or cell lysate, 40 µg protein (**2**) treated with anti-F_{420}-hydrogenase antiserum (1:500 dilution). A 12% gel was used. α, β, and γ refer to the M_r=44,000, 37,000, and 29,000 subunits, respectively.

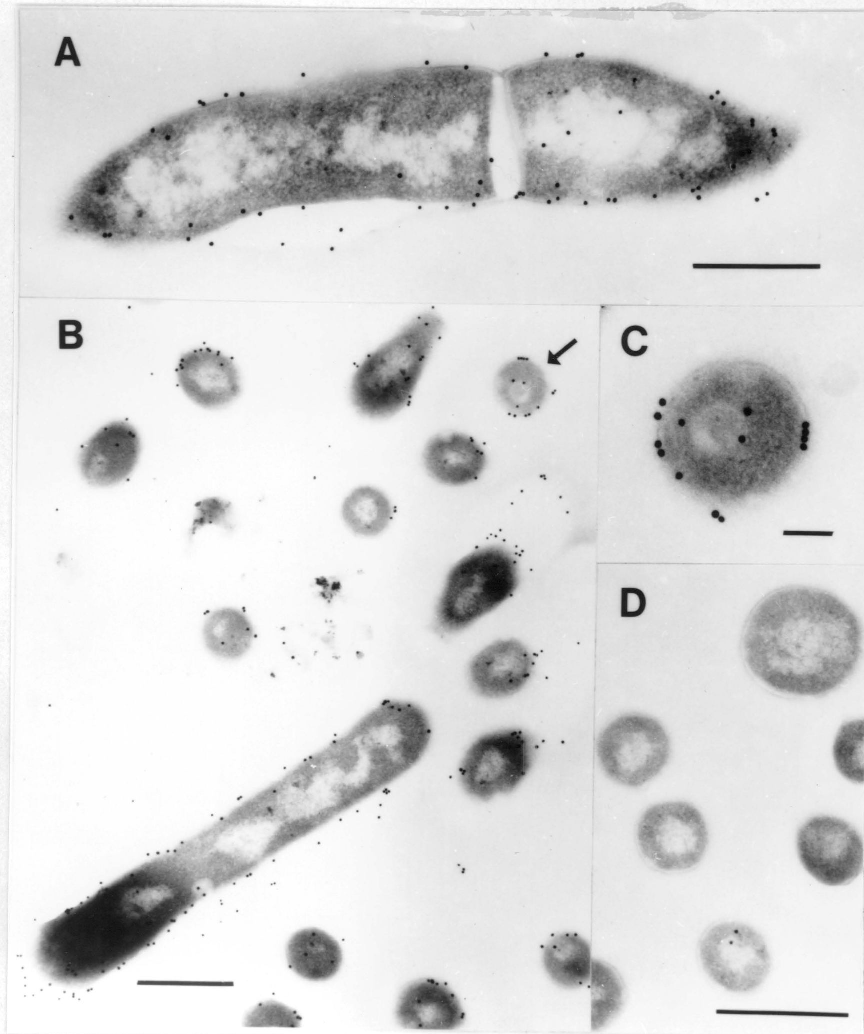

Figure 2. Immunogold labeling of thin sections of <u>M</u>. <u>formicicum</u> whole cells with anti-formate dehydrogenase antiserum. (**A**) Longitudinal section of a labeled cell; Bar = 0.5 µm. (**B**) Longitudinal and cross sections of several labeled cells; Bar = 0.5 µm. (**C**) Enlarged photograph of the cell marked with an arrow in panel (**B**), showing details of the cell membrane; Bar = 0.1 µm. (**D**) Cells incubated with control serum instead of antibody; Bar = 0.5 µm.

Thin sections of cells treated with anti-F_{420}-hydrogenase antiserum were also marked by gold particles at the cell membrane (Fig. 3). A survey of 41 cells revealed that 78.4% of the label was distributed in the immediate vicinity of the membrane and only 21.6% in the cytoplasm. These results indicate that the formate dehydrogenase and F_{420}-hydrogenase are membrane-associated in whole cells.

Osmotic shock experiments. Whole cells had minimal F_{420}-dependent formate dehydrogenase activity but comparatively more MV-dependent formate dehydrogenase activity. The shock fluid from osmotically treated cells contained negligible F_{420}-dependent and MV-dependent formate dehydrogenase activity, while lysate from these cells had formate dehydrogenase activities comparable to that of cell extract from untreated cells (Table 1).

Whole cells had low F_{420}-dependent hydrogenase activity but significant MV-dependent hydrogenase activity. The shock fluid from osmotically treated cells contained negligible F_{420}-dependent or MV-dependent hydrogenase activity as compared to the lysate of the shocked cells (Table 1). The hydrogenase activities of the shocked cell lysate were 29-45% lower than that of the untreated cell lysate, possibly due to the 10-fold dilution of the cells used to prepare the former. These results suggest that the formate dehydrogenase and F_{420}-hydrogenase of *M.*

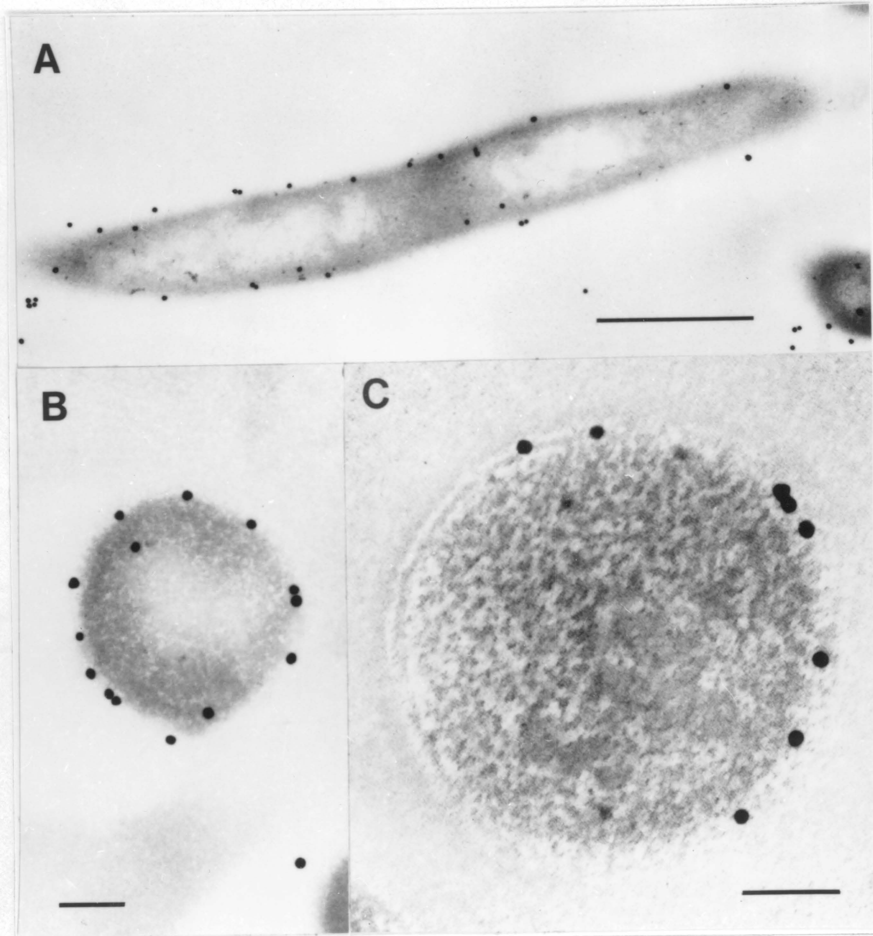

Figure 3. Immunogold labeling of thin sections of M. formicicum whole cells with anti-F_{420}-hydrogenase antiserum. (**A**) Longitudinal section of a labeled cell; Bar = 0.5 µm. (**B**) Cross section of a labeled cell; Bar = 0.5 µm. (**C**) cross section of a labeled cell showing details of the cell membrane; Bar = 0.1 µm.

Table 1. Distribution of formate dehydrogenase and hydrogenase activities after osmotic shocking of *M. formicicum* whole cells.

Fraction[a]	Formate dehydrogenase activities[b]				Hydrogenase activities[2]			
	F_{420}-dependent		MV-dependent		F_{420}-dependent		MV-dependent	
	Total units	%[c]	Total units	%[c]	Total units	%[c]	Total units	%[c]
Whole cells	0.097	1.7	3.92	17.2	0.178	6.1	150	46.3
Shock fluid	0.022	0.4	0.22	1.0	0.014	0.5	0.8	0.4
Shocked cell lysate	5.71	99.6	22.6	99.0	2.89	99.5	323	99.7
Untreated cell lysate	5.93	103	34.0	149	3.76	129	469	145

[a]The total protein contents of the shock fluid, shocked cell lysate, and untreated cell lysate were 0.08, 7.81, and 7.56 mg, respectively. Protein content of the whole cells was not determined.

[b]Total protein and activities were calculated on the basis of an 8 ml volume; those for the untreated cell lysate were calculated on the basis of a 0.8 ml volume.

[c]Percent of the total activity of shock fluid plus shocked cell lysate.

formicicum are not soluble periplasmic proteins.

Hydropathy analysis of the formate dehydrogenase. The deduced amino acid sequences of the *fdhA* and *fdhB* genes encoding the α and β subunits of the formate dehydrogenase (33) were analyzed according to Kyte and Doolittle (17). Regions of ≥19 amino acid residues with an average hydropathic index >1.6, and not interrupted by hydrophilic residues, were considered membrane spanning domains based on data from known membrane-bound proteins (17). A hydropathy plot of the α subunit (Fig. 4) showed no membrane spanning regions and an approximately equal distribution of hydrophobic and hydrophilic segments. Residues 24-49 of the β subunit had an average hydropathic index of 1.5; however, this region was interrupted by several hydrophilic residues (Fig. 4). The remainder of the β subunit had an approximately equal distribution of hydrophobic and hydrophilic segments. The average hydropathic index of the α subunit was -0.545 and that of the β subunit, -0.152.

