JOURNAL OF BACTERIOLOGY, July 1989, p. 3854–3859
0021-9193/89/073854-06$02.00/0

Vol. 171, No. 7

Reconstitution and Properties of a Coenzyme F_{420}-Mediated Formate Hydrogenlyase System in *Methanobacterium formicicum*

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Received 14 December 1988/Accepted 6 April 1989

Formate hydrogenlyase activity in a 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 added flavin adenine dinucleotide (FAD) for maximal activity. 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 the cell extract greatly reduced formate hydrogenlyase activity; addition of the purified enzymes restored activity. The formate hydrogenlyase activity was reversible, since both the cell extract and the reconstituted system produced formate from H_2 plus CO_2 and HCO_3^-.

Methanobacterium formicicum is one of several methanogenic bacteria which can use either formate or H_2 plus CO_2 for growth and methanogenesis (1). During growth on either substrate, this organism synthesizes a hydrogenase and formate dehydrogenase which both reduce the physiological electron acceptor coenzyme F_{420} (F_{420}) (28). The F_{420}-reducing formate dehydrogenase of *M. formicicum* has been characterized (2, 3, 17, 29–32), the genes encoding its two subunits (α and β) have been cloned and sequenced (33), and its regulation has been investigated (16, 23). *M. formicicum* and other H_2-oxidizing methanogens have two distinct hydrogenases (11, 12, 20). One (F_{420}-hydrogenase) reduces F_{420} and methyl viologen (MV); the other (MV-hydrogenase) reduces MV but not F_{420}. Several F_{420}-hydrogenases have been purified and characterized (5, 9, 18, 34, 39). Component A1 of the H_2-coupled methyl coenzyme M methylreductase system of *Methanobacterium thermoautotrophicum* has F_{420}-dependent hydrogenase activity (19), suggesting a role for F_{420}-hydrogenase in H_2 uptake. A function for F_{420}-hydrogenase in H_2 production has not been investigated.

Cell extracts of methanogenic bacteria contain a formate hydrogenlyase system which requires F_{420} for activity (8, 21, 38). However, a requirement for additional electron carriers has not been investigated, and the system has not been reconstituted in vitro with purified components. This system is reversible, since formate is synthesized from H_2 plus CO_2 or HCO_3^- in cell extracts (21) and whole cells (7).

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

MATERIALS AND METHODS

Cell extract preparation. General anaerobic procedures were as described before (28), 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. The cell extract of *M. formicicum* was obtained by anaerobic centrifugation (30,000 × *g*, 20 min, 4°C) of an anaerobically prepared French pressure cell lysate (5). The cell extract was depleted of F_{420} and other highly anionic materials by anaerobic DEAE-cellulose chromatography, essentially as described by Tzeng et al. (38). However, bound protein was eluted with equilibration buffer containing 0.35 M KCl, and F_{420} and other highly anionic material was eluted with 2 M NaCl. Boiled cell extract was prepared anaerobically as described before (19).

Enzyme purification. The F_{420}-hydrogenase of *M. formicicum* was purified aerobically as described before (5). The MV-hydrogenase of *M. formicicum* was partially purified aerobically 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 and electroelution. The formate dehydrogenase of *M. formicicum* was purified anaerobically (31), and low-molecular-weight compounds were removed by anaerobic ultrafiltration as described before (30).

Enzyme assays. Hydrogen uptake (20) and formate dehydrogenase (30) activities were assayed spectrophotometrically at 35°C and pH 7.5 as described before. One unit of activity was defined as the reduction of 1 μmol of acceptor per min. Initial velocities were used for calculations. Hydrogenase samples for H_2 uptake assays were vacuum degassed and reductively reactivated as described before (5). Protein was determined by the dye-binding assay (micromethod) of Bradford (6), with bovine serum albumin as the standard.

