A BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION
OF COENZYME F₄₂₀-REDUCING HYDROGENASE
FROM METHANOBACTERIUM FORMICICUM

by

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Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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in

ANAEROBIC MICROBIOLOGY

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A BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION
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Committee Chairman: Dr. James G. Ferry

Anaerobic Microbiology

(ABSTRACT)

The coenzyme F420-reducing hydrogenase of
Methanobacterium formicicum was purified 87-fold to
electrophoretic homogeneity. The enzyme formed aggregates
(1,000 kd) of a coenzyme F420-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 F420-reducing hydrogenase during reactivation
with H2 and coenzyme F420, unless KCl was present, yielding
coenzyme F420-inactive apoenzyme. The hydrogenase
catalyzed H2 production at a rate 3-fold less than that for
H₂ uptake. Specific antiserum inhibited the coenzyme F₄₂₀-dependent activity but not the methyl viologen-dependent activity of the purified enzyme.

Cell extract of *M. formicicium* contained a coenzyme F₄₂₀-mediated formate hydrogenlyase system. Formate hydrogenlyase activity was reconstituted with coenzyme F₄₂₀-reducing hydrogenase, coenzyme F₄₂₀-reducing formate dehydrogenase, and coenzyme F₄₂₀, all purified from *M. formicicium*. The reconstituted system required FAD for maximal activity (kinetic Kₐ = 4 µM). Without FAD, the formate dehydrogenase and hydrogenase rapidly lost coenzyme F₄₂₀-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 F₄₂₀-reducing hydrogenase and formate dehydrogenase of *M. formicicium* 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:

Purification and properties of the membrane-associated coenzyme F420-reducing hydrogenase from
(submitted).


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


DEDICATION

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

I am very grateful to Dr. James G. (Greg) Ferry for his enduring support and guidance in my academic career. I also thank my committee members: Dr. T. D. Wilkins, Dr. J. L. Johnson, Dr. B. M. Anderson, and Dr. D. R. Bevan for their support and interest.

I thank (University of Florida, Gainesville), for his contribution to the manuscript in Section V and , and for their contributions in Section VI. I gratefully acknowledge financial support from the National Science Foundation, the Commonwealth of Virginia, and the College of Agriculture and Life Sciences. I am very grateful to , , , and for their secretarial help and for always brightening things up with a smile.

To my coworkers: , , , , , , , , , , , , , , , and , I extend many thanks for knowledge and good times shared, which made my stay at the Anaerobe Lab very enriching. I also thank for his friendship, especially during my first two years here.

Finally, I thank my parents, my brother and sisters, and my wife, for their encouragement and support during my academic endeavours.
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SECTION I. GENERAL INTRODUCTION

*Methanobacterium formicicum* is one of several methanogenic bacteria which obtain energy for growth by reducing CO₂ to methane with electrons derived from H₂ or formate. The oxidation of formate in this organism is catalyzed by formate dehydrogenase, which reduces the physiological electron acceptor, coenzyme F₄₂₀ (F₄₂₀). The oxidation of H₂ is catalyzed by hydrogenase, two types of which have been identified in H₂-oxidizing methanogenic bacteria. One (F₄₂₀-hydrogenase) reduces F₄₂₀. The other (MV-hydrogenase) reduces the artificial electron acceptor, methyl viologen but not F₄₂₀, and its physiological electron acceptor is unknown. The F₄₂₀-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 H₂ and CO₂. Formate hydrogenlyase in coliform bacteria consists of a formate dehydrogenase, a hydrogenase, and one or more intermediate electron carriers. F₄₂₀ 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 \textit{M. formicicum} and reconstitute the system; ii) to determine the cellular locations of its enzymatic components; and iii) to characterize the hydrogenase component.
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 CO₂ 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 CO₂ 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 H₂, CO₂, alcohols, fatty acids (formate, acetate, propionate, butyrate, etc.) and other organic acids (e.g., succinate and lactate). Second, H₂-producing acetogenic bacteria oxidize alcohols and higher fatty acids and produce acetate, H₂, and CO₂. Finally, methanogenic bacteria reduce CO₂ to methane with H₂ as electron donor and convert substrates including formate, acetate, methanol, and methylamines to methane and CO₂.

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, \( \text{H}_2/\text{CO}_2 \) is the major methane precursor in the rumen (61), and formate accounts for about 18% of the methane produced (62). Formate and \( \text{H}_2/\text{CO}_2 \) are equivalent substrates for methanogenesis, yielding about the same standard free energy change per mole of methane produced (87). About one half of all \( \text{H}_2 \)-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 \( \text{H}_2 \)-producing acetogenic bacteria through a process termed 'interspecies \( \text{H}_2 \) transfer'. For example, the degradation of propionate to acetate and \( \text{H}_2 \) by the \( \text{H}_2 \)-producing acetogenic bacteria is endergonic but is exergonic when coupled to methanogenesis from \( \text{H}_2 \) plus \( \text{CO}_2 \) (18). Therefore, growth of these bacteria depends entirely upon the rapid removal of \( \text{H}_2 \) 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 \( \text{H}_2 \) production via hydrogenase, which is thermodynamically unfavorable under standard conditions (18):
\[
\text{NADH} + \text{H}^+ \rightleftharpoons \text{NAD}^+ + \text{H}_2 \quad \Delta G^\circ' = +18.0\text{kJoules}
\]

Hydrogenase

In pure cultures of fermentative microorganisms, H\textsubscript{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\textsubscript{2} is maintained at low levels, and proportionally more of the oxidized products, CO\textsubscript{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\textsubscript{420} (38), methanofuran (70), methanopterin (138), coenzyme M (128), factor F\textsubscript{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 formyl-methanofuran, 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 CO₂ to methane with H₂ as electron donor and proposed a pathway in which CO₂ is sequentially reduced to methane. Sparling and Daniels (125) demonstrated that formate is not reduced directly to methane but is first oxidized to CO₂, suggesting that the same CO₂ 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 H₂, catalyzed by hydrogenase, or of formate, catalyzed by formate dehydrogenase. CO₂ is initially bound to methanofuran (MFR) and is reduced to the formyl level, yielding formyl-MFR (78). The formyl moiety is transferred to tetrahydromethanopterin (H₄MPT), yielding 5-formyl-H₄MPT (35), which is then dehydrated to 5,10-methenyl-H₄MPT (34). This product is reduced to methylene-H₄MPT with reduced coenzyme F₄₂₀ (F₄₂₀) as the electron donor (57). Methylene-H₄MPT is reduced to methyl-H₄MPT and the methyl moiety then transferred to coenzyme M (HS-CoM) (110).