DISCUSSION

The results show that the formate dehydrogenase of *Methanobacterium formicicum* was associated with the cytoplasmic membrane in whole cells, although the enzyme is present in the soluble fraction of cell extract prepared by

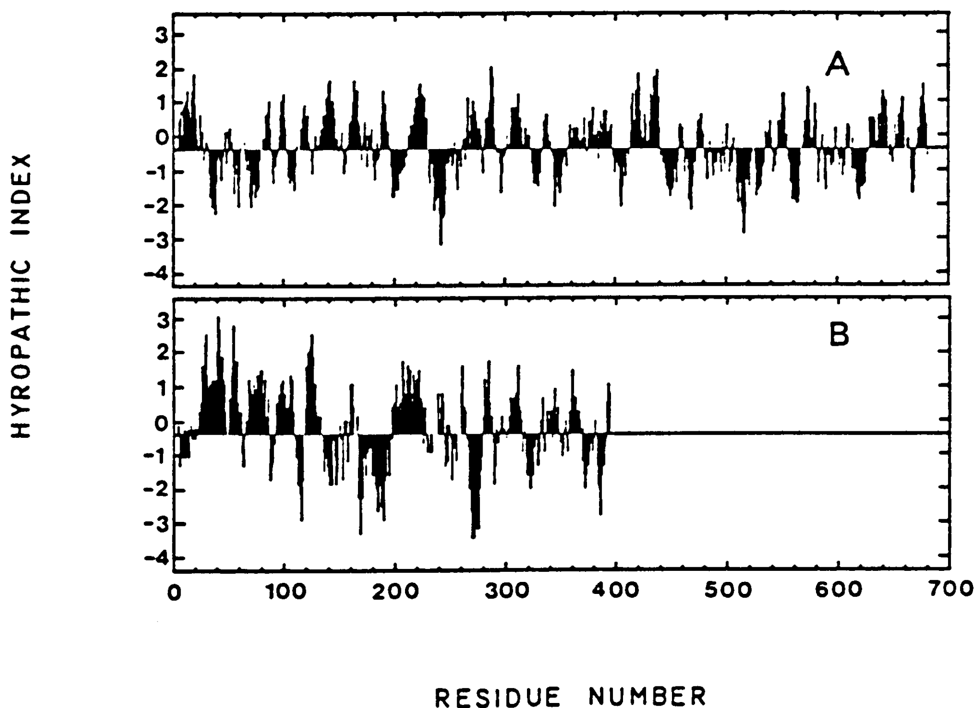

Figure 4. Hydropathy plots of the deduced amino acid sequences of the α and β subunits of the formate dehydrogenase of *M. formicicum*. (A) α subunit; (B) β subunit. A positive hydropathic index indicates hydrophobicity and a negative index, hydrophilicity.

mechanical cell disruption (28). These results, combined with the hydropathy analyses reported here, suggest that the formate dehydrogenase of *M. formicicum* is peripherally associated with the cytoplasmic membrane and is easily dislodged by physical methods of cell disruption. Cytochemical staining of whole cells reveals that the formate dehydrogenase of *M. formicicum* is located within internal membrane vesicles (S. F. Baron, N. L. Schauer, and J. G. Ferry (1985) Abstr Ann Meet Am Soc Microbiol I19 p. 149); however, as reported for *Methanobacterium thermoautotrophicum* (2), internal membrane vesicles may be artifacts of electron microscopic fixation procedures.

The results of this immunocytochemical study were consistent with the previous report that the F_{420}-hydrogenase of *M. formicicum* is membrane-associated after subcellular fractionation of cell extract (6). The enzyme is apparently not intrinsic or tightly bound to the cell membrane, since 40% of the F_{420}-dependent hydrogenase activity in *M. formicicum* cell extracts appears in the soluble fraction after subcellular fractionation (6). Although operationally soluble, the F_{420}-hydrogenase of *Methanococcus voltae* has recently been shown to be located at the cell membrane by the immunogold labelling technique (22). The hydrophobicity of the F_{420}-hydrogenases of *M. formicicum* (6) and other methanogens (11,23,34,36)

suggests that the enzyme associates with the membrane through hydrophobic interactions. Although the results presented here suggest that the F_{420}-hydrogenase and formate dehydrogenase are not soluble periplasmic proteins, no conclusions can be drawn regarding the membrane sidedness of these enzymes.

The cell membrane of Escherichia coli is impermeable to oxidized or reduced MV (16). Assuming the same for M. formicicum, the observed MV-dependent hydrogenase activity of whole cells (Table 1) could be explained in two ways: i) a membrane-bound electron transport chain could mediate electron flow from hydrogenase on the inside to MV on the outside of the membrane. ii) a hydrogenase with high MV-dependent activity but low F_{420}-dependent activity could be tightly bound to the outer side of the cell membrane. The specific MV-dependent activity of the MV-hydrogenase of M. formicicum is at least 100-fold greater than that of the F_{420}-hydrogenase (S.F. Baron, unpublished data). However, the MV-hydrogenase is probably not tightly bound to the membrane, since it remains in the soluble fraction of cell extract after sucrose gradient ultracentrifugation (6).

The H_2-coupled methyl coenzyme M reductase system of M. thermoautotrophicum has been resolved into 4 protein components: A1, A2, A3, and C; and 4 soluble factors: component B, FAD, Mg^{+2} and ATP (24). Component C is

thought to catalyze the reduction of methyl coenzyme M to methane; component A1 has F_{420}-dependent hydrogenase activity. Blaut and Gottschalk (9) demonstrated that methanogenesis from methanol plus H_2 in *Methanosarcina barkeri* is coupled to ATP synthesis and proposed that one or more components of the methyl coenzyme M reductase system is involved in proton translocation across the membrane. Methyl coenzyme M reductase activity in *M. thermoautotrophicum* appears in the supernatant fraction after ultracentrifugation of cell extract (12), suggesting that some or all of the methylreductase components are soluble. However, the results of immunogold labeling experiments indicate that component C in *M. thermoautotrophicum* (1) and *M. voltae* (26) is located at the cell membrane. A membrane-bound ATPase has been demonstrated in methanogenic bacterium strain Göl by immunogold labeling with antibodies against the β subunit of the F_0F_1-ATPase of *E. coli* (21). Thus, three enzymes of electron transfer in methanogens (formate dehydrogenase, F_{420}-hydrogenase, and component C of the methylreductase system) have been shown to be located at the cell membrane. These observations are consistent with the hypothesis that methanogenesis from various electron donors (e.g., formate or H_2) is coupled to ATP synthesis by formation of a proton gradient across the membrane.

M. formicicum has a formate hydrogenlyase system composed of the formate dehydrogenase, the F_{420}-hydrogenase, and F_{420} (S.F. Baron and J.G. Ferry, manuscript in preparation). It is interesting that the component enzymes of this system are both membrane-associated, but the physiological significance of their location requires further study.

ACKNOWLEDGEMENTS

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LITERATURE CITED

1. Aldrich, H. C., D. B. Beinborm, M. Bokranz, and P. Schönheit. 1987. Immunocytochemical localization of methyl-coenzyme M reductase in *Methanobacterium formicicum*. Arch. Microbiol. **147**:190-194.

2. Aldrich, H. C., D. B. Beimborn, and P. Schönheit. 1987. Creation of artifactual internal membranes during fixation of *Methanobacterium thermoautotrophicum*. Can. J. Microbiol. **33**:844-849.

3. Armbruster, B. L., and E. Kellenberger. 1986. Low temperature embedding, **In**: Aldrich HC, Todd WJ (eds), Ultrastructure techniques for microorganisms. Plenum Press, New York and London, pp 267-295.

4. Barber M. J., H. D. May, and J. G. Ferry. 1986. Inactivation of formate dehydrogenase from *Methanobacterium formicicum* by cyanide. Biochemistry. **25**:8150-8155.

5. Barber, M. J., L. M. Siegel, N. L. Schauer, H. D. May, and J. G. Ferry. 1983. Formate dehydrogenase from Methanobacterium formicicum. Electron paramagnetic resonance spectroscopy of the molybdenum and iron-sulfur centers. J. Biol. Chem. **258**:10839-10845.

6. Baron, S. F., D. P. Brown, and J. G. Ferry. 1987. Locations of the hydrogenases of Methanobacterium formicicum after subcellular fractionation of cell extract. J. Bacteriol. **169**:3823-3825.

7. Bell, G. R., J. LeGall, and H. D. Peck, Jr. 1974. Evidence for the periplasmic location of hydrogenase in Desulfovibrio gigas. J. Bacteriol. **120**:994-997.

8. Bendayan, M. 1984. Enzyme-gold electron microscopic cytochemistry, a new affinity approach for the ultrastructural localization of macromolecules. J. Electr. Microsc. Tech. **1**:349-372.

9. Blaut, M., and G. Gottschalk. 1985. Evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria. TIBS **10**:486-489.

10. Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

11. Fox, J. A., D. J. Livingston, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 1. Purification and characterization. Biochemistry. **26**:4219-4227.

12. Gunsalus, R. P., and R. S. Wolfe. 1978. ATP activation and properties of the methyl coenzyme M reductase system in Methanobacterium thermoautotrophicum. J. Bacteriol. **135**:851-857.

13. Hartzell, P. L., G. Zvilius, J. C. Escalante-Semerena, and M. I. Donnelly. 1985. Coenzyme F_{420} dependence of the methylenetetrahydromethanopterin dehydrogenase of Methanobacterium thermoautotrophicum. Biochem. Biophys. Res. Commun. **133**:884-890.

14. Jacobson, F. S., L. Daniels, J. A. Fox, C. T. Walsh, and W. H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazaflavin- reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. **257**:3385-3388.

15. Jin, S. -L. C., D. K. Blanchard, and J. S. Chen. 1983. Two hydrogenases with distinct electron-carrier specificity and subunit composition in Methanobacterium formicicum. Biochim. Biophys. Acta. **748**:8-20.

