A Reversible Electron-Bifurcating Ferredoxin- and NAD-Dependent [FeFe]-Hydrogenase (HydABC) in *Moorella thermoacetica*

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Moorella thermoacetica was long the only model organism used to study the biochemistry of acetogenesis from CO₂. Depending on the growth substrate, this Gram-positive bacterium can either form H₂ or consume it. Despite the importance of H₂ in its metabolism, a hydrogenase from the organism has not yet been characterized. We report here the purification and properties of an electron-bifurcating [FeFe]-hydrogenase from *M. thermoacetica* and show that the cytoplasmic enzyme efficiently catalyzes both H₂ formation and H₂ uptake. The purified heterotrimeric iron-sulfur flavoprotein (HydABC) catalyzed the coupled reduction of ferredoxin (Fd) and NAD⁺ with H₂ at 55°C at pH 7.5 at a specific rate of about 100 μmol min⁻¹ mg protein⁻¹ and the reverse reaction, the coupled reduction of protons to H₂ with reduced ferredoxin and NADH, at a specific rate of about 10 μmol min⁻¹ mg protein⁻¹ in the stoichiometry $\text{Fd}_{\text{ox}} + \text{NAD}^+ + 2\text{H}_2 \rightleftharpoons \text{Fd}_{\text{red}}^{2-} + \text{NADH} + 3\text{H}^+$. When ferredoxin from *Clostridium pasteurianum*, NAD⁺, and the enzyme were incubated at pH 7.0 under 100% H₂ in the gas phase ($E_0' = -414$ mV), more than 95% of the ferredoxin ($E_0' = -400$ mV) was reduced, which indicated that ferredoxin reduction with H₂ is driven by the exergonic reduction of NAD⁺ ($E_0' = -320$ mV) with H₂. In the absence of NAD⁺, ferredoxin was not reduced. We identified the genes encoding HydABC within the transcriptional unit *hydCBAX* and mapped the transcription start site.

Until recently, *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) (1) had been the only model organism for the study of the biochemistry of acetogenesis. What we know of the mechanism of the total synthesis of acetate from CO₂ is derived mostly from studies of the enzymes in this bacterium by the groups of Wood (2), Ljungdahl (3), Ragsdale (4), Drake (5), and Lindahl (6). Acetate synthesis from CO₂ is associated with ATP synthesis, as evidenced by growth of acetogens on H₂ and CO₂ (7–9). However, despite more than 40 years of intensive investigations, the mechanism of energy conservation is not understood (10).

Acetobacterium woodii was recently introduced as another model organism for the study of energy conservation in acetogens. With this organism, considerable progress in the understanding of the mechanism of energy conservation during growth on H₂ and CO₂ has been made (11–13). However, *A. woodii* differs from *M. thermoacetica* in many respects. *M. thermoacetica* contains cytochromes, menaquinone, and an electron-bifurcating NAD-dependent reduced ferredoxin:NADP⁺ oxidoreductase complex (NfnAB) (10, 14, 15), which are not found in *A. woodii* (12, 16). On the contrary, *A. woodii* contains an energy-conserving reduced ferredoxin:NAD⁺ oxidoreductase complex (RnfABCDEG [RnfA–G]) and is sodium ion dependent (11, 16), two important properties not shared by *M. thermoacetica* (4, 17). Apparently, the cytochrome-containing and cytochrome-free acetogens differ in their mechanism of energy conservation, as do cytochrome-containing and cytochrome-free methanogenic archaea (18).

M. thermoacetica ferments glucose to three acetic acids with the concomitant formation of small amounts of H₂ (5, 19, 20). The Gram-positive anaerobic bacterium can also grow on H₂ and CO₂, forming acetic acid as an end product (8, 9). The hydrogenase(s) involved in H₂ formation and H₂ uptake has not yet been characterized, although it is central to the understanding of the energy metabolism. What is known from biochemical studies is that cell

extracts of glucose-grown cells catalyze the coupled reduction of ferredoxin (Fd) and NAD⁺ with H₂, and cell extracts from H₂-grown cells additionally catalyze the reduction of NADP⁺ with H₂ (10). The presence of two hydrogenases in *M. thermoacetica* was shown decades ago by using viologen dyes as an electron acceptor (8, 19, 21).