The last step of the CO₂ 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 CO₂ to methane. Taken from reference 110. MFR, methanofuran; H₄MPT, tetrahydromethanopterin; HS-CoM, coenzyme M; HS-HTP, 7-mercaptoheptanoyl-threonine phosphate; F₄₃₀, factor F₄₃₀; F₄₂₀ox, F₄₂₀red, oxidized and reduced coenzyme F₄₂₀; e⁻, electron.
The $\text{H}_2$-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-$\text{S}$-$\text{CoM}$ 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-$\text{S}$-$\text{CoM}$ is component B (101), recently identified as 7-mercaptoheptanoyl-threonine phosphate (HS-$\text{HTP}$, see Fig. 1) (100), and the oxidized product of this reaction is a mixed disulfide of HS-$\text{CoM}$ and HS-$\text{HTP}$ (CoM-$\text{S-S}$-$\text{HTP}$) (16,41). The disulfide can be reductively cleaved back to HS-$\text{CoM}$ and HS-$\text{HTP}$ by an $\text{H}_2$-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 $\text{H}_2$ by a membrane-associated hydrogenase (135) coupled to the methylreductase reaction (31). $\text{H}_2$-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 H₂ drives ATP synthesis in *Methanosarcina barkeri* and *Methanosphaera stadtmaniae*. The F₄₂₀-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 H₂-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 F₄₂₀

Coenzyme F₄₂₀ (F₄₂₀) has been found in all methanogenic bacteria examined (38). Structurally, F₄₂₀ 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). F₄₂₀ 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 F₄₂₀ 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), F₄₂₀:NADP oxidoreductase (68,137), pyruvate synthase, α-ketoglutarate synthase (50,151), and methylene-H₄MPT dehydrogenase (57).

Hydrogenase

A. Definition and occurrence in nature.

The activation of H₂ is carried out by the enzyme hydrogenase, which catalyzes the reversible reaction:

\[ \text{H}_2 \Leftrightarrow 2\text{H}^+ + 2\text{e}^- \]

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 H₂ as an energy source.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Representative genus</th>
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<tr>
<td>Desulfovibrio\textsuperscript{b}</td>
<td>$4\text{H}_2 + \text{SO}_4^{2-} \rightarrow \text{S}^{-2} + 4\text{H}_2\text{O}$</td>
<td>10</td>
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<tr>
<td>Methanobacterium\textsuperscript{b}</td>
<td>$4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$</td>
<td>71</td>
</tr>
<tr>
<td>Acetobacterium\textsuperscript{b}</td>
<td>$4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O}$</td>
<td>120</td>
</tr>
<tr>
<td>Escherichia\textsuperscript{c}</td>
<td>$\text{H}_2 + \text{Fumarate} \rightarrow \text{Succinate}$</td>
<td>14</td>
</tr>
<tr>
<td>Escherichia\textsuperscript{c}</td>
<td>$\text{H}_2 + (\text{CH}_3)_3\text{NO} \rightarrow (\text{CH}_3)_3\text{N} + \text{H}_2\text{O}$</td>
<td>80</td>
</tr>
<tr>
<td>Escherichia\textsuperscript{c}</td>
<td>$\text{H}_2 + (\text{CH}_3)_2\text{SO} \rightarrow (\text{CH}_3)_2\text{S} + \text{H}_2\text{O}$</td>
<td>80</td>
</tr>
<tr>
<td>Campylobacter\textsuperscript{c}</td>
<td>$\text{H}_2 + \text{S}^0 \rightarrow \text{H}_2\text{S}$</td>
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</tr>
<tr>
<td>Alcaligenes\textsuperscript{d}</td>
<td>$\text{H}_2 + \frac{1}{2}\text{O}_2 \rightarrow \text{H}_2\text{O}$</td>
<td>9</td>
</tr>
<tr>
<td>Paracoccus\textsuperscript{d}</td>
<td>$5\text{H}_2 + 2\text{NO}_3^- + 2\text{H}^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O}$</td>
<td>117</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Modified from reference 117.

\textsuperscript{b}Obligately anaerobic

\textsuperscript{c}Facultatively anaerobic or microaerophilic

\textsuperscript{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. H₂ 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, CO₂, and H₂ (64). NADH generated during glycolysis can be reoxidized either through H₂ 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 H₂ allows more acetyl-CoA to be used for ATP synthesis. This mechanism is most effective when H₂ is rapidly removed by interspecies H₂ 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 H₂ and CO₂ (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
**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 H₂ cycling by Desulfovibrio during growth on lactate and sulfate. ECP, electron carrier proteins; C₃OX,rd, oxidized and reduced forms of cytochrome c₃; e⁻, electron; H₂ase, 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 \([2\text{Fe}-2\text{S}]\), \([3\text{Fe}-\text{XS}]\), or \([4\text{Fe}-4\text{S}]\) 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 \((\text{NiFe hydrogenases})\) (5,6,12,22,29,42, 75,92,130,131,132); ii) those which contain nickel, iron-sulfur centers, and selenium \((\text{NiFeSe hydrogenases})\) (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 H₂ 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 H₂ uptake, H₂ evolution, and proton-deuterium exchange activities (79).