16. Jones, R. W., T. A. Gray, and P. B. Garland. 1976. A study of the permeability of the cytoplasmic membrane of Escherichia coli to reduced and oxidized benzyl viologen and methyl viologen cations: complications in the use of viologens as redox mediators for membrane-bound enzymes. Biochem. Soc. Trans. **4**:671-673.

17. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105-132.

18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

19. May, H. D., P. S. Patel, and J. G. Ferry. 1988. Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in Methanobacterium formicicum. J. Bacteriol. **170**:3384-3389.

20. May, H. D., N. L. Schauer, and J. G. Ferry. 1986. Molybdopterin cofactor from Methanobacterium formicicum formate dehydrogenase. J. Bacteriol. **166**:500-504.

21. Mayer, F., A. Jussofie, M. Salzmann, M. Lubben, M. Rohde, and G. Gottschalk. 1987. Immunoelectron microscopic demonstration of ATPase on the cytoplasmic membrane of the methanogenic bacterium strain Göl. J. Bacteriol. **169**:2307-2309.

22. Muth, E. 1988. Localization of the F_{420}-reducing hydrogenase in Methanococcus voltae by immuno-gold technique. Arch. Microbiol. **150**:205-207

23. Muth, E., E. Mörschel, and A. Klein. 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium *Methanococcus voltae*. Eur. J. Biochem. **169**:571-577.

24. Nagle, Jr. D. P., and R. S. Wolfe. 1983. Component A of the methyl coenzyme M methylreductase system of *Methanobacterium*: resolution into four components. Proc. Natl. Acad. Sci. USA **80**:2151-2155.

25. Nelson, M. J. K., D. P. Brown, and J. G. Ferry. 1984. FAD requirement for the reduction of coenzyme F420 by hydrogenase from *Methanobacterium formicicum*. Biochem. Biophys. Res. Commun. **120**:775-781.

26. Ossmer, R., T. Mund, P. Hartzell, U. Konheiser, G. W. Kohring, A. Klein, R. S. Wolfe, G. Gottschalk, and F. Mayer. 1986. Immunocytochemical localization of component C of the methylreductase system in *Methanococcus voltae* and *Methanobacterium thermoautotrophicum*. Proc. Natl. Acad. Sci. USA **83**:5789-5792.

27. Patel, P. S., and J. G. Ferry. 1988. Characterization of the upstream region of the formate dehydrogenase operon of *Methanobacterium formicicum*. J. Bacteriol. **170**:3390-3395.

28. Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. **142**:800-807.

29. Schauer, N. L., and J. G. Ferry. 1982. Properties of formate dehydrogenase in *Methanobacterium formicicum*. J. Bacteriol. **150**:1-7 (Erratum **151**:1642).

30. Schauer, N. L., and J. G. Ferry. 1983. FAD requirement for the reduction of coenzyme F_{420} by formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. **155**:467-472.

31. Schauer, N. L., and J. G. Ferry. 1986. Composition of the coenzyme F_{420}-dependent formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. **165**:405-411.

32. Schauer, N. L., J. G. Ferry, J. G. Honek, W. H. Orme-Johnson, and C. T. Walsh. 1986. Mechanistic studies of the coenzyme F_{420}-reducing formate dehydrogenase from Methanobacterium formicicum. Biochemistry. **25**:7163-7168.

33. Shuber, A. P., E. C. Orr, M. A. Recny, P. F. Schendel, H. D. May, N. L. Schauer, and J. G. Ferry. 1986. Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from Methanobacterium formicicum. J. Biol. Chem. **261**:12942-12947.

34. Sprott, G. D., K. M. Shaw, and T. J. Beveridge. 1987. Properties of the particulate enzyme F_{420}-reducing hydrogenase isolated from Methanospirillum hungatei. Can. J. Microbiol. **33**:896-904.

35. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.

36. Yamazaki, S. 1982. A selenium-containing hydrogenase from Methanococcus vannielii. J. Biol. Chem. **257**:7926-7929.

SECTION VII. GENERAL SUMMARY

Properties and functions of the F_{420}-hydrogenase

F_{420}-hydrogenases from six H_2-oxidizing methanogenic bacteria have now been purified, and their properties are summarized and compared in Table 1. All form large aggregates, ranging from 720K-1300K in molecular weight. The enzymes from *Methanobacterium formicicum* (SECTION III), *Methanobacterium thermoautotrophicum* (49), and *Methanococcus vannielii* (95) also exhibit a smaller, F_{420}-active species of molecular weight approximately 110K. All F_{420}-hydrogenases have at least two subunits, except that of *Methanosarcina barkeri*, which has only a 60K subunit (42). Those examined contain nickel, iron, and acid-labile sulfide, the latter two presumably in 4Fe-4S clusters (42,81). The enzymes of *Methanococcus voltae* (95) and *Methanococcus vannielii* (150) also contain selenium.

FAD is present in the F_{420}-hydrogenases of *M. thermoautotrophicum* (49), *M. formicicum* (SECTION III,97), and *M. voltae* (95); the enzyme from *M. barkeri* contains FMN or riboflavin (42). Similarly, the NAD^+-reducing hydrogenase of *Alcaligenes eutrophus* (119) and NAD^+-reducing formate dehydrogenase of *Pseudomonas oxalaticus* (93) contain FMN, and the F_{420}-reducing formate

Table 1. Properties of F_{420}-hydrogenases purified from methanogenic bacteria.

	Methanobacterium		*Methanococcus*		*Methanospirillum hungatei*	*Methanosarcina barkeri*
	thermoautotrophicum	*formicicum*	*voltae*	*vannielii*		
Location	Particulate	Membrane	Membrane		Particulate	Soluble
Mol. Wt. Complex[a]	800	1,020	745	1,300	720	800
Monomer[a]	115	109	110	340		
Subunits[b]	1x47 1x31 1x26	1x44 1x37 1x29	55 45 37 27	56 42 35 27	5x51 15x31	60
Cofactors/mol[c]						
Ni	1	1	1	2	6-7	1
Se		0	1	4		
Fe	13-14	12-14	5			8-10
S^{-2}		11				present
Flavin	1FAD	1FAD	1FAD			1FMN or Riboflavin
Fe/S cluster	[4Fe-4S]					[4Fe-4S]
Isoelectric point	5.7	5.6				
Specific activity[d]	49.0	52.7	10.0		0.85	
pH optimum	6.5-7.5	7.5	7.0			
H_2 evolution[e]	FOH_2	$F_{420}H_2$				MV_r
Reference	49,81,82,141	this work	94,95	150	126	42

[a]Molecular weight in thousands.
[b]Number of subunits in monomer or complex (if available) x molecular weight in thousands.
[c]All are based on the monomer molecular weight, except that of *M. barkeri*, which is based on the 60K subunit.
[d]Maximum H_2 uptake activity reported (µmoles F_{420} reduced/min/mg protein).
[e]With the indicated electron donor. MV_r=reduced methyl viologen.

dehydrogenase of *M. formicicum* (115) contains FAD. Since flavins can be reduced in two single electron steps, they are proposed to transfer electrons from iron sulfur clusters (obligate one electron carriers) to the obligate two electron (hydride ion) acceptors, F_{420} and NAD^+ (66,142). Indeed, FAD is required for F_{420}-reducing activity of the F_{420}-hydrogenase (97) and F_{420}-reducing formate dehydrogenase (114,115) of *M. formicicum*. FAD dissociates from these enzymes during hydrophobic interaction chromatography under reduced conditions, yielding apoenzymes which reduce methyl viologen but not F_{420} (97,114). FAD also dissociates from the formate dehydrogenase when the enzyme is reduced with formate or dithionite (115). The results in SECTION III show a similar dissociation of FAD from the F_{420}-hydrogenase when it was reduced with its substrates, H_2 and F_{420}. F_{420}-reducing activity could be stabilized by addition of potassium salts or exogenous FAD. These results suggest that when the F_{420}-hydrogenase is reduced, the FAD binding site becomes exposed, allowing FAD to dissociate. Perhaps potassium salts keep the enzyme in the proper conformation to cover the FAD site. The ability of various salts to stabilize F_{420}-dependent activity was directly proportional to the atomic or molecular radius of the cation used, with potassium and ammonium salts being the most effective. The

inability of $FADH_2$ to stabilize F_{420}-dependent activity suggests that the F_{420}-hydrogenase has a lower affinity for $FADH_2$ than FAD. In fact, FAD is a planar molecule, while $FADH_2$ has a 'puckered' conformation (142). Binding of FAD to the F_{420}-hydrogenase may require the adenosine side chain, since FMN and riboflavin were considerably less effective than FAD in stabilizing F_{420}-dependent activity during reactivation.

As discussed in SECTION III, the F_{420}-hydrogenase of M. formicicum catalyzed F_{420}-dependent H_2 production at a rate about 3-fold less than that for F_{420}-dependent H_2 uptake. The F_{420}-hydrogenase of M. thermoautotrophicum also catalyzes H_2 production from reduced FO (the riboflavin analogue of F_{420}) (82), and that of Methanosarcina barkeri from reduced methyl viologen (42). Thus, F_{420}-hydrogenases appear to be bidirectional. For comparison, the bidirectional hydrogenases of the obligately anaerobic bacteria Clostridium pasteurianum, Megasphaera elsdenii, and Desulfovibrio vulgaris have ratios of H_2 production (from reduced methyl viologen) to H_2 uptake (with methylene blue or benzyl viologen) of 0.23, 0.78, and 0.21 (2). In contrast, the uptake hydrogenases of C. pasteurianum (24) and Azotobacter vinelandii (123) have H_2 production/H_2 uptake ratios (reduced methyl viologen/methylene blue) of 0.0003 and 0.021, respectively.

Unlike other bidirectional hydrogenases, which contain only iron-sulfur clusters (2), F_{420}-hydrogenases also contain nickel and sometimes selenium (refer to Table 1).