The genome sequence of *M. thermoacetica* indicates the presence of two gene clusters that each encode a cytoplasmic heteromeric [FeFe]-hydrogenase and genes encoding a membrane-bound [NiFe]-hydrogenase of the Ech type in association with a formate dehydrogenase (10, 17). The deduced amino acid sequence of one of the heteromeric [FeFe]-hydrogenases shows similarity to the amino acid sequences of the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenases from *Thermotoga maritima* (22) and from *A. woodii* (13); the deduced amino acid sequence of the other heteromeric [FeFe]-hydrogenase shows sequence similarity to the amino acid sequence of NADP⁺-reducing [FeFe]-hydrogenase from *Desulfovibrio fructosovorans* (23). The two [FeFe]-hydrogenases also show sequence similarity to each other, which prohibits a prediction of which gene cluster encodes the electron-bifurcating [FeFe]-hydrogenase and which encodes the NADP-reducing [FeFe]-hydrogenase.

To date, two electron-bifurcating [FeFe]-hydrogenases have been characterized, one from *T. maritima* (22, 24) and one from

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A. woodii (13). The purified enzyme from *T. maritima* is a heterotrimer (HydABC) and catalyzes the formation of two H_2 molecules from 1 NADH and 1 reduced ferredoxin (Fd_{red}^{2-}), which is thought to be the physiological function in the hyperthermophile growing on glucose. Whether the enzyme can also catalyze the reverse reaction has not been reported. HydA harbors the H cluster (active site), three [4Fe4S] clusters, and two [2Fe2S] clusters; HydB harbors three [4Fe4S] clusters, one [2Fe2S] cluster, and one flavin mononucleotide (FMN); and HydC harbors one [2Fe2S] cluster (24). The purified enzyme from *A. woodii* is a heterotetrameric (HydABCD) iron-sulfur flavoprotein containing 36 irons and 1 FMN and catalyzes the coupled reduction of NAD^+ and ferredoxin with H_2 , which is thought to be the physiological function in the mesophilic acetogen growing on H_2 and CO_2 . Whether the enzyme can also catalyze the formation of H_2 from NADH and Fd_{red}^{2-} has not been investigated (13). Also not answered is the question of whether these heteromeric [FeFe]-hydrogenases from *T. maritima* and *A. woodii* catalyze reactions that are not only stoichiometrically but also energetically coupled. Is an endergonic reaction under the experimental conditions used really driven by an exergonic reaction, or are only two exergonic reactions linked to each other? In the case of the enzyme from *T. maritima*, this question has not been addressed experimentally. In the case of the enzyme from *A. woodii*, experimental conditions were used (pH 8 and 100% H_2 in the gas phase) where the reductions of both ferredoxin from *Clostridium pasteurianum* ($E_0' = -400$ mV [pH independent between pH 6.4 and 8.7]) (25) and NAD^+ [$E_0^{(pH\ 8)} = -350$ mV] with H_2 [$E_0^{(pH\ 8)} = -480$ mV] were exergonic.

Flavin-based electron bifurcation was first discovered only several years ago (26, 27). The first example was the cytoplasmic butyryl coenzyme A (CoA) dehydrogenase electron-transferring flavoprotein complex Bcd-EtfAB from *Clostridium kluyveri*, which catalyzes the coupled reduction of crotonyl-CoA and ferredoxin with 2 NADH, a reaction which appears to be irreversible (28). Also apparently irreversible is the reaction catalyzed by the cytoplasmic electron-bifurcating MvhADG-HdrABC complex from methanogenic archaea that couples the endergonic reduction of ferredoxin with H_2 to the exergonic reduction of the heterodisulfide CoM-S-S-CoB with H_2 (29). In contrast, the coupled reduction of NAD^+ and ferredoxin with 2 NADPH catalyzed by the cytoplasmic electron-bifurcating NfnAB complex from *C. kluyveri* and *M. thermoacetica* is fully reversible (10, 30). Only in the cases of the MvhADG-HdrABC complex and the NfnAB complex has it been shown that these two enzymes couple the two reactions not only stoichiometrically but also energetically.