The H₂-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 H₂ oxidation involves a heterolytic cleavage: nickel binds H₂ 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 H₂ molecule during deprotonation (129). The oxidation of H₂ 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 H₂ 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 H₂ 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 O₂.

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 (F₄₂₀-hydrogenase) reduces F₄₂₀ and the artificial electron acceptor, methyl viologen. The other (F₄₂₀-non-reactive or MV-hydrogenase) reduces methyl viologen but not F₄₂₀, 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.

F₄₂₀-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 F₄₂₀-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 F₄₂₀-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:

$$
\text{HCOO}^- + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + 2\text{e}^- + 2\text{H}^+
$$

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 COENZYMЕ F$_{420}$-REDUCING HYDROGENASE FROM MЕTHАNОBАКТЕРИУМ FORMИССУМ

SUMMARY

The membrane-associated coenzyme F$_{420}$-reducing hydrogenase of Methanobacterium formicicum was purified 87-fold to electrophoretic homogeneity. The enzyme contained $\alpha$, $\beta$, and $\gamma$ 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 $\alpha$ 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 H₂ production at a rate 3-fold less than that for H₂ uptake and reduced coenzyme F₄₂₀, methyl viologen, flavins, and 7,8-didemethyl-8-hydroxy-5-deazariboflavin. Maximal coenzyme F₄₂₀-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 F₄₂₀-dependent but not the methyl viologen-dependent activity of the purified enzyme.

INTRODUCTION

The methanogenic bacteria that oxidize H₂ and reduce carbon dioxide (4H₂ + CO₂ ↔ CH₄ + 2H₂O) obtain energy for growth by a chemiosmotic mechanism that couples ATP synthesis to electron transport (8). At least one step in the CO₂ reduction pathway (16) is driven by the reduced form of the physiological electron acceptor, coenzyme F₄₂₀ (F₄₂₀). Many H₂-oxidizing methanogens studied have two distinct hydrogenases (19,20,29). One (F₄₂₀-hydrogenase) reduces F₄₂₀, and also the artificial electron acceptor, methyl viologen (MV). The other (F₄₂₀-non-reactive hydrogenase or MV-hydrogenase) reduces MV but not F₄₂₀. F₄₂₀-hydrogenases from several H₂-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; 15 mM 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 FAO73) at 138 MPa (20,000 lb/in^2). The cell lysate was collected into serum bottles under N₂ and stored as frozen pellets in liquid N₂. 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 MgCl₂, 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). F₄₂₀-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 F₄₂₀-dependent to MV-dependent activity were pooled and made to 40% saturation (0°C) with (NH₄)₂SO₄, followed by centrifugation (19,000 x g, 30 min, 4°C). The supernatant solution was made to 70% saturation (final concentration) with (NH₄)₂SO₄ 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 μM 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 (Rf=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 N2. The purified enzyme could also be stored in air at -20°C for at least 1 month without loss of activity. Anti-F420-hydrogenase antiserum was prepared as described (6).
**Hydrogenase assay.** Samples were made to 48 μM in F420 and 30 μM in FAD, vacuum degassed with N2, flushed with H2 for 1 min, and then incubated for 30-60 min at 35°C. H2 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 F420 or 20 mM MV, under 82 KPa H2. The standard assay mixture without F420 or MV was also used to assay for reduction of alternative electron acceptors by the F420-hydrogenase. The following extinction coefficients were used (all in mM⁻¹cm⁻¹ at pH 7.5): FAD, ε₄₅₀=11.3; FMN or riboflavin, ε₄₅₀=12.2; benzyl viologen, ε₅₅₀=8.1; NAD or NADP, ε₃₃₉=6.22; and 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (2), ε₄₂₀=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 F420-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 \( \mu \)g 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), \( \beta \)-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 deflavoenzyme present in the purified F₄₂₀-hydrogenase preparation. A sample (128 µl) in buffer A containing 0.5 M KCl, 48 µM F₄₂₀, and 30 µM FAD, was vacuum degassed with N₂, flushed with H₂ for 1 min, and incubated for 30 min at 35°C. After removal of H₂ by vacuum degassing with N₂, 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 F₄₂⁰-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.** F₄₂⁰ 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. FADH₂ was prepared by reducing FAD with zinc dust under N₂. All other chemicals were obtained from commercial sources.

**RESULTS**

**Purification, molecular weight, and subunit composition.** A representative purification of the F₄₂⁰-hydrogenase from *M. formicicum* is shown in Table 1. Chromatography on DEAE-cellulose and Phenyl Sepharose CL-4B resolved the F₄₂⁰-hydrogenase from the MV-hydrogenase, as previously reported (29). H₂-producing activity copurified with H₂ 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
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<th>Yield (%)</th>
<th>Activity ratios</th>
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<td>87.1</td>
<td>1.8</td>
<td>0.266 / 0.224</td>
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</table>

aF420-dependent H2 uptake activity determined with the standard assay. Samples were reactivated before assay as described in Materials and Methods.

bBased on F420-dependent H2 uptake activity.

cRatio of F420-dependent to MV-dependent H2 uptake.

dRatio of F420-dependent H2 production to F420-dependent H2 uptake (standard assay). Activities are reported as µmol H2 produced or oxidized/min/mg protein. H2-producing activity was determined with the reconstituted formate hydrogenylase as described (5), except that the hydrogenase sample to be assayed was substituted for purified F420-hydrogenase. Each assay contained 114 µg of purified formate dehydrogenase and ~0.06 units of F420-dependent H2 uptake activity from the hydrogenase sample to be assayed.