Formate hydrogenlyase activity in cell extracts was assayed anaerobically at 35°C in 2-ml serum vials (no. 223713; Wheaton Scientific, Millville, N.J.) filled with O_2-free N_2 (82 kPa) and sealed with black butyl rubber bungs. The complete reaction mixture (0.1 ml) contained 48 μM F_{420}, 30 μM flavin adenine dinucleotide (FAD), 50 μM sodium 2-bromoethane sulfonate, 30 mM NaHCOO, and anaerobically prepared cell

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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 O_2-free N_2, cell extract was added anaerobically, and flushing was continued for 3 min. The reaction was initiated with formate. Gas samples (50 μl) were removed at intervals with a Pressure-Lok syringe (Supelco, Inc., Bellefonte, Pa.) and assayed for H_2 by thermal conductivity gas chromatography as described before (15). Activities were calculated from the linear portion of the time course data obtained.

The reconstituted formate hydrogenlyase was assayed anaerobically at 35°C in 5-ml serum vials (no. 223738; Wheaton Scientific) filled with O_2-free N_2 (82 kPa) and sealed with black butyl rubber bungs. The complete reaction mixture (0.5 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 30 mM sodium formate, 30 μM FAD, 10 mM NaN_3, 20 mM 2-mercaptoethanol, and 48 μM F_{420}. Purified F_{420}-hydrogenase was vacuum degassed with N_2, added to vacuum-degassed reaction mixture, and incubated at 35°C for 30 s, followed by the addition of formate dehydrogenase. Gas samples (0.2 ml) were withdrawn at intervals and assayed for H_2 as above.

Formate hydrogenlyase activity in the reverse direction (formate formation) was assayed anaerobically at 35°C in 5-ml serum vials as described above. The complete reaction mixture (0.5 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 60 mM $NaHCO_3$, 30 μM FAD, 10 mM NaN_3, 20 mM 2-mercaptoethanol, and 48 μM F_{420} (final pH, 7.5). When the cell extract was assayed, 50 μM sodium 2-bromoethane sulfonate was included. The reaction mixture was vacuum degassed with N_2, and $NaHCO_3$ (1 M stock solution) was added anaerobically through the stopper. Just before assay, the vials were flushed for 3 min with O_2-free H_2-CO_2 (4:1). For assay of the 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 s at 35°C. The reaction was then initiated with purified F_{420}-hydrogenase which had previously been vacuum degassed and reactivated by incubation for 30 min at 35°C in the presence of 82 kPa of H_2, 48 μM F_{420}, and 0.7 M KCl. Samples (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 high-pressure liquid chromatography as described before (15), except that the flow rate was 0.5 ml/min. The detection limit was about 50 μM.

Enzyme stability in the reconstituted formate hydrogenlyase. The formate hydrogenlyase system was reconstituted with or without 30 μM FAD as described above. Samples (20 μl) were withdrawn from the reaction mixture at intervals; 10 μl was used to assay for F_{420}-dependent activity, and the other 10 μl was used to assay for MV-dependent activity of the formate dehydrogenase or (in separate experiments) the hydrogenase. The standard formate dehydrogenase and H_2 uptake assays were used. However, the formate dehydrogenase assays were initiated with enzyme sample rather than formate, and endogenous activity was not determined. Because 0.6 mM formate was carried over from the formate hydrogenlyase assay to the hydrogenase assay, 10 mM NaN_3 was included in the hydrogenase assay mixtures to inhibit residual formate dehydrogenase activity (29). Hydrogenase activity was not affected by azide at this concentration.

Immunoadsorption. Anti-F_{420}-hydrogenase antiserum (5a) and anti-formate dehydrogenase antiserum (33) were prepared as described before. All of the following steps were performed in an anaerobic glove bag with an atmosphere of 95% N_2 and 5% H_2. 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 portions. Each portion was allowed to drain completely into the bed and incubate at 25°C for 5 to 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 portions as above, and the eluates were collected in chilled 5-ml amber serum vials. Residual extract was removed from the columns by centrifugation and pooled with the rest of the eluate. The treated extracts were stored in liquid N_2.