Here we report the purification and properties of the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase from *M. thermoacetica* and show that the reaction catalyzed by the cytoplasmic heterotrimeric enzyme is both reversible and energy coupled. We identified the encoding gene cluster via sequence information obtained by MALDI-TOF MS (matrix-assisted laser desorption-ionization time of flight mass spectrometry) of the trypsin-digested purified enzyme and characterized the transcriptional units. The role of this energy-converting enzyme complex in the acetogenesis of *M. thermoacetica* is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. thermoacetica* DSM 521 was cultivated anaerobically at 55°C on glucose with 100% CO_2 as the gas phase (10). The cells were harvested by centrifugation under N_2 when the

optical density at 660 nm (OD_{660}) was about 2.5 and were stored at $-80^\circ C$ until use. *C. pasteurianum* DSM 525 was grown anaerobically at 37°C on a glucose-ammonium medium (31), and the cells were harvested in the mid-exponential phase.

Biochemicals and enzymes. NAD^+ , NADH, $NADP^+$, flavin adenine dinucleotide (FAD), FMN, pyruvate, thiamine pyrophosphate, coenzyme A, and methyl viologen were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). All materials for protein purification were obtained from GE Healthcare (Freiburg, Germany). Pyruvate:ferredoxin oxidoreductase was purified from *M. thermoacetica* (32). Ferredoxin (Fd) (33) and ferredoxin-dependent monomeric [FeFe]-hydrogenase (28) were purified from *C. pasteurianum* DSM 525.

Purification of the electron-bifurcating ferredoxin- and NAD-dependent hydrogenase. The enzyme was purified under strictly anoxic conditions at room temperature in a type B vinyl anaerobic chamber (Coy, Grass Lake, MI), which was filled with 95% N_2 –5% H_2 and contained a palladium catalyst for O_2 reduction with H_2 . All buffers were boiled, flushed with N_2 , supplemented with 2 mM dithiothreitol (DTT), and maintained under a slight overpressure of N_2 . Frozen wet cells of *M. thermoacetica* (7.6 g) were suspended in 15 ml of 50 mM Tris-HCl (pH 7.6) containing 2 mM DTT, 5 μM FAD, and 5 μM FMN (buffer A). After adding 2 mg DNase I and 0.5 mM $MgCl_2$, the cell suspension was passed through a prechilled French pressure cell three times at 120 MPa. Unbroken cells and cell debris were removed by centrifugation at $10,000 \times g$ at 4°C for 30 min. Membranes were isolated by ultracentrifugation at $150,000 \times g$ at 4°C for 60 min. The supernatant obtained by ultracentrifugation at $150,000 \times g$ containing the cytoplasmic fraction with approximately 33 mg protein ml^{-1} was used for the purification of ferredoxin- and NAD-dependent hydrogenase. The membrane fraction was resuspended in 15 ml buffer A using a Teflon Potter homogenizer and subsequently centrifuged at $150,000 \times g$ at 4°C for 60 min. After a second wash via the same procedure, the membrane fraction was homogenized in 2 ml buffer A for enzyme assays.