 três 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 I and 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 $\alpha$ subunit, less of the $\gamma$ subunit, and none of the $\beta$ subunit (Fig. 2). The results suggest that the $F_{420}$-hydrogenase was purified to electrophoretic homogeneity 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 $\alpha$, $\beta$, and $\gamma$ 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 electrophoreosed on native polyacrylamide gradient (4-15%) tube gels (5 mm inside diameter) using the asparagines-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 F420-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 F420-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 F420-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 F420-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 F42O-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 F42O-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 F42O-hydrogenase from *M. formicicum.*

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (mol/mol of enzyme)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD (as purified enzyme)</td>
<td>0.73 ± 0.08 (3)(^b)</td>
</tr>
<tr>
<td>FAD (reconstituted enzyme)</td>
<td>0.83 ± 0.06 (3)(^b)</td>
</tr>
<tr>
<td>Ni</td>
<td>0.65 ± 0.03 (2)(^c)</td>
</tr>
<tr>
<td>Fe</td>
<td>11.9 ± 0.3 (4)(^d)</td>
</tr>
<tr>
<td></td>
<td>13.7 ± 0.9 (2)(^c)</td>
</tr>
<tr>
<td>S(^-2)</td>
<td>10.9 ± 2.3 (5)(^d)</td>
</tr>
<tr>
<td>Zn</td>
<td>0.55 ± 0.06 (2)(^c)</td>
</tr>
<tr>
<td>Se</td>
<td>&lt;0.015(^e)</td>
</tr>
</tbody>
</table>

\(^a\)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 F42O-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 O₂ and required reactivation before assay. Untreated samples of F₄₂₀-hydrogenase (as purified) reduced F₄₂₀ 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 O₂ greatly reduced the lag period, as did extensive vacuum degassing of the enzyme solution with N₂. These results imply that residual O₂ 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 H₂ increased maximal activities about 2-fold. A 30 min preincubation of vacuum degassed samples under H₂ in the presence of F₄₂₀ 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 F₄₂₀-dependent activity relative to MV-dependent activity unless KCl was present (Table 3). FADH₂, FMN, and riboflavin could not substitute for FAD (data not shown). When KCl was replaced with K₂SO₄, KCH₃COO, (NH₄)₂SO₄,
Figure 4. Reactivation of F_{420}-hydrogenase from M. formicicum. Aliquots (5 µl, 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 KC1 on reactivation of $F_{420}$-hydrogenase from M. formicicum.

<table>
<thead>
<tr>
<th>Addition to reactivation mixture$^a$</th>
<th>Activity$^b$ (units/ml) with:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{420}$</td>
<td>$MV$</td>
<td>Ratio$^c$</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.067 ± 0.014</td>
<td>5.00 ± 0.40</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>0.8 M KC1</td>
<td>0.970 ± 0.103</td>
<td>4.31 ± 0.29</td>
<td>0.225</td>
<td></td>
</tr>
<tr>
<td>30 μM FAD</td>
<td>1.58 ± 0.23</td>
<td>4.96 ± 0.76</td>
<td>0.319</td>
<td></td>
</tr>
<tr>
<td>30 μM FAD, 0.8 M KC1</td>
<td>1.67 ± 0.05</td>
<td>5.25 ± 0.42</td>
<td>0.318</td>
<td></td>
</tr>
</tbody>
</table>

$^a$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.
NH₄Cl, or NaCl (all at 0.8N) the F₄₂₀-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 F₄₂₀-dependent activity during reactivation. Previous results have shown that the F₄₂₀-hydrogenase (29) and formate dehydrogenase of M. formicicum (33,34) require FAD to reduce F₄₂₀ but not MV. The results in Table 4 show that FAD dissociated from the F₄₂₀-hydrogenase under conditions of reactivation (F₄₂₀ plus H₂); KCl prevented the dissociation. This dissociation was apparently complete, since boiling the enzyme released nearly the same amount of FAD as reduction with H₂ in the presence of F₄₂₀ (Table 4).

The optimum temperature for F₄₂₀-dependent activity of the F₄₂₀-hydrogenase was 55°C, and the activation energy was calculated to be 10.0 Kcal/mol from an Arrhenius plot. F₄₂₀-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 MgCl₂ into the assay mixture stimulated F₄₂₀-dependent activity by about 2-fold. The effect of pH on F₄₂₀-dependent H₂ uptake and H₂
### TABLE 4. Dissociation of FAD from F₄₂₀-hydrogenase of M. formicicum.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FAD released (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 µM F₄₂₀, N₂ atm.</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>48 µM F₄₂₀, 0.8 M KCl, H₂ atm.</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>H₂ atm.</td>
<td>0.050 ± 0.050</td>
</tr>
<tr>
<td>48 µM F₄₂₀, H₂ atm.</td>
<td>0.564 ± 0.151</td>
</tr>
<tr>
<td>100°C, 15 min</td>
<td>0.534 ± 0.046</td>
</tr>
</tbody>
</table>

---

**a**Samples (0.2 ml, 0.5 mg protein) of F₄₂₀-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 N₂, flushed with N₂ or H₂ 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; O, 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 H₂ evolution to H₂ 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 F₄₂₀-hydrogenase reduced F₄₂₀, FO (the riboflavin analogue of F₄₂₀) (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 F₄₂₀, while that for MV was 42-fold greater.