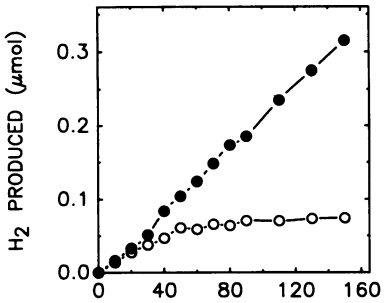

FIG. 1. Reconstitution of the formate hydrogenlyase of *M. formicicum* with purified components. Purified formate dehydrogenase (14 μg of protein) was added to the reaction mixture first and allowed to incubate for 5 min. The reaction was then initiated with purified F_{420}-hydrogenase (0.7 μg of protein). Symbols: ●, complete reaction mixture; ○, FAD omitted.

Chemicals. Coenzyme F_{420} was purified aerobically from the cell extract of *M. formicicum* by DEAE-cellulose chromatography, followed by gel filtration as described before (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 the cell extract of *M. formicicum* was abolished when highly anionic material was removed by DEAE-cellulose chromatography. However, addition of purified F_{420} restored activity to a level (47 nmol of H_2 produced per min per mg of protein) similar to that of untreated cell extract. FAD slightly stimulated formate hydrogenlyase activity when present with F_{420} but did not restore activity by itself. Flavin mononucleotide, NAD, or NADP (each at 30 μM) could not replace F_{420} either alone or in combination with FAD. These results are similar to those obtained with F_{420}-depleted cell extracts from other methanogenic bacteria (8, 21, 38).

F_{420}-hydrogenase, formate dehydrogenase, and F_{420}, all purified from *M. formicicum*, together reconstituted formate hydrogenlyase activity (3.26 μmol of H_2 per min per mg of F_{420}-hydrogenase) (Fig. 1). No activity was observed if any of these components or formate was omitted (data not shown). The K_m for F_{420} in the reconstituted system was 12 μM. Optimal activity was obtained between pH 6.5 and 7.5, and no H_2 was produced below pH 5.0 (5). Activity was dependent on the amount of purified hydrogenase or formate

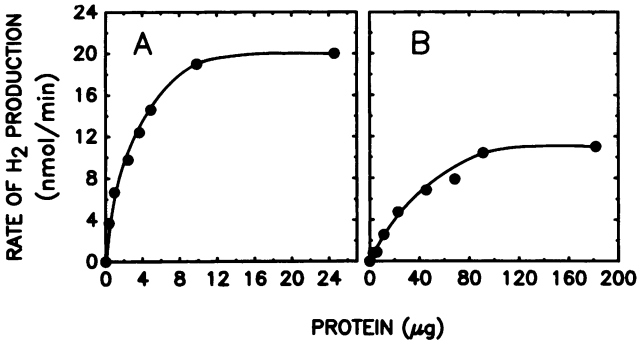

FIG. 2. Dependence of the reconstituted formate hydrogenlyase activity on the amount of formate dehydrogenase and F_{420}-hydrogenase added. (A) F_{420}-hydrogenase varied, formate dehydrogenase held constant (114 μg of protein); (B) formate dehydrogenase varied, F_{420}-hydrogenase held constant (2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

dehydrogenase added (Fig. 2). The system was routinely reconstituted with the formate dehydrogenase activity in excess, and under these conditions, F_{420} remained fully reduced for at least 60 min.

FAD requirement for formate hydrogenlyase activity. FAD is a component of the formate dehydrogenase (31) and F_{420}-hydrogenase (5) of *M. formicicum*. Both enzymes lose bound FAD under reduced conditions (5, 20, 30, 31), yielding apoenzymes which can reduce MV but not F_{420}. FAD was also required for maximal activity of the reconstituted formate hydrogenlyase system; the rate of H_2 production decreased after 25 min in its absence (Fig. 1). Maximal activity was obtained with 30 to 50 μM FAD (Fig. 3). Boiled cell extract could substitute for FAD, but $FADH_2$, flavin mononucleotide, riboflavin, and NAD(P) could not (data not shown). At 25 min into the formate hydrogenlyase reaction, the F_{420}-dependent and MV-dependent activities of the formate dehydrogenase were 0.40 and 2.2 U/ml, respectively, in the presence of FAD; in the absence of FAD, they were 0.05 and 2.0 U/ml, respectively. Similarly, after 25 min, the F_{420}-dependent and MV-dependent activities of the F_{420}-hydrogenase were 0.34 and 1.4 U/ml, respectively, in the presence of FAD, while in the absence of FAD, they