F_{420}-hydrogenases are generally assumed to function primarily as H_2 uptake enzymes to provide electrons for the reduction of CO_2 to methane. The partially purified component A1 of the H_2-driven methylreductase system of M. thermoautotrophicum has F_{420}-dependent hydrogenase activity, and reduced F_{420} can serve as electron donor for this system (96), although an absolute requirement for either has not yet been demonstrated. The bidirectionality of the M. formicicum F_{420}-hydrogenase and its involvement in the formate hydrogenlyase system (SECTION IV) suggest an additional role for the enzyme in H_2 production. Although its electron acceptor is unknown, the MV-hydrogenase of M. formicicum reduces methyl viologen at rates up to 100-fold greater than the F_{420}-hydrogenase in cell extract (see SECTION V). Moreover, the MV-hydrogenase could not support formate hydrogenlyase activity in F_{420}-hydrogenase-depleted cell extract (SECTION IV). These observations suggest a role for this enzyme primarily in H_2 uptake. Other organisms also have both uptake and bidirectional hydrogenases. For example, the N_2-fixing bacterium, C. pasteurianum, has a ferredoxin-dependent bidirectional hydrogenase (25) and a ferredoxin-

reducing uptake hydrogenase (24). The former is involved in H_2 production during fermentation, while the latter may supply reduced ferredoxin for N_2 fixation (24). *Escherichia coli* (111) and *Salmonella typhimurium* (112) have three membrane-bound hydrogenase isoenzymes (1, 2, and 3). Isoenzyme 1 functions in H_2 uptake during fermentative growth, isoenzyme 2 in respiratory H_2 uptake, and isoenzyme 3 in H_2 production *via* the formate hydrogenlyase system.

Properties and functions of the formate hydrogenlyase system

The reconstitution of formatehydrogenlyase described in SECTION IV represents the first time that this system from any organism has been fully defined. The *M. formicicum* system consisted of only two membrane-associated protein components: F_{420}-hydrogenase and formate dehydrogenase; a soluble component: F_{420}; and FAD, required for F_{420}-dependent activity of the component enzymes. This system is apparently simpler than others which have been described. For example, the formate hydrogenlyase present in cell extracts of the H_2-producing acetogenic 'S organism' consists of NAD^+-dependent formate dehydrogenase, NADH:ferredoxin oxidoreductase, ferredoxin, and ferredoxin-dependent hydrogenase (108). The formate hydrogenlyase of *D. vulgaris* is mediated by the low potential cytochrome c_3 and a cytochrome c_3 reductase (J. P. Williams, J. T.

Davidson, and H. D. Peck, Jr., Bacteriol. Proc., p. 110, 1964); low potential cytochromes may also be involved in the formate hydrogenlyase of *E. coli* (53).

Formate hydrogenlyase activity in methanogenic bacteria may just be due to the coexistence of formate dehydrogenase and hydrogenase linked by the same electron acceptor. On the other hand, the formate hydrogenlyase system may also serve a physiological function. Unlike fermentative bacteria, which can regulate the intracellular redox balance by means of branched fermentation pathways (135), methanogenic bacteria can only make methane as an end product. H_2 production *via* formate hydrogenlyase would provide an alternative route for disposal of electrons during growth on formate. In fact, *M. formicicum* produces small amounts of H_2 during growth on formate under optimal conditions, and large amounts when methanogenesis is impaired by high temperature (113). Because formate hydrogenlyase in methanogenic bacteria is reversible (SECTION IV,36,99), the organisms may similarly dispose of electrons as formate during growth on H_2/CO_2. The reverse formate hydrogenlyase could allow H_2-oxidizing methanogenic bacteria to fix CO_2 as formate for biosynthesis. Mutants of *M. thermoautotrophicum* have recently been isolated which require formate for growth on H_2/CO_2 (R. S. Tanner and D. P. Nagle, Abstr. Ann. Meet. Am. Soc. Microbiol., I-10, p.

182, 1988). Certain clostridia use formate generated by a ferredoxin-dependent CO_2 reductase for the biosynthesis of purines, pyrimidines, and methionine (134).

H_2 produced by the formate hydrogenlyase system could be an obligate intermediate in methanogenesis from formate, analogous to H_2-cycling during lactate-driven sulfate reduction in *Desulfovibrio* sp. (102; see SECTION II). As illustrated in Fig. 1, formate would be dissimilated to bicarbonate and H_2 by the F_{420}-mediated formate hydrogenlyase system at the internal side of the membrane. The H_2 produced would be reoxidized by an external hydrogenase and the electrons generated used to reduce bicarbonate to methane. The net uptake of protons in the cytoplasm and release in the periplasmic space would generate a protonmotive force for ATP synthesis.

Cellular locations of F_{420}-hydrogenase and formate dehydrogenase

As discussed in SECTION V, the F_{420}-hydrogenase of *M. formicicum* sedimented with membrane material after sucrose gradient fractionation of cell extract. This sedimentation could simply be due to the large size and particulate nature of the enzyme, especially if it were complexed with other proteins. However, the results of immunogold labelling experiments in SECTION VI clearly show that the

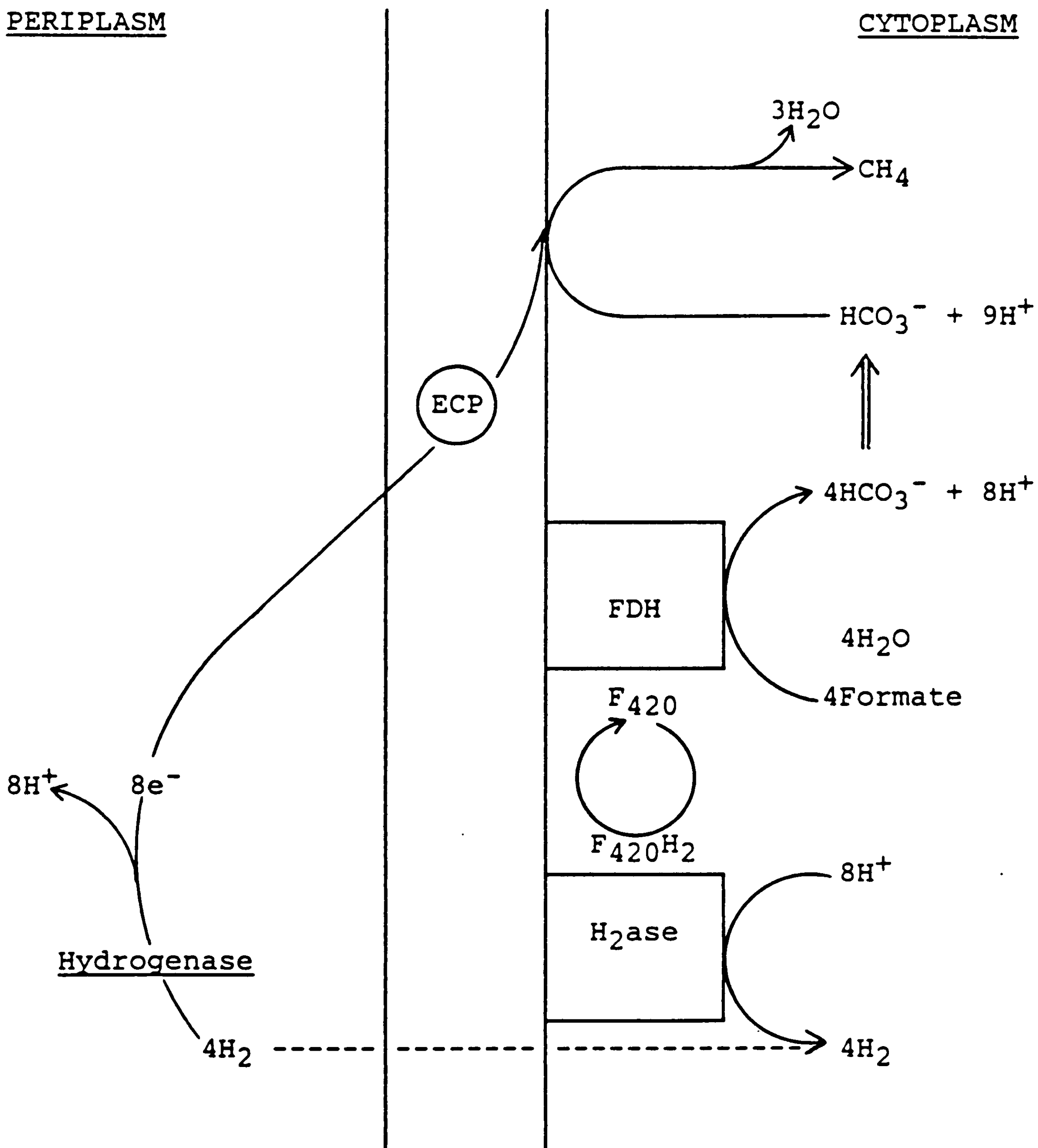

Figure 1. Hypothetical mechanism for H_2 cycling during methanogenesis from formate. FDH, formate dehydrogenase; H_2ase, F_{420}-hydrogenase; ECP, electron carrier protein(s).

F_{420}-hydrogenase is associated with the cytoplasmic membrane in whole cells. The F_{420}-hydrogenase of M. voltae is also located at the cytoplasmic membrane in whole cells (94) although the enzyme is soluble in cell extracts (95). McKellar and Sprott (90) reported a particulate hydrogenase in Methanobacterium strain G2R which reduces methyl viologen but not F_{420}. The membrane location of F_{420}-hydrogenase is consistent with the hypothesis that H_2-driven methanogenesis generates a protonmotive force, as discussed in SECTION II.