**Immunoinhibition.** Anti-F₄₂₀-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 F₄₂₀-hydrogenase with the antiserum inhibited F₄₂₀-dependent H₂ 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 μg) 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); ($ε_{401} = 26.9 \text{ mM} \cdot \text{cm}^{-1}$). Each assay contained 2.6 μg purified $F_{420}$-hydrogenase.
<table>
<thead>
<tr>
<th>Acceptor^a</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F420</td>
<td>37</td>
<td>97.6</td>
</tr>
<tr>
<td>FO</td>
<td>80</td>
<td>98.6</td>
</tr>
<tr>
<td>FAD</td>
<td>87</td>
<td>53.5</td>
</tr>
<tr>
<td>FMN</td>
<td>61</td>
<td>121</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>89</td>
<td>78.0</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>1560</td>
<td>172</td>
</tr>
</tbody>
</table>

^aEach assay contained 0.9-1.4 μg of purified, reactivated F420-hydrogenase. The standard H2 uptake assay was used except that the following electron acceptors were substituted (concentration ranges used indicated in parentheses): F420 (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 F42O-hydrogenase from M. formicicum by specific antiserum. (A) Aliquots (10 μl, 1.7 μg protein) of purified, reactivated F42O-hydrogenase were mixed with aliquots (10 μl) of vacuum degassed anti-F42O-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 F42O-dependent (●) and MV-dependent (○) H2 uptake using the standard assays. 100% = 1.54 units/ml F42O-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 F42O-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 F₄₂⁰-hydrogenase from M. formicicum resembles the enzymes from M. thermoautotrophicum (15) and M. voltae (28) in containing a minimal F₄₂⁰-reducing unit of molecular weight approximately 110K comprised of α, β, and γ subunits. In contrast, the F₄₂⁰-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 F₄₂⁰-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 F₄₂⁰-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 F₄₂⁰-reducing αβγ trimers. Circular structures like those of the M. thermoautotrophicum enzyme were observed in electron micrographs of purified F₄₂⁰-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 F₄₂₀-dependent H₂ 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-F₄₂₀-hydrogenase antiserum to inhibit F₄₂₀-dependent but not MV-dependent activity of purified F₄₂₀-hydrogenase (Fig. 7) suggests that the sites where these electron acceptors are reduced are spatially separated, as proposed for the *M. thermoautotrophicum* enzyme (24).

F₄₂₀-hydrogenases in H₂-utilizing CO₂-reducing methanogenic bacteria apparently function to oxidize H₂ to provide electrons for at least one reductive step in the pathway (16). However, H₂ is produced during the utilization of formate as reductant in *M. formicicum* (31) implying the presence of a bidirectional hydrogenase in this organism. The F₄₂₀-hydrogenase described here has been shown to function as a bidirectional enzyme and to participate in the formate hydrogenlyase system of this organism (5).
ACKNOWLEDGEMENTS

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REFERENCES


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 $K_d = 4 \mu M$). 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 \( \text{H}_2/\text{CO}_2 \) 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 \( \text{F}_4\text{2O} \) (\( \text{F}_4\text{2O} \)). The \( \text{F}_4\text{2O} \)-reducing formate dehydrogenase of *M. formicicum* has been characterized (2,3,17,29,30,31,32), the genes encoding its two subunits (\( \alpha \) and \( \beta \)) have been cloned and sequenced (33), and its regulation has been investigated (18,24). *M. formicicum* and other \( \text{H}_2 \)-oxidizing methanogens have two distinct hydrogenases (12,13,21). One (\( \text{F}_4\text{2O} \)-hydrogenase) reduces \( \text{F}_4\text{2O} \) and methyl viologen (MV); the other (MV-hydrogenase) reduces MV but not \( \text{F}_4\text{2O} \). Several \( \text{F}_4\text{2O} \)-hydrogenases have been purified and characterized (5,10,19,34,39). Component A1 of the \( \text{H}_2 \)-coupled methyl coenzyme M methylreductase system of *Methanobacterium thermoautotrophicum* has \( \text{F}_4\text{2O} \)-dependent hydrogenase activity (20), suggesting a role for \( \text{F}_4\text{2O} \)-hydrogenase in \( \text{H}_2 \) uptake. A function for \( \text{F}_4\text{2O} \)-hydrogenase in \( \text{H}_2 \) production has not been investigated.

Cell extracts of methanogenic bacteria contain a formate hydrogenlyase system which requires \( \text{F}_4\text{2O} \) for activity (9,22,38). However, a requirement for additional
electron carriers has not been investigated, and the system has not been reconstituted \textit{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 \textit{M. formicicum} (5). Here, we report that F$_{420}$-hydrogenase, formate dehydrogenase and F$_{420}$ purified from \textit{M. formicicum} together reconstitute formate hydrogenlyase activity. We examine some properties of the reconstituted system and address its physiological role in formate metabolism.

\section*{MATERIALS AND METHODS}

\textbf{Cell extract preparation.} Cell extract of \textit{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 \textit{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).

\textbf{Enzyme purification.} The F$_{420}$-hydrogenase of \textit{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 H₂-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 F₄₂₀, 30 µM FAD, 50 µM sodium 2-bromoethane sulfonate (BES), 30 mM NaHCO₃, 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 N₂, 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 H₂ 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 NaN₃, 20 mM 2-mercaptoethanol, and 48 μM F₄₂₀. Purified, vacuum degassed F₄₂₀-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 H₂ 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 NaHCO₃, 30 μM FAD, 10 mM NaN₃, 20 mM 2-mercaptoethanol, and 48 μM F₄₂₀ (final pH 7.5). 50 μM sodium 2-bromoethane
sulfonate was included when cell extract was assayed. The reaction mixture was vacuum degassed with N₂, and NaHCO₃ (1 M stock solution) was added anaerobically through the stopper. Just before assay, the vials were flushed for 3 min with H₂:CO₂ (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 F₄₂₀-hydrogenase which had previously been reactivated by incubation for 30 min at 35°C in the presence of 82 kPa H₂, 48 μM F₄₂₀, 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 F₄₂₀-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 H₂
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 NaN₃ 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-F₄₂₀-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 N₂.