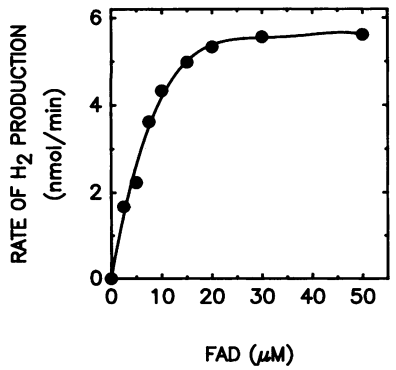

FIG. 3. FAD dependence of the reconstituted formate hydrogenlyase from *M. formicicum*. Each assay mix contained 114 μg of purified formate dehydrogenase, 2.6 μg of F_{420}-hydrogenase, and the indicated concentrations of FAD. H_2 production rates were calculated by using tangents to the time courses at 45 min. The rates plotted were corrected for the activity obtained without FAD (1.5 nmol/min).

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

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

[a] Hydrogenase and formate dehydrogenase were assayed spectrophotometrically. Formate hydrogenlyase was assayed by gas chromatography; 1 U indicates the production of 1 nmol of H_2 per min.

[b] Cell extract was treated with the indicated antiserum or control adsorbed to protein A-Sepharose CL-4B. Activities reported are means from at least three assays. Parentheses indicate the percentage of activity remaining after immunoadsorption (control-serum-treated value taken as 100%).

were 0.07 and 0.90 U/ml, respectively. 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.

Effect of immunoadsorption of F_{420}-hydrogenase or formate dehydrogenase from cell extract on formate hydrogenlyase activity. Western immunoblot analysis of the cell extract reveals that anti-F_{420}-hydrogenase and anti-formate dehydrogenase antisera react specifically with the respective enzymes (5a). Immunoadsorption of F_{420}-hydrogenase from the 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 the cell extract, while the MV-hydrogenase, which contributes about 98% of the MV-dependent H_2 uptake activity in cell extracts (5), was not removed. The immunoadsorption of F_{420}-hydrogenase did not significantly affect formate dehydrogenase activity (Table 1). Immunoadsorption of formate dehydrogenase from the cell extract substantially decreased F_{420}-dependent and MV-dependent formate dehydrogenase activity (Table 1), indicating removal of the formate dehydrogenase. This treatment slightly decreased F_{420}-dependent but not MV-dependent H_2 uptake activity. Immunoadsorption of F_{420}-hydrogenase or formate dehydrogenase from the cell extract substantially reduced formate hydrogenlyase activity (Table 1), and addition of purified F_{420}-hydrogenase or purified formate dehydrogenase restored activity fully (Fig. 4). Addition of partially purified MV-hydrogenase to F_{420}-hydrogenase-depleted extract did not restore formate hydrogenlyase activity (Fig. 4). These results indicate that the F_{420}-hydrogenase is the only hydrogenase in the cell extract capable of supporting formate hydrogenlyase activity. The results also confirm the requirement for the formate dehydrogenase in this system; moreover, only one formate dehydrogenase is known to be present in *M. formicicum* (31).