The supernatant obtained by ultracentrifugation at $150,000 \times g$ was loaded onto a DEAE Sepharose Fast Flow column (2.6 cm by 14 cm) equilibrated with buffer A. The column was washed with 150 ml buffer A. Protein was eluted with a 0 to 1 M NaCl linear gradient at a flow rate of 8 $ml\ min^{-1}$. The hydrogenase activity was eluted at around 0.24 M NaCl. The fractions containing activity were pooled, concentrated, and desalted by using an Amicon cell with a 50-kDa-cutoff membrane. The concentrate was then applied onto a Q Sepharose high-performance column (2.6 cm by 13 cm) equilibrated with buffer A. The column was washed with 70 ml buffer A. Protein was eluted with a NaCl gradient at a flow rate of 5 $ml\ min^{-1}$ (stepwise 0.1 and 0.2 M, linear between 0.2 and 0.3 M, and stepwise 0.3, 0.4, and 0.5 M; 90 ml each in buffer A). The hydrogenase activity was recovered in fractions eluting at around 0.26 M NaCl. The fractions were brought to 0.8 M $(NH_4)_2SO_4$ and loaded onto a Phenyl Sepharose high-performance column (1.6 cm by 15 cm) equilibrated with buffer A containing 0.8 M $(NH_4)_2SO_4$. Protein was eluted with a stepwise $(NH_4)_2SO_4$ gradient (0.8, 0.6, 0.4, 0.2, 0.1, and 0 M; 50 ml each in buffer A) at a flow rate of 4 $ml\ min^{-1}$. The hydrogenase activity eluted in a peak at 0 M $(NH_4)_2SO_4$. The pooled fractions were concentrated and desalted with an Amicon cell with a 50-kDa-cutoff membrane. The concentrate was loaded onto a HiTrap Q HP column (5 ml) equilibrated with buffer A containing 5% glycerol. The protein was eluted with a 0 to 1 M NaCl linear gradient (80 ml) at a flow rate of 4 $ml\ min^{-1}$. The enzyme eluted in a single peak at around 0.26 M NaCl. The fraction was concentrated, desalted with a 50-kDa-cutoff Amicon filter, and then stored at 4°C in buffer A under an atmosphere of 95% N_2 –5% H_2 until use. During purification, both methyl viologen reduction activity with H_2 and NAD^+ -dependent ferredoxin reduction activity with H_2 were monitored.

Enzyme assays. Enzyme activities were measured under strictly anoxic conditions; 1.5-ml anoxic cuvettes (H_2 uptake) or 6.5-ml serum bottles (H_2 formation) were sealed with rubber stoppers and filled with 0.8-ml reaction mixtures (see below). N_2 (100%) or H_2 (100%) at 1.2×10^5 Pa was the gas phase. After the start of the reaction with enzyme or

NAD⁺, NAD(P)⁺ reduction was monitored spectrophotometrically at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$), ferredoxin reduction was monitored at 430 nm ($\epsilon_{\Delta\text{ox-red}} \approx 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$), and methyl viologen reduction was monitored at 578 nm ($\epsilon = 9.8 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit was defined as the transfer of 2 μmol electrons min^{-1} .

For the assays, a temperature of 45°C was routinely chosen because the handling of the anoxic cuvettes at 45°C was easier than at 55°C, the growth temperature optimum of *M. thermoacetica* (at 55°C, you burn your fingers). In a few cases, however, the measurements were done at 55°C, and at this temperature, the activity was found to be twice as high as that at 45°C. Most enzymes increase their activity by a factor of 2 when the temperature is increased by 10°C (Q10 rule). Q₁₀ is the temperature coefficient representing the factor, by which the rate of a reaction increases for every 10°C rise in temperature.

H₂ uptake activity. For H₂ uptake activity assays, the reaction mixture contained 100 mM Tris-HCl (pH 7.5) or potassium phosphate (pH 7.5) and, where indicated, 2 mM DTT and 10 μM FMN. The gas phase was 100% H₂. For NAD⁺-dependent ferredoxin reduction activity, the reaction mixture was supplemented with about 30 μM *C. pasteurianum* ferredoxin and 1 mM NAD⁺. For methyl viologen or NAD(P)⁺ reduction, the reaction mixture was supplemented with 10 mM methyl viologen and/or 1 mM NAD(P)⁺.