Chemicals. Coenzyme F₄₂₀ 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 F₄₂₀ restored activity (Fig. 1) to a level (47 nmoles H₂ produced/min/mg protein) similar to that of untreated cell extract. FAD stimulated formate hydrogenlyase activity when present with F₄₂₀ but did not restore activity by itself (Fig. 1). FMN, NAD, or NADP (all at 30 μM) did not replace F₄₂₀, either alone or in combination with FAD. These results are similar to those obtained with F₄₂₀-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.
F₄₂₀-hydrogenase, formate dehydrogenase, and F₄₂₀, all purified from *M. formicicum*, together reconstituted formate hydrogenlyase activity (3.26 μmoles H₂/min/mg F₄₂₀-hydrogenase) (Fig. 2). No activity was observed if any of these components or formate was omitted (data not shown). The Kₘ for F₄₂₀ in the reconstituted system was 12 μM. Optimal activity was obtained between pH 6.5 and 7.5, and no H₂ 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, F₄₂₀ remained fully reduced for at least 60 min.

**FAD requirement for formate hydrogenlyase activity.**

FAD is a component of the formate dehydrogenase (31) and F₄₂₀-hydrogenase (5) of *M. formicicum*. Both enzymes lose bound FAD under reduced conditions (5,21,30,31), yielding apoenzymes which can reduce MV but not F₄₂₀. FAD was also required for maximal activity of the reconstituted formate hydrogenlyase system; the rate of H₂ production decreased after 25 min in its absence (Fig. 2). Boiled cell extract substituted for FAD, but FADH₂, FMN, riboflavin, or NAD(P) did not (data not shown). As illustrated in Fig. 4, after 25 min into the formate hydrogenlyase reaction, the F₄₂₀-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 F₄₂₀-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. formicicium. Each assay contained 114 µg of purified formate dehydrogenase, 2.6 µg of F420-hydrogenase, and the indicated concentration of FAD. H₂ production rates were calculated from the slope of the time courses at 45 min. Inset: a Lineweaver-Burk plot of the data.
<table>
<thead>
<tr>
<th>Enzyme assayed</th>
<th>Activity (units/mg protein) after treatment of cell extract with:</th>
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<td></td>
<td>Control serum</td>
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<tr>
<td>Hydrogenase:</td>
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<tr>
<td>(\text{F}_{420})-dependent</td>
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<tr>
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<td>Methyl viologen-dependent</td>
<td>4.36</td>
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<tr>
<td>Formate hydrogenlyase</td>
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*Hydrogenase and formate dehydrogenase were assayed spectrophotometrically; one unit is the reduction of 1 mmole of acceptor per min. Formate hydrogenlyase was assayed using gas chromatography; one unit is the production of 1 mmole of \(\text{H}_2\) 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 immunoabsorption (control serum-treated=100%).*
MV-hydrogenase, which contributes about 98% of the MV-dependent \( \text{H}_2 \) uptake activity in cell extract (5), was not removed. The immunoadsorption of \( \text{F}_{420} \)-hydrogenase did not significantly affect formate dehydrogenase activity (Table 1). Immunoadsorption of formate dehydrogenase from cell extract substantially decreased \( \text{F}_{420} \)-dependent and MV-dependent formate dehydrogenase activity (Table 1), indicating a removal of the formate dehydrogenase. This treatment slightly decreased \( \text{F}_{420} \)-dependent but not MV-dependent \( \text{H}_2 \) uptake activity. Immunoadsorption of \( \text{F}_{420} \)-hydrogenase or formate dehydrogenase from cell extract substantially reduced formate hydrogenlyase activity (Table 1), and addition of purified \( \text{F}_{420} \)-hydrogenase or purified formate dehydrogenase fully restored activity (Fig. 6). Addition of partially purified MV-hydrogenase to \( \text{F}_{420} \)-hydrogenase-depleted extract did not restore formate hydrogenlyase activity (Fig. 6). These results indicate that the \( \text{F}_{420} \)-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 \text{M. formicicum} (31).

Reversibility of the formate hydrogenlyase. \( \text{F}_{420} \)-depleted cell extract produced formate from \( \text{H}_2 \) plus
Figure 6. Restoration of formate hydrogenlyase activity in formate dehydrogenase-depleted or F₄₂₀-hydrogenase-depleted cell extract of M. formicicum by addition of the purified enzymes. F₄₂₀-hydrogenase or formate dehydrogenase was removed from cell extract by immunoadsorption. Vacuum degassed solutions of F₄₂₀-hydrogenase, partially purified MV-hydrogenase, or formate dehydrogenase were added to the assay vials just prior to initiation of the assays. (A) F₄₂₀-hydrogenase-depleted extract with: ○, no additions; 3.6 (△) or 9.6 (□) µg F₄₂₀-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 \textit{M. formicicum}. Formate production from H\textsubscript{2} plus CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} was assayed as described in MATERIALS AND METHODS. (●, ○), DEAE-cellulose-treated extract, 0.46 mg protein (in 0.1 ml) per assay. (▲, Δ), reconstituted formate hydrogenlyase; each assay contained 5.4 µg purified formate dehydrogenase and 15.5 µg of purified, reactivated F\textsubscript{420}-hydrogenase. Symbols: ●, ▲, complete reaction mixture; ○, Δ, FAD omitted.
soluble intermediate electron carrier (F420), and FAD. The function of FAD in the M. formicicium formate hydrogenlyase was not as a free electron carrier between formate dehydrogenase and F420-hydrogenase, since no activity was obtained in the presence of FAD and absence of F420; rather, FAD appears to stabilize F420-dependent activity of the component enzymes. An F420: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 F420. Although M. formicicium has both F420-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 F420-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 F₄₂₀-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 H₂ 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 H₂ relative to CH₄ at pH 7.6 and 37°C (28). However, when the growth temperature is raised to 63°C, more H₂ is produced than CH₄ (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 H₂/CO₂ may also allow the cell to dispose of excess reducing potential during growth on either substrate.

However, the involvement of H₂ as an obligatory intermediate in one or more reductive steps during formate-dependent CO₂ reduction to methane can not be ruled out. Likewise, H₂ 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 H₂ partial pressure were decreased by a membrane-dependent H₂-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 H₂/CO₂ (28), even though these two substrates yield virtually the same free energy change per mole of CH₄ produced.