Reversibility of the formate hydrogenlyase. F_{420}-depleted cell extract produced formate from H_2 plus CO_2 and HCO_3^- when purified F_{420} was added (Fig. 5), indicating that F_{420}-

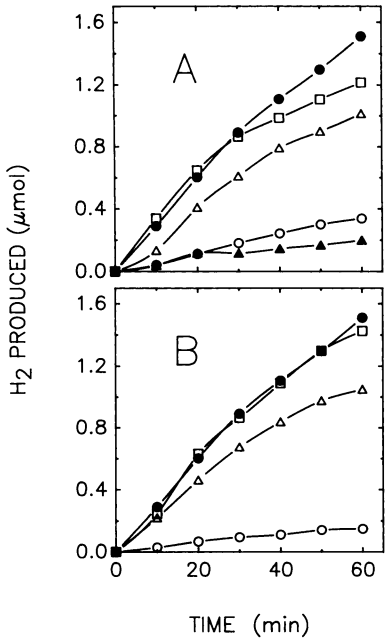

FIG. 4. Restoration of formate hydrogenlyase activity in formate dehydrogenase-depleted or F_{420}-hydrogenase-depleted cell extract of *M. formicicum* by addition of the purified enzymes. F_{420}-hydrogenase or formate dehydrogenase was removed from the cell extract by immunoadsorption. Vacuum-degassed solutions of F_{420}-hydrogenase, partially purified MV-hydrogenase, or formate dehydrogenase were added to the assay vials just prior to initiation of the assays. (A) F_{420}-hydrogenase-depleted extract with (○) no additions, 3.6 (△) or 9.6 (□) μg of F_{420}-hydrogenase added; (▲) 101 μg of partially purified MV-hydrogenase added; or (●) control serum added. (B) Formate dehydrogenase-depleted extract with (○) no additions; 11 (△) or 41 (□) μg of formate dehydrogenase added; or (●) control serum added.

mediated formate hydrogenlyase activity was reversible. No formate was detected when H_2, CO_2 plus HCO_3^-, or F_{420} was omitted (data not shown). FAD stimulated formate production 6.3-fold when present with F_{420} (Fig. 5) but did not mediate activity without F_{420} (data not shown).

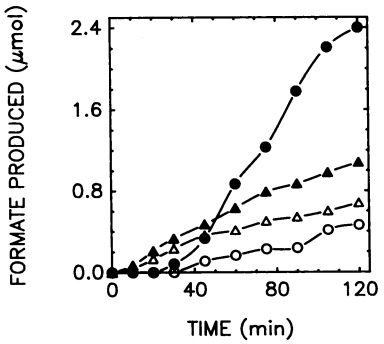

FIG. 5. Reversibility of the formate hydrogenlyase of *M. formicicum*. Formate production from H_2 plus CO_2 and HCO_3^- was assayed as described in Materials and Methods. Symbols: ●,○, DEAE-cellulose-treated extract, 0.46 mg of protein (in 0.1 ml) per assay; ▲,△, reconstituted formate hydrogenlyase (each assay contained 5.4 μg of purified formate dehydrogenase and 15.5 μg of purified, reactivated F_{420}-hydrogenase). Solid symbols, Complete reaction mixture; open symbols, FAD omitted.

The reconstituted formate hydrogenlyase also produced formate from H_2 plus CO_2 and HCO_3^- (Fig. 5). In these assays, hydrogenase activity was 5.5-fold in 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 plus HCO_3^- was omitted (data not shown). FAD stimulated activity about 1.3-fold in the presence of F_{420} (Fig. 5) but could not replace F_{420} (data not shown).

DISCUSSION

Formate hydrogenlyase systems function in a variety of anaerobic bacteria (10); however, none of these systems have been completely defined. The formate hydrogenlyase system of *M. formicicum* consisted of only two protein components (F_{420}-hydrogenase and formate dehydrogenase), a soluble intermediate electron carrier (F_{420}), and FAD. The function of FAD in the *M. formicicum* formate hydrogenlyase was not as a free electron carrier between formate dehydrogenase and F_{420}-hydrogenase, since no activity was obtained in the presence of FAD and absence of F_{420}; rather, FAD appears to stabilize the F_{420}-dependent activity of the component enzymes. An F_{420}:NADP oxidoreductase is present in several methanogenic bacteria (13, 29, 38); however, NADP was not required for formate hydrogenlyase activity, nor were any other intermediate electron carriers besides F_{420}. Although *M. formicicum* has both F_{420}-hydrogenase and MV-hydrogenase activities (11, 12, 20), the latter could not support formate hydrogenlyase activity, since addition of the partially purified enzyme did not restore formate hydrogenlyase activity in an F_{420}-hydrogenase-depleted cell extract.