The circular structures present in electron micrographs of F_{420}-hydrogenases (SECTION III,95,126,141) are proposed to consist of two stacked rings, each containing four $\alpha\beta\gamma$ trimers (141), as illustrated in Fig. 2. Based on electron microscopic evidence, Mayer et al. (86) proposed that components of the methylreductase system of methanogen strain Göl are assembled in a large, membrane-bound sacculus called a methanoreductosome, as illustrated in Fig. 2. The sacculus consists of an incomplete, hollow sphere (R_c) forming the head of the structure, a ring shaped moiety (R_m) forming the neck, and a membrane attachment site (R_t). R_c contains several copies of component C of the methylreductase, while the identities of the other two components are unknown. However, R_c strikingly resembles the circular structures observed in

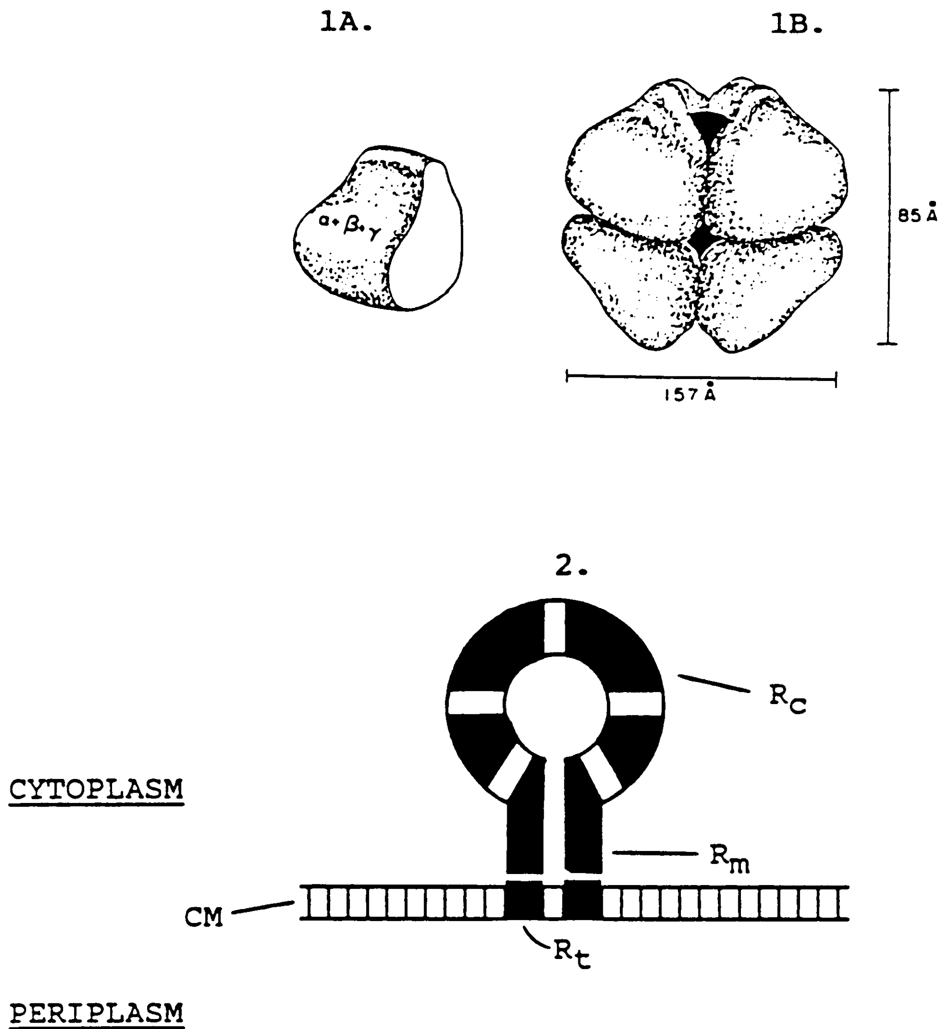

Figure 2. Proposed models for structures of F_{420}-hydrogenase (141) and the methanoreductosome complex (86). 1A, building block: αβγ trimer of F_{420}-hydrogenase; 1B, F_{420}-hydrogenase aggregate composed of 8 αβγ trimers arranged as two stacked rings. 2, Methanoreductosome structure. R_c, head assembly containing methylreductase component C; R_m, stem structure; R_t, membrane attachment protein; CM, cell membrane.

electron micrographs of F_{420}-hydrogenases. An association of F_{420}-hydrogenase and component C together at the cytoplasmic membrane might be expected if this hydrogenase were indeed part of the methylreductase system and involved in proton translocation.

The formate dehydrogenase of *M. formicicum* was shown to be membrane-associated in SECTION VI. The association is apparently loose, since the enzyme is soluble in cell extracts (113), and no potential membrane attachment sites were detected in the amino acid sequence of the enzyme (SECTION VI). The formate dehydrogenases from *Wolinella succinogenes* (74) and *Campylobacter sputorum* (98) are also membrane-associated. *E. coli* has two selenium-containing formate dehydrogenases (28). A membrane-bound species reduces phenazine methosulfate and is involved in nitrate respiration. A benzyl viologen-reducing species is soluble or loosely membrane-associated and is involved in the formate hydrogenlyase system.

SECTION VIII. LITERATURE CITED

(for Sections I, II, and VII)

1. Adams, M. W. W., S. -L. C. Jin, J. -S. Chen, and L. E. Mortenson. 1986. The redox properties and activation of the F_{420}-non-reactive hydrogenase of Methanobacterium formicicum. Biochim. Biophys. Acta. **869**:37-47.

2. Adams, M. W. W., M. K. Johnson, I. C. Zambrano, and L. E. Mortenson. 1986. On the novel H_2-activating iron-sulfur center of the "Fe-only" hydrogenases. Biochimie. **68**:35-41.

3. Adams, M. W. W., and L. E. Mortenson. 1984. The physical and catalytic properties of hydrogenase II of Clostridium pasteurianum. J. Biol. Chem. **259**:7045-7055.

4. Adams, M. W. W., L. E. Mortenson, and J. -S. Chen. 1981. Hydrogenase. Biochim. Biophys. Acta. **594**:105-176.

5. Albracht, S. P. J., E. G. Gradt, and R. K. Thauer. 1982. The ESR properties of nickel in hydrogenase from Methanobacterium thermoautotrophicum. FEBS Lett. **140**:311-313.

6. Albracht, S. P. J., J. W. van der Zwaan, and R. D. Fontijn. 1984. EPR spectrum at 4,9, and 35 GHz of hydrogenase from Chromatium vinosum. Direct evidence for spin-spin interaction between NiIII and the iron-sulphur cluster. Biochim. Biophys. Acta. **766**:245-258.

7. Aldrich, H. C., D. B. Beinborm, M. Bokranz, and P. Schönheit. 1987. Immunocytochemical localization of methyl-coenzyme M reductase in Methanobacterium formicicum. Arch. Microbiol. **147**:190-194.

8. Anthony, C. 1982. Methanogens and methanogenesis, p. 296-379. **In:** C. Anthony (ed.), The biochemistry of methylotrophs. Academic Press, London.

9. Aragno, M. and H. G. Schlegel. 1981. The hydrogen-oxidizing bacteria, p. 865-893. **In:** M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (eds.), The prokaryotes: a handbook on habitats, isolation and identification of bacteria. Vol. 1. Springer Verlag, New York.

10. Badziong, W., and R. K. Thauer. 1980. Vectorial electron transport in Desulfovibrio vulgaris (Marburg) growing on hydrogen plus sulfate as sole energy source. Arch. Microbiol. **125**:167-174.

11. Barker, H. A., S. Ruben, and M. D. Kamen. 1940. The reduction of radioactive carbon dioxide by methane-producing bacteria. Proc. Natl. Acad. Sci. U.S.A. **26**:426-430.

12. Bell, S. H., D. P. E. Dickinson, R. Rieder, R. Cammack, D. S. Patil, D. O. Hall, and K. K. Rao. 1984. Spectroscopic studies of the nature of the iron clusters in the soluble hydrogenase from Desulfovibrio desulfuricans (strain Norway 4). Eur. J. Biochem. **145**:645-651.

13. Berlier, Y., G. D. Fauque, J. LeGall, E. S. Choi, H. D. Peck, Jr., and P. A. Lespinat. 1987. Inhibition studies of three classes of Desulfovibrio hydrogenase: application to the further characterization of the multiple hydrogenases found in Desulfovibrio vulgaris Hildenborough. Biochem. Biophys. Res. Commun. **146**:147-153.

14. Bernhard, T., and G. Gottschalk. 1978. The hydrogenase of Escherichia coli, purification, some properties and the function of the enzyme, p. 199-208. **In:** H. G. Schlegel and K. Schneider (eds.), Hydrogenases: their catalytic activity, structure and function. Erich Goltze KG, Göttingen.

15. Blaut, M. and G. Gottschalk 1985. Evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria. T.I.B.S. **7**:354-357.

16. Bobik, T. A., K. D. Olson, K. M. Noll, and R. S. Wolfe. 1987. Evidence that the heterdisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate is a product of the methylreductase reaction in Methanobacterium. Biochem. Biophys. Res. Commun. **149**:455-460.

17. Boone, D. R., and M. P. Bryant. 1980. Propionate-degrading bacterium Syntrophobacter wolinii sp. nov., gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. **40**:626-632.

18. Bryant, M. P. 1979. Microbial methane production-theoretical aspects. J. Anim. Sci. **48**:193-201.

19. Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe. 1967. Methanobacillus omelianskii, a symbiotic association of two species of bacteria. Arch. Microbiol. **59**:20-31.

20. Cammack, R., V. M. Fernandez, and K. Schneider. 1986. Activation and active sites of nickel-containing hydrogenases. Biochimie. **68**:85-91.

21. Cammack, R., D. O. Hall, and K. K. Rao. 1985. Hydrogenases: structure and applications in hydrogen production, p. 75-102. **In:** R. K. Poole and C. Dow (eds.), Microbial gas metabolism: mechanistic, metabolic and biotechnological aspects. Academic Press, Inc., New York.

22. Cammack, R., D. S. Patil, R. Aguirre, and E. C. Hatchikian. 1982. Redox preperties of the ESR-detectable nickel in hydrogenase from Desulfovibrio gigas. FEBS Lett. **142**:289-292.

23. Cammack, R., K. K. Rao, J. Serra, and M. J. Llama. 1986. The redox properties of the iron-sulphur cluster in hydrogenase from Chromatium vinosum, strain D. Biochimie. **68**:93-96.