The reversibility of the formate hydrogenlyase suggests that this system may allow the cell to fix CO₂ as formate for biosynthesis. In fact, formate dehydrogenase is synthesized at high levels in M. formicicum cells grown on H₂/CO₂ alone (28). Recently, mutants of Methanobacterium thermoautotrophicum have been isolated which require formate for growth on H₂/CO₂ (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 H₂-producing activity of the F₄₂₀-hydrogenase; because formate
and formate dehydrogenase activity were in excess during the reaction, a constant supply of reduced F420 was available for H2 production by the hydrogenase. The ratio of H2-evolution to H2-uptake for the F420-hydrogenase was 0.30 (5), indicating that the enzyme is bidirectional. The optimal pH for H2 production by hydrogenases is often more acidic than that for H2 oxidation (15). However, the optimal pH for both H2 production and H2 oxidation by the M. formicicum F420-hydrogenase was near pH 7.5 (5).

ACKNOWLEDGMENTS

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LITERATURE CITED


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

SUMMARY

The F420-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 F420-hydrogenase was strongly hydrophobic, suggesting that it associates with isolated membranes through hydrophobic interactions.

INTRODUCTION

Two hydrogenases have been reported in H2-oxidizing methanogens (6,7,9,15). One enzyme (F420-hydrogenase) reduces coenzyme F420 (F420) and methyl viologen (MV). The other enzyme (MV-hydrogenase) reduces MV but not F420. 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, since no $F_{420}$-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 $\mu$M in $F_{420}$ and 30 $\mu$M in FAD and incubated in the dark for 1 h at 35°C. Under these
conditions, the F42O-hydrogenase is stabilized toward F42O-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 F42O or MV per min. F42O 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 F₄₂₀-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 F₄₂₀-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 F₄₂₀-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 F₄₂₀-dependent activity (I, Fig. 1A) could be accounted for in III and IV (Fig. 1B) after solubilization of membranes.

These results show that the F₄₂₀-hydrogenase of *M. formicicum* is associated with isolated membranes and suggest that the enzyme may be located on membranes in the intact cell. The F₄₂₀-hydrogenases studied to date are monomers which form aggregates of up to Mr 800,000 (5,6,7,15), including the enzyme from *M. formicicum* for which aggregates never exceed Mr 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: $\bullet$, $F_{420}$-dependent activity; $\bigcirc$
, MV-dependent activity; $\triangle$, 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 F420-hydrogenase which may have dissociated from the membranes. The carbon monoxide dehydrogenase complex of *Methanosarcina thermophila* (Mr 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 F420-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 F420-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 F420-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 F420-dependent activity, indicating that they represented the MV-hydrogenase. These results show that the F420-hydrogenase
Figure 2. Hydrophobic interaction chromatography of the F420-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: ○, F420-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 stadtmannae* is driven by a protonmotive force generated across the membrane by methanogenesis from methanol and H₂ (1,2), and membrane preparations from the H₂-oxidizing methanogen, *Methanobacterium thermoautotrophicum*, carry out H₂-driven ATP synthesis (4). Our results indicate that the F₄₂₀-hydrogenase of *M. formicicum* is associated with membranes, and therefore may be involved in ATP synthesis in this and other H₂-oxidizing methanogens.

The F₄₂₀-hydrogenases of *Methanobacterium* spp. are reported to be unstable (8,9). The F₄₂₀-hydrogenase from *M. formicicum* JF-1 contains bound FAD required for reduction of F₄₂₀ (9). Hydrophobic interaction chromatography converts the enzyme to a deflavo-species which reduces MV but not F₄₂₀; preincubation of the enzyme with FAD restores the F₄₂₀-dependent activity (9). Similarly, when the enzyme is reactivated by incubation under H₂ with F₄₂₀, it loses all F₄₂₀-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 F₄₂₀-hydrogenase and reconstituted any deflavo-enzyme present. The ratio of F₄₂₀-dependent to
MV-dependent activity of the peak in Fig. 2A was 21-fold greater than that of the peak of F₄₂₀-dependent activity in Fig. 2B. The reason for this is unknown; perhaps the dissociated form of the F₄₂₀-hydrogenase is irreversibly converted to deflavo-enzyme, while the conversion of the membrane-associated form is reversible.

ACKNOWLEDGMENTS

We thank S. for growing the cells. This work was supported by Grant DMB-8409558 from the National Science Foundation and by project 2124920 from the Commonwealth of Virginia. S. F. B. was the recipient of a Pratt Animal Nutrition Fellowship from the College of Agriculture and Life Sciences.

LITERATURE CITED


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 H₂ as the electron donor for CO₂ 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 F₄₂⁰-reducing formate dehydrogenase of *M. formicicum* has been characterized (4,5,20,29,31,32), 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 H₂-oxidizing methanogens have two distinct hydrogenases (14,15,25,36). One (F₄₂⁰-hydrogenase) reduces F₄₂⁰ and methyl viologen (MV); the other (MV-hydrogenase) reduces MV but not F₄₂⁰. Several F₄₂⁰-hydrogenases have been purified and characterized (11,23,34,36, S. F. Baron and J. G. Ferry, manuscript in preparation). The F₄₂⁰-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-\textit{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}\text{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
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 N₂) polycarbonate screw cap centrifuge tube, which was kept anaerobic during transfers by flushing with a stream of N₂. Cell pellets were resuspended by vortexing. Cell paste (2.0 g) from a late log phase culture (A₅₅₀=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 NaN₃; 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 MgCl₂ containing 10 mM NaN₃ 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 H₂, 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 F₄₂₀ 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 Mr 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 Mr 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 *M. formicicum* 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\textsubscript{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\textsubscript{420}-hydrogenase are membrane-associated in whole cells.