The formate hydrogenlyase systems of *Escherichia coli* (24) and *Rhodopseudomonas palustris* (26) consist of a soluble formate dehydrogenase, a particulate hydrogenase, and one or more unidentified intermediate electron carriers. Cytochrome c_3 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 (5a) and F_{420}-hydrogenase (4, 5a) of *M. formicicum* are both membrane associated, suggesting a role in electron transport. However, the physiological function of formate hydrogenlyase in methanogenic bacteria is not known. Cultures of *Methanococcus vannielii* produce increasing amounts of H_2 as the pH rises from 7.6 to 8.8 during growth on formate (35). Cultures of *M. formicicum* growing on formate produce only small amounts of H_2 relative to CH_4 at pH 7.6 and 37°C (28). However, when the growth temperature is raised to 63°C, more H_2 is produced than CH_4 (28). These observations are consistent with a role for the formate hydrogenlyase system in maintaining the redox balance in formate-grown cells, especially when methanogenesis is impaired. The ability to interconvert formate and H_2 plus CO_2 may also allow the cell to dispose of excess reducing potential during growth on either substrate.

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

reaction may be required for reductive biosynthesis. Because the reaction has a standard free energy change of +1.3 kJ (37), the cell may not be able to derive additional energy unless the H_2 partial pressure is decreased by a membrane-dependent H_2-cycling mechanism similar to that proposed for the sulfate-reducing bacteria (22).

Interestingly molar growth yields of *M. formicicum* grown with formate are about 1.4-fold greater than those for cells grown on H_2 plus CO_2 (28), even though these two substrates yield virtually the same free energy change per mole of CH_4 produced.

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

Reconstitution of the *M. formicicum* formate hydrogenlyase offered a convenient assay for the H_2-producing activity of the F_{420}-hydrogenase; because formate and formate dehydrogenase activity were in excess during the reaction, a constant supply of reduced F_{420} was available for H_2 production by the hydrogenase. The ratio of H_2 evolution to H_2 uptake for the F_{420}-hydrogenase was 0.30 (5), indicating that the enzyme acts bidirectionally. The optimal pH for H_2 production by hydrogenases is often more acidic than that for H_2 oxidation (14). However, the optimal pH for both H_2 production and H_2 oxidation by the *M. formicicum* F_{420}-hydrogenase was near pH 7.5 (5).

Reduction of the formate dehydrogenase or F_{420}-hydrogenase of *M. formicicum* causes bound FAD to dissociate, yielding F_{420}-inactive apoenzymes (5, 20, 30, 31). These results suggest that flavin binding is redox dependent, as also shown for the NAD-dependent hydrogenase from *Alcaligenes eutrophus* (25). FAD greatly stimulated H_2 evolution from formate by the reconstituted formate hydrogenlyase system (Fig. 1 and 3). Although FAD greatly stimulated formate formation from H_2 and CO_2 with crude extracts (Fig. 5), a similar amount of FAD stimulation was not obtained with the reconstituted system (Fig. 5). This apparent discrepancy with the reconstituted system cannot be explained by the data presented but may result from a difference in the redox states during turnover of F_{420}-hydrogenase and formate dehydrogenase which prevented binding of exogenously supplied FAD.

ACKNOWLEDGMENTS

This work was supported by funds from the National Science Foundation (grant DMB-8409558) and the Commonwealth of Virginia (Project 2124920). S.F.B. was the recipient of a Pratt Animal Nutrition Fellowship from the College of Agriculture and Life Sciences.

We thank Neil Schauer and Hal May for generous gifts of purified formate dehydrogenase and anti-formate dehydrogenase antiserum. We also thank Pete Jablonski for growing the cells and for help with photography.

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