24. Chen, J. -S., and D. K. Blanchard. 1978. Purification and properties of the H_2-oxidizing (uptake) hydrogenase of the N_2-fixing anaerobe Clostridium pasteurianum W5. Biochem. Biophys. Res. Commun. **84**:1144-1150.

25. Chen, J. -S., and L. E. Mortenson. 1974. Purification and properties of hydrogenase from Clostridium pasteurianum W5. Biochim. Biophys. Acta. **371**:283-298.

26. Chen, M., and M. J. Wolin. 1977. Influence of CH_4 production by Methanobacterium ruminantium on the fermentation of glucose and lactate by Selenomonas ruminantium. Appl. Environ. Microbiol. **34**:756-759.

27. Chung, K. T. 1976. Inhibitory effects of H_2 on growth of Clostridium cellobioparum. Appl. Environ. Microbiol. **31**:342-348.

28. Cox, J. C., E. S. Edwards, and J. A. DeMoss. 1981. Resolution of distinct selenium-containing formate dehydrogenases from Escherichia coli. J. Bacteriol. **145**:1317-1324.

29. Czechowski, M. H., S. H. He, M. Nacro, D. V. DerVartanian, H. D. Peck, Jr., and J. LeGall. 1984. A cytoplasmic nickel-iron hydrogenase with high specific activity from Desulfovibrio multispirans sp. nov., a new species of sulfate reducing bacterium. Biochem. Biophys. Res. Commun. **125**:1025-1032.

30. Daniels, L., G. Fuchs, R. K. Thauer, and J. G. Zeikus. 1977. Carbon monoxide oxidation by methanogenic bacteria. J. Bacteriol. **132**:118-126.

31. Daniels, L., R. Sparling, and G. D. Sprott. 1984. The bioenergetics of methanogenesis. Biochim. Biophys. Acta. **768**:113-163.

32. Diekert, G., U. Könheiser, K. Piechulla, and R. K. Thauer. 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. J. Bacteriol. **148**:459-464.

33. Doddema, H. J., C. van der Drift, G. D. Vogels, and M. Veenhuis. 1979. Chemiosmotic coupling in Methanobacterium thermoautotrophicum: hydrogen-dependent adenosine 5'-triphosphate synthesis by subcellular particles. J. Bacteriol. **140**:1081-1089.

34. Donnelly, M. I., J. C. Escalante-Semerena, K. L. Rinehart, Jr., and R. S. Wolfe. 1985. Methenyl-tetrahydromethanopterin cyclohydrolase. Arch. Biochem. Biophys. **242**:430-439

35. Donnelly, M. I., and R. S. Wolfe. 1986. The role of formylmethanofuran:tetrahydromethanopterin formyl transferase in methanogenesis from carbon dioxide. J. Biol. Chem. **261**:16653-16659.

36. Eguchi, S. Y., N. Nishio, and S. Nagai. 1985. Formic acid production from H_2 and bicarbonate by a formate-utilizing methanogen. Appl. Microbiol. Biotechnol. **22**:148-151.

37. Eirich, L. D., G. D. Vogels, and R. S. Wolfe. 1978. Proposed structure for coenzyme F_{420} from Methanobacterium. Biochemistry. 17:4583-4593.

38. Eirich, L. D., G. D. Vogels, and R. S. Wolfe. 1979. Distribution of coenzyme F_{420} and properties of its hydrolytic fragments. J. Bacteriol. 140:20-27.

39. Ellefson, W. L., W. B. Whitman, and R. W. Wolfe. 1982. Nickel-containing factor F_{430}: chromophore of the methylreductase of Methanobacterium. Proc. Natl. Acad. Sci. U.S.A. 79:3707-3710.

40. Ellefson, W. L., and R. S. Wolfe. 1980. Role of component C in the methylreductase system of Methanobacterium. J. Biol. Chem. 255:8388-8389.

41. Ellerman, J., R. Hedderich, R. Böcher, and R. K. Thauer. 1988. The final step in methane formation. Investigations with highly purified methyl-CoM reductase (component C) from Methanobacterium thermoautotrophicum (strain Marburg). Eur. J. Biochem. 172:669-677.

42. Fauque, G., M. Texeira, I. Moura, P. A. Lespinat, A. V. Xavier, D. V. derVartanian, H. D. Peck, Jr., J. LeGall, and J. G. Moura. 1984. Purification, characterization and redox properties of hydrogenase from Methanosarcina barkeri (DSM 800). Eur. J. Biochem. 142:21-28.

43. Fernandez, V. M., E. C. Hatchikian, and R. Cammack. 1985. Properties and reactivation of two different deactivated forms of Desulfovibrio gigas hydrogenase. Biochim. Biophys. Acta. 832:69-79.

44. Fernandez, V. M., E. C. Hatchikian, D. S. Patil, and R. Cammack. 1986. ESR-detectable nickel and iron-sulphur centres in relation to the reversible activation of Desulfovibrio gigas hydrogenase. Biochim. Biophys. Acta. 883:145-154.

45. Fernandez, V. M., K. K. Rao, M. A. Fernandez, and R. Cammack. 1986. Activation and deactivation of the membrane-bound hydrogenase from Desulfovibrio desulfuricans, Norway strain. Biochime. 68:43-48.

46. Ferry, J. G., and R. S. Wolfe. 1977. Nutritional and biochemical characterization of Methanospirillum hungatei. Appl. Environ. Microbiol. **34**:371-376.

47. Fisher, J., R. Spencer, and C. T. Walsh. 1976. Enzyme-catalyzed redox reactions with the flavin analogues 5-deazariboflavin, 5-deazariboflavin-5'-phosphate, and 5-deazariboflavin-5'-diphosphate,5'-5'-adenosine ester. Biochemistry. **15**:1054-1064.

48. Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen, and C. R. Woese. 1980. The phylogeny of prokaryotes. Science. **209**:457-463.

49. Fox, J. A., D. J. Livingston, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 1. Purification and characterization. Biochemistry USA. **26**:4219-4227.

50. Fuchs, G., and E. Stupperich. 1982. Autotrophic CO_2 fixation pathway in Methanobacterium thermoautotrophicum. Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. 1 Orig. Reihe C. **3**:277-288.

51. Gow, L. A., I. P. Pankhania, S. P. Ballantine, D. H. Boxer, and W. A. Hamilton. 1986. Identification of a membrane-bound hydrogenase of Desulfovibrio vulgaris (Hildenborough). Biochim. Biophys. Acta. **851**:57-64.

52. Grande, H. J., W. R. Dunham, B. Averill, C. vanDijk, and R. M. Sands. 1983. Electron paramagnetic resonance and other properties of hydrogenases isolated from Desulfovibrio vulgaris (strain Hildenborough) and Megasphaera elsdenii. Eur. J. Biochem. **136**:201-207.

53. Gray, C. T., and H. Gest. 1965. Biological formation of molecular hydrogen. Science. **148**:186-192.

54. Gunsalus, R. P., and R. S. Wolfe. 1978. ATP activation and properties of the methyl coenzyme M reductase system in Methanobacterium thermoautotrophicum. J. Bacteriol. **135**:851-857.

55. Hagen, W. R., A. vanBerkel-Arts, K. M. Krüse-Wolters, W. R. Dunham, and C. Veeger. 1986. EPR of a novel high spin component in activated hydrogenase from Desulfovibrio vulgaris (Hildenborough). FEBS Lett. **201**:158-162.

56. Hagen, W. R., A. vanBerkel-Arts, K. M. Krüse-Wolters, G. Voordouw, and C. Veeger. 1986. The iron-sulfur composition of the active site of hydrogenas from Desulfovibrio vulgaris (Hildenborough) deduced from its subunit structure and total iron-sulfur content. FEBS Lett. **203**:59-63.

57. Hartzell, P. L., G. Zvilius, J. C. Escalante-Semerena, and M. I. Donnelly. 1985. Coenzyme F_{420} dependence of the methylenetetrahydromethanopterin dehydrogenase of Methanobacterium thermoautotrophicum. Biochem. Biophys. Res. Commun. **133**:884-890.

58. Hausinger, R. P. 1987. Nickel utilization by microorganisms. Microbiol. Rev. **51**:22-42.

59. Hedderich, R., and R. K. Thauer. Methanobacterium thermoautotrophicum contains a soluble enzyme that specifically catalyzes the reduction of the heterodisulfide of coenzyme M and 7-mercapto-heptanolythreonine phosphate with H_2. FEBS Lett. **234**:223-227.

60. Hilpert, R., J. Winter, W. Hammes, and O. Kandler. 1981. The sensitivity of archaebacteria to antibiotics. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe C. **2**:11-20.

61. Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. Arch. Microbiol. **59**:158-164.

62. Hungate, R. E., W. Smith, T. Bandrop, T. Yu, and J. C. Rabinowitz. 1967. Formate as an intermediate in the bovine rumen fermentation. J. Bacteriol. **102**:389-397.

63. Huynh, B. H., M. H. Czechowski, H. -J. Krüger, D. V. DerVartanian, H. D. Peck, Jr., and J. LeGall. 1984. Desulfovibrio vulgaris hydrogenase: a nonheme iron enzyme lacking nickel that exhibits anomalous EPR and Mössbauer spectra. Proc. Natl. Acad. Sci. U.S.A. **81**:3728-3732.

64. Ianotti, E. L., D. Katkewitz, M. J. Wolin, and M. P. Bryant. 1973. Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: changes caused by interspecies transfer of H_2. J. Bacteriol. **114**:1231-1240.

65. Jacobson, F. S., L. Daniels, J. A. Fox, C. T. Walsh, and W. H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Biol. Chem. **257**:3385-3388.

66. Jacobson, F. S., and C. T. Walsh. 1984. Properties of 7,8-didemethyl-8-hydroxy-5-deazariboflavins relevant to redox coenzyme function in methanogen metabolism. J. Biol. Chem. **257**:3385-3388.