**Osmotic shock experiments.** Whole cells had minimal F\textsubscript{420}-dependent formate dehydrogenase activity but comparatively more MV-dependent formate dehydrogenase activity. The shock fluid from osmotically treated cells contained negligible F\textsubscript{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\textsubscript{420}-dependent hydrogenase activity but significant MV-dependent hydrogenase activity. The shock fluid from osmotically treated cells contained negligible F\textsubscript{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\textsubscript{420}-hydrogenase of M.
Figure 3. Immunogold labeling of thin sections of *M. formicicum* whole cells with anti-F₄₂₀-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>
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<tr>
<th>Fraction</th>
<th>Formate dehydrogenase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hydrogenase activity&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
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<tr>
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<td>F₄20-dependent</td>
<td>MV-dependent</td>
</tr>
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<td>Whole cells</td>
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<td>Shock fluid</td>
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<td>0.6</td>
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<td>Shaken cell lysate</td>
<td>5.71</td>
<td>99.6</td>
</tr>
<tr>
<td>Untreated cell lysate</td>
<td>5.93</td>
<td>103</td>
</tr>
</tbody>
</table>

<sup>a</sup>The total protein contents of the shock fluid, shaken cell lysate, and untreated cell lysate were 0.06, 7.81, and 7.36 mg, respectively. Protein content of the whole cells was not determined.

<sup>b</sup>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 5.8 ml volume.

<sup>c</sup>Percent of the total activity of shock fluid plus shaken 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 F₄₂₀-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 F₄₂₀-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 F₄₂₀-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 H₂-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, Mg²⁺ and ATP (24). Component C is
thought to catalyze the reduction of methyl coenzyme M to methane; component A1 has F420-dependent hydrogenase activity. Blaut and Gottschalk (9) demonstrated that methanogenesis from methanol plus H2 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 F0F1-ATPase of E. coli (21). Thus, three enzymes of electron transfer in methanogens (formate dehydrogenase, F420-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 H2) 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\textsubscript{420}-hydrogenase, and F\textsubscript{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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Properties and functions of the F420-hydrogenase

F420-hydrogenases from six H2-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, F420-active species of molecular weight approximately 110K. All F420-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 F420-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 F420-reducing formate
Table 1. Properties of $F_{420}$-hydrogenases purified from methanogenic bacteria.

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<tr>
<th>Location</th>
<th>Methanobacterium</th>
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<th>Methanosporillum</th>
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<td>thermoautotrophic</td>
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<td>volatii</td>
<td>barkeri</td>
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<td>$H_2$ evolution</td>
<td>$PO_2$</td>
<td>$F_{420}H_2^+$</td>
<td>$MV_5^+$</td>
<td>$MV_5^+$</td>
</tr>
<tr>
<td>Reference</td>
<td>49,81,82,141</td>
<td>this work</td>
<td>94.95</td>
<td>150</td>
</tr>
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</table>

*aMolecular weight in thousands.

*bNumber of subunits in monomer or complex (if available) ± molecular weight in thousands.

*cAll are based on the monomer molecular weight, except that of $M$. barkeri, which is based on the 60K subunit.

*dMaximum $H_2$ uptake activity reported (complex $F_{420}$ reduced/mg protein).

*eWith the indicated electron donor. $MV_5^+$ 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. formicicium 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_{42O}-hydrogenases also contain nickel and sometimes selenium (refer to Table 1).

F_{42O}-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_{42O}-dependent hydrogenase activity, and reduced F_{42O} 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_{42O}-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_{42O}-hydrogenase in cell extract (see SECTION V). Moreover, the MV-hydrogenase could not support formate hydrogenlyase activity in F_{42O}-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 H₂ production during fermentation, while the latter may supply reduced ferredoxin for N₂ fixation (24).

Escherichia coli (111) and Salmonella typhimurium (112) have three membrane-bound hydrogenase isoenzymes (1, 2, and 3). Isoenzyme 1 functions in H₂ uptake during fermentative growth, isoenzyme 2 in respiratory H₂ uptake, and isoenzyme 3 in H₂ 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: F₄₂₀-hydrogenase and formate dehydrogenase; a soluble component: F₄₂₀; and FAD, required for F₄₂₀-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 H₂-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 c₃ and a cytochrome c₃ 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. H₂ 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 H₂ 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 H₂/CO₂. The reverse formate hydrogenlyase could allow H₂-oxidizing methanogenic bacteria to fix CO₂ as formate for biosynthesis. Mutants of M. thermoautotrophicum have recently been isolated which require formate for growth on H₂/CO₂ (R. S. Tanner and D. P. Nagle, Abstr. Ann. Meet. Am. Soc. Microbiol., I-10, p.
Certain clostridia use formate generated by a ferredoxin-dependent CO₂ reductase for the biosynthesis of purines, pyrimidines, and methionine (134).

H₂ produced by the formate hydrogenlyase system could be an obligate intermediate in methanogenesis from formate, analogous to H₂-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 H₂ by the F₄₂₀-mediated formate hydrogenlyase system at the internal side of the membrane. The H₂ 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 F₄₂₀-hydrogenase and formate dehydrogenase**

As discussed in SECTION V, the F₄₂₀-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 H₂ cycling during methanogenesis from formate. FDH, formate dehydrogenase; H₂ase, F₄₂₀-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 F₄₂₀-hydrogenase (141) and the methanoreductosome complex (86). 1A, building block: αβγ trimer of F₄₂₀-hydrogenase; 1B, F₄₂₀-hydrogenase aggregate composed of 8 αβγ trimers arranged as two stacked rings. 2, Methanoreductosome structure. Rᵥ, head assembly containing methylreductase component C; Rₘ, stem structure; Rₜ, membrane attachment protein; CM, cell membrane.
electron micrographs of $\text{F}_{420}$-hydrogenases. An association of $\text{F}_{420}$-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)


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