67. Jin, S.-L. C., D. K. Blanchard, and J. -S. Chen. 1983. Two hydrogenases with distinct electron-carrier specificity and subunit composition in Methanobacterium formicicum. Biochim. Biophys. Acta. **748**:8-20.

68. Jones, J. B., and T. C. Stadtman. 1980. Reconstitution of a formate-$NADP^+$ oxidoreductase from formate dehydrogenase and a 5-deazaflavin-linked $NADP^+$ reductase isolated from Methanococcus vannielii. J. Biol. Chem. **255**:1049-1053.

69. Jones, J. B., and T. C. Stadtman. 1981. Selenium-dependent and selenium-independent formate dehydrogenases of Methanococcus vannielii. Separation of the two forms and characterization of the purified selenium-independent form. J. Biol. Chem. **256**:656-663.

70. Jones, W. J., M. I. Donnelly, and R. S. Wolfe. 1985. Evidence of a common pathway of carbon dioxide reduction to methane in methanogens. J. Bacteriol. **163**:126-131.

71. Jones, W. J., D. P. Nagle, Jr., and W. B. Whitman. 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51**:135-177.

72. Jungermann, K, H. Kirchniawy, and R. K. Thauer. 1979. Ferredoxin dependent CO_2 reduction to formate in Clostridium pasteurianum. Biochem. Biophys. Res. Commun. **41**:682-689.

73. Kandler, O., and H. König. 1985. Cell envelopes of archaebacteria, p. 413-457. **In:** C. R. Woese and R. S. Wolfe (eds.), The bacteria. Vol. 8. Academic Press, Inc., New York.

74. Kröger, A., E. Winkler, A. Innerhofer, H. Hackenberg, and H. Schagger. 1979. The formate dehydrogenase involved in electron transport from formate to fumarate in Vibrio succinogenes. Eur. J. Biochem. **94**:465-475.

75. Krüger, H. -J., B. H. Huynh, P. O. Ljungdahl, A. V. Xavier, D. V. DerVartanian, I. Moura, H. D. Peck, Jr., M. Texeira, J. J. G. Moura, and J. LeGall. 1982. Evidence for nickel and a three-iron center in the hydrogenase of Desulfovibrio desulfuricans. J. Biol. Chem. **257**:14620-14623.

76. Latham, M. J., and M. J. Wolin. 1977. Fermentation of cellulose by Ruminococcus flavefaciens in the presence and absence of Methanobacterium ruminantium. Appl. Environ. Microbiol. **34**:297-301.

77. LeGall, J., J. J. G. Moura, H. D. Peck, Jr., and A. V. Xavier. 1982. Hydrogenase and other iron-sulfur proteins from sulfate-reducing and methane-forming bacteria, p. 177-248. **In:** T. G. Spiro (ed.), Iron-sulfur proteins. John Wiley & Sons, Inc., New York.

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

79. Lespinat, P. A., Y. Berlier, G. Fauque, M. Czechowski, B. Dimon, and J. LeGall. 1986. The pH dependence of proton-deuterium exchange, hydrogen production and uptake catalyzed by hydrogenases from sulfate-reducing bacteria. Biochimie. **68**:55-61.

80. Lin, E. C. C., and D. R. Kuritzkes. 1987. Pathways for anaerobic electron transport, p. 201-221. **In:** F. C. Neidhardt, J. L. Ingraham, K. Brooks-Low, B. Magasanik, M. Schaeter, and H. E. Umbarger (eds.), Escherichia coli and Salmonella typhimurium. Cellular and molecular biology. American Society for Microbiology, Washington, D. C.

81. Lindahl, P. A., N. Kojima, R. P. Hausinger, J. A. Fox, B. C. Teo, C. T. Walsh, and W. H. Orme-Johnson. 1984. Nickel and iron EXAFS of F_{420}-reducing hydrogenase from Methanobacterium thermoautotrophicum. J. Am. Chem. Soc. **106**:3062-3065.

82. Livingston, D. J., J. A. Fox, W. H. Orme-Johnson, and C. T. Walsh. 1987. 8-Hydroxy-5-deazaflavin-reducing hydrogenase from Methanobacterium thermoautotrophicum: 2. Kinetic and hydrogen-transfer studies. Biochemistry USA. **26**:4228-4236

83. Ljungdahl, L. G., and H. G. Wood. 1969. Total synthesis of acetate from CO_2 by heterotrophic bacteria. Annu. Rev. Microbiol. **23**:515-538.

84. Makula, R. A., and M. E. Singer. 1978. Ether-containing lipids of methanogenic bacteria. Biochem. Biophys. Res. Commun. **82**:716-722.

85. Mayer, F., A. Jussofie, M. Salzmann, M. Lubben, M. Rohde, and G. Gottschalk. 1987. Immunoelectron microscopic demonstration of ATPase on the cytoplasmic membrane of the methanogenic bacterium strain Göl. J. Bacteriol. **169**:2307-2309.

86. Mayer, F., M. Rohde, M. Salzmann, A. Jussofie, and G. Gottschalk. 1988. The methanoreductosome: a high-molecular-weight enzyme complex in the methanogenic bacterium strain Göl that contains components of the methylreductase system. J. Bacteriol. **170**:1438-1444.

87. McInerney, M. J., and M. P. Bryant. 1981. Review of methane fermentation fundamentals, p. 20-40. **In:** D. L. Wise (ed.), Fuel gas production from biomass. Chemical Rubber Co. Press, Inc., West Palm Beach, Fla.

88. McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton. 1981. Syntrophomonas wolfei gen. nov., sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. **41**:1029-1039.

89. McInerney, M. J., M. P. Bryant, and N. Pfennig. 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122**:129-135.

90. McKellar, R. C. and G. D. Sprott. 1979. Solubilization and properties of a particulate hydrogenase from Methanobacterium strain G2R. J. Bacteriol. **139**:231-238.

91. Mountfort, D. O., E. Mörschel, D. B. Beimborn, and P. Schönheit. 1986. Methanogenesis and ATP synthesis in a protoplast system of Methanobacterium thermoautotrophicum. J. Bacteriol. **168**:892-900.

92. Moura, J. J. G., I. Moura, B. -H. Huynh, H. -J. Krüger, M. Texeira, R. C. Duvarney, D. V. DerVartanian, A. V. Xavier, H. D. Peck, Jr., and J. LeGall. 1982. Unambiguous identification of the nickel signal in ^{61}Ni-enriched Desulfovibrio gigas hydrogenase. Biochem. Biophys. Res. Commun. **108**:1388-1393.

93. Müller, U., P. Willnow, U. Ruschig, and T. Höpner. 1978. Formate dehydrogenase from Pseudomonas oxalaticus. Eur. J. Biochem. **83**:485-498.

94. Muth, E. 1988. Localization of the F_{420}-reducing hydrogenase in Methanococcus voltae by immuno-gold technique. Arch. Microbiol. **150**:205-207

95. Muth, E., E. Mörschel, and A. Klein. 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium Methanococcus voltae. Eur. J. Biochem. **169**:571-577.

96. Nagle Jr., D. P., and R. S. Wolfe. 1983. Component A of the methyl coenzyme M methylreductase system of Methanobacterium: resolution into four components. Proc. Natl. Acad. Sci. U.S.A. **80**:2151-2155.

97. Nelson, M. J. K., D. P. Brown, and J. G. Ferry. 1984. FAD requirement for the reduction of coenzyme F420 by hydrogenase from Methanobacterium formicicum. Biochem. Biophys. Res. Commun. **120**:775-781.

98. Niekus, H. G. D., M. Veenhuis, and A. H. Stouthamer. 1980. Formate oxidation in Campylobacter sputorum subspecies bubulus. FEMS Microbiol. Lett. **9**:1-6.

99. Nishio, N., S. Y. Eguchi, H. Kawashima, and S. Nagai. 1983. Mutual conversion between H_2 plus CO_2 and formate by a formate-utilizing methanogen. J. Ferment. Technol. **61**:557-561.

100. Noll, K. M., K. L. Rinehart, Jr., R. S. Tanner, and R. S. Wolfe. 1986. Structure of component B (7-mercaptoheptanoylthreonine phosphate) of the methylcoenzyme M methylreductase system of Methanobacterium thermoautotrophicum. Proc. Natl. Acad. Sci. U.S.A. **83**:4238-4242.

101. Noll, K. M., and R. S. Wolfe. 1987. The role of 7-mercaptoheptanoylthreonine phosphate in the methylcoenzyme M methylreductase system from Methanobacterium thermoautotrophicum. Biochem. Biophys. Res. Commun. **145**:204-210.

102. Odom, J. M., and H. D. Peck, Jr. 1981. Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, Desulfovibrio spp. FEMS Microbiol. Lett. **12**:47-50.

103. Ossmer, R., T. Mund, P. Hartzell, U. Konheiser, G. W. Kohring, A. Klein, R. S. Wolfe, G. Gottschalk, and F. Mayer. 1986. Immunocytochemical localization of component C of the methylreductase system in Methanococcus voltae and Methanobacterium thermoautotrophicum. Proc. Natl. Acad. Sci. USA **83**:5789-5792.

104. Pankhania, I. P., L. A. Gow, and W. A. Hamilton. 1986. Extraction of periplasmic hydrogenase from Desulfovibrio vulgaris (Hildenborough). FEMS Microbiol. Lett. **35**:1-4.

105. Perski, H. J., P. Schönheit, and R. K. Thauer. 1982. Sodium dependence of methane formation in methanogenic bacteria. FEBS Lett. **143**:323-326.

106. Ragsdale, S. W., and L. G. Ljungdahl. 1984. Hydrogenase from Acetobacterium woodii. Arch. Microbiol. **139**:361-366.

107. Reddy, C. A., M. P. Bryant, and M. J. Wolin. 1972. Characteristics of S organism isolated from Methanobacillus omelianskii. J. Bacteriol. **108**:539-545.

108. Reddy, C. A., M. P. Bryant, and M. J. Wolin. 1972. Ferredoxin and nicotinamide adenine dinucleotide-dependent H_2 production from ethanol and formate in extracts of S organism isolated from "Methanobacillus omelianskii". J. Bacteriol. **110**:126-132.

109. Rieder, R., R. Cammack, and D. O. Hall. 1984. Purification and properties of the soluble hydrogenase from Desulfovibrio desulfuricans (strain Norway 4). Eur. J. Biochem. **145**:637-643.

110. Rouvière, P. E., and R. S. Wolfe. 1988. Novel biochemistry of methanogenesis. J. Biol. Chem. **263**:7913-7916.

111. Sawers, R. G., S. P. Ballantine, and D. H. Boxer. 1985. Differential expression of hydrogenase isoenzymes in Escherichia coli K-12: evidence for a third isoenzyme. J. Bacteriol. **164**:1324-1331.

112. Sawers, R. G., D. J. Jamieson, C. F. Higgins, and D. H. Boxer. 1986. Characterization and physiological roles of membrane-bound hydrogenase isoenzymes from Salmonella typhimurium. J. Bacteriol. **168**:398-404.

113. Schauer, N. L., and J. G. Ferry. 1980. Metabolism of formate in Methanobacterium formicicum. J. Bacteriol. **142**:800-807.

114. Schauer, N. L., and J. G. Ferry. 1983. FAD requirement for the reduction of coenzyme F_{420} by formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **155**:467-472.

115. Schauer, N. L., and J. G. Ferry. 1986. Composition of the coenzyme F_{420}-dependent formate dehydrogenase from Methanobacterium formicicum. J. Bacteriol. **165**:405-411.

116. Schink, B., and H. G. Schlegel. 1979. The membrane-bound hydrogenase of Alcaligenes eutrophus. I. Solubilization, purification, and biochemical properties. Biochim. Biophys. Acta. **567**:315-324.

117. Schlegel, H. G., and K. Schneider. 1978. Introductory report: distribution and physiological role of hydrogenase in microorganisms, p. 15-44. **In:** Hydrogenases: their catalytic activity, structure, and function. Erich Goltze KG, Göttingen.

118. Schneider, K., and H. G. Schlegel. 1976. Purification and properties of soluble hydrogenase from Alcaligenes eutrophus H16. Biochim. Biophys. Acta. **452**:66-80.

119. Schneider, K., and H. G. Schlegel. 1978. Identification and quantitative determination of the flavin component of soluble hydrogenase from Alcaligenes eutrophus. Biochem. Biophys. Res. Commun. **84**:564-571.

120. Schobert, S. 1978. Acetic acid from H_2 and CO_2, formation of acetate by cell extracts of Acetobacterium woodii. Arch. Microbiol. **114**:143-148.

121. Schönheit, P., and D. B. Beimborn. 1985. Presence of a Na^+/H^+ antiporter in Methanobacterium thermoautotrophicum and its role in Na^+ dependent methanogenesis. Arch. Microbiol. **142**:354-361.

122. Scott, R. A., S. A. Wallin, M. Czechowski, D. V. DerVartanian, J. LeGall, H. D. Peck, Jr., and I. Moura. 1984. X-ray absorption spectroscopy of nickel in the hydrogenase from Desulfovibrio gigas. J. Am. Chem. Soc. **106**:6864-6865.

123. Seefeldt, L. C., and D. J. Arp. 1986. Purification to homogeneity of Azotobacter vinelandii hydrogenase: a nickel and iron containing αβ dimer. Biochimie. **68**:25-34.

124. Seefeldt, L. C., L. C. McCollum, C. M. Doyle, and D. J. Arp. 1987. Immunological and molecular evidence for a membrane-bound, dimeric hydrogenase in Rhodopseudomonas capsulata. Biochim. Biophys. Acta. **914**:299-303.

125. Sparling, R., and L. Daniels. 1986. Source of carbon and hydrogen in methane produced from formate by Methanococcus thermolithotrophicus. J. Bacteriol. **168**:1402-1407.

126. Sprott, G. D., K. M. Shaw, and T. J. Beveridge. 1987. Properties of the particulate enzyme F_{420}-reducing hydrogenase isolated from Methanospirillum hungatei. Can. J. Microbiol. **33**:896-904.

127. Stephenson, M., and L. H. Stickland. 1931. Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. Biochem. J. **25**:205-214.

128. Taylor, C. D., and R. S. Wolfe. 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3^-$). J. Biol. Chem. **249**:4879-4885.

129. Texeira, M., G. Fauque, I. Moura, P. A. Lespinat, Y. Berlier, B. Prickril, H. D. Peck, Jr., A. V. Xavier, J. LeGall, and J. J. G. Moura. 1987. Nickel-[iron-sulfur]-containing hydrogenases from Desulfovibrio baculatus (DSM 1743). Redox centers and catalytic properties. Eur. J. Biochem. **167**:47-58.

130. Texeira, M., I. Moura, G. Fauque, M. Czechowski, Y. Berlier, P. A. Lespinat, J. LeGall, A. V. Xavier, and J. J. G. Moura. 1986. Redox properties and activity studies on a nickel-containing hydrogenase isolated from a halophilic sulfate reducer Desulfovibrio salexigens. Biochimie. **68**:75-84.

131. Texeira, M., I. Moura, A. V. Xavier, D. V. DerVartanian, J. LeGall, H. D. Peck, Jr., B. H. Huynh, and J. J. G. Moura. 1983. Desulfovibrio gigas hydrogenase: redox properties of the nickel and iron-sulfur centers. Eur. J. Biochem. **130**:481-484.

132. Texeira, M., I. Moura, A. V. Xavier, B. H. Huynh, D. V. DerVartanian, H. D. Peck, Jr., J. LeGall, and J. J. G. Moura. 1985. EPR studies on the mechanism of activation and catalytic cycle of the nickel-containing hydrogenase from Desulfovibrio gigas. J. Biol. Chem. **260**:8942-8950.

133. Texeira, M., I. Moura, A. V. Xavier, J. J. G. Moura, G. Fauque, B. Prickril, and J. LeGall. 1985. Nickel-iron-sulfur-selenium-containing hydrogenases isolated from Desulfovibrio baculatus strain 9974. Rev. Port. Quim. **27**:194-195.

134. Thauer, R. K., G. Fuchs, and K. Jungermann. 1977. Role of iron-sulfur proteins in formate metabolism, p. 121-156. **In:** W. Lovenberg (ed.), Iron-sulfur proteins. Vol. III. Academic Press, New York.

135. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.

136. Tornabene, T. G., and T. A. Langworthy. 1978. Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science. **203**:51-53.

137. Tzeng, S. -F., M. P. Bryant, and R. S. Wolfe. 1975. Factor F_{420}-dependent pyridine nucleotide-linked formate metabolism of Methanobacterium ruminantium. J. Bacteriol. **121**:192-196.

138. van Beelen, P., A. P. M. Stassen, J. W. G. Bosch, G. D. Vogels, W. Guijt, and C. A. G. Haasnoot. 1984. Elucidation of the structure of methanopterin, a coenzyme from Methanobacterium thermoautotrophicum, using two dimensional nuclear magnetic resonance techniques. Eur. J. Biochem. **138**:563-571.

139. van Dijk, C., H. J. Grande, S. G. Mayhew, and C. Veeger. 1980. Properties of the hydrogenase of Megasphaera elsdenii. Eur. J. Biochem. **107**:251-261.

140. van Dijk, C., A. van Berkel-Arts, and C. Veeger. 1983. The effect of reoxidation on the reduced hydrogenase of Desulfovibrio vulgaris strain Hildenborough and its oxygen stability. FEBS Lett. **156**:340-344.

141. Wackett, L. P., E. A. Hartwieg, J. A. King, W. H. Orme-Johnson, and C. T. Walsh. 1987. Electron microscopy of nickel-containing methanogenic enzymes: methyl reductase and F_{420}-hydrogenase. J. Bacteriol. **169**:718-727.

142. Walsh, C. T. 1980. Flavin coenzymes: at the crossroads of biological redox chemistry. Acc. Chem. Res. **13**:148-155.

143. Walsh, C. T., and W. H. Orme-Johnson. 1987. Nickel enzymes. Biochemistry USA. **26**:4901-4906.

144. Wang, G. M. J. Benecky, B. H. Huynh, J. F. Cline, M. W. W. Adams, L. E. Mortenson, B. M. Hoffman, and E. Münck. 1984. Mössbauer and electron nuclear double resonance study of oxidized bidirectional hydrogenase from Clostridium pasteurianum W5. J. Biol. Chem. **259**:14328-14331.

145. Weimer, P. J., and J. G. Zeikus. 1977. Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Appl. Environ. Microbiol. **33**:289-297.

146. Woese, C. R., and G. E. Fox. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. U.S.A. **74**:5088-5090.

147. Woese, C. R., L. J. Magrum, and G. E. Fox. 1978. Archaebacteria. J. Mol. Evol. **11**:245-252.

148. Wolfe, R. S., and N. Pfennig. 1977. Reduction of sulfur by Spirillum 5175 and syntrophism with Chlorobium. Appl. Environ. Microbiol. **33**:427-433.

149. Yagi, T., K. Kimura, and H. Inokuchi. 1985. Analysis of the active center of hydrogenase from Desulfovibrio vulgaris Miyazaki by magnetic measurements. J. Biochem. (Tokyo). **97**:181-187.

150. Yamazaki, S. 1982. A selenium-containing hydrogenase from Methanococcus vannielii. J. Biol. Chem. **257**:7926-7929.

151. Zeikus, J. G., G. Fuchs, W. Kenealy, and R. K. Thauer. 1977. Oxidoreductases involved in cell carbon synthesis of Methanobacterium thermoautotrophicum. J. Bacteriol. **132**:604-613.

152. Zillig, W., K. O. Stetter, R. Schnabel, and M. Thomm. 1985. DNA-dependent RNA polymerases of the archaebacteria, p. 494-524. **In:** C. R. Woese and R. S. Wolfe (eds.), The bacteria. Vol. 8. Academic Press, Inc., New York.

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