H₂ formation activity. For H₂ formation activity assays, the reaction mixture contained 100 mM potassium phosphate (pH 7.0) or, as indicated, 2 mM DTT, 10 μM FMN, 1 mM NADH, and a reduced ferredoxin-regenerating system (10 mM pyruvate, 0.1 mM thiamine pyrophosphate, 1 mM coenzyme A, 12 μM *C. pasteurianum* ferredoxin, and 0.3 U pyruvate:ferredoxin oxidoreductase). The gas phase was 100% N₂. The reaction was started with enzyme or NADH, and the serum bottle was then continuously shaken at 200 rpm to ensure H₂ transfer from the liquid phase into the gas phase. Gas samples (0.2 ml) were withdrawn every 1 min, and H₂ was quantified by gas chromatography.

Pyruvate:ferredoxin oxidoreductase. The reaction mixture for pyruvate:ferredoxin oxidoreductase assays contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, 10 mM pyruvate, 0.1 mM thiamine pyrophosphate, 1 mM coenzyme A, and about 30 μM *C. pasteurianum* ferredoxin or 10 mM methyl viologen as the electron acceptor. The gas phase was 100% N₂.

Analytical methods. Protein concentration was determined by using the Bio-Rad protein assay (Munich, Germany), with bovine serum albumin as the standard. SDS-PAGE analysis was performed on 12% Mini-Protein TGX precast gels (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. The gels were stained with Coomassie Brilliant Blue G250.

The SDS-PAGE protein bands were excised and digested with sequencing-grade modified trypsin (Promega, Mannheim, Germany). The resulting peptide mixture was injected onto a PepMap100 C₁₈ RP nanocolumn (Dionex, Idstein, Germany) and separated on an UltiMate 3000 liquid chromatography system (Dionex) in a continuous acetonitrile gradient. A Probot microfraction collector (Dionex) was used to spot liquid chromatography-separated peptides onto a MALDI target, mixed with matrix solution (alpha-cyano-4-hydroxycinnamic acid). MALDI-TOF-TOF analysis was carried out on a 4800 Proteomics analyzer (Applied Biosystems/MDS Sciex, Foster City, CA). Tandem mass spectrometry (MS/MS) data were searched against an in-house protein sequence database by using Mascot (Matrixscience, United Kingdom) embedded into GPS explorer software.

The relative molecular mass of the purified hydrogenase was measured by gel filtration on a Superdex 200HR column (1.0 cm by 30 cm) calibrated with Gel Filtration Calibration Kit HMW (high molecular weight; GE Healthcare, Freiburg, Germany), equilibrated with buffer A containing 300 mM NaCl and run at a flow rate of 0.5 ml min^{-1} .

The iron content of the purified enzyme was determined colorimetrically with 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazinedisodium trihydrate (Ferene; Sigma) (30). For identification of flavin and measurement of the flavin content of the enzyme, the purified enzyme was first

washed by ultrafiltration with a 20-fold volume of flavin-free buffer A. The washed enzyme was then heat denatured in a boiling-water bath for 10 min in the dark. After cooling, the denatured protein was removed by centrifugation at $13,000 \times g$ at 4°C for 20 min. For identification of the flavin in the enzyme, the supernatant was analyzed by thin-layer chromatography (TLC) on an RP-18 F254 aluminum sheet (Merck, Darmstadt, Germany), with FAD and FMN as the standards. The TLC plate was developed with an aqueous solution containing 85% 5 mM ammonium formate and 15% methanol. The presence of flavin was determined by irradiation with UV light. For quantification of flavin in the enzyme, the UV-visible (UV-Vis) spectrum of the supernatant of the denatured protein was recorded on a UV-1650PC spectrophotometer (Shimadzu, Japan), and the amount of FMN was calculated by using a molar extinction coefficient at 446 nm of $12,200 \text{ M}^{-1} \text{ cm}^{-1}$ (34).

The H₂ content in the gas phase was measured on a gas chromatograph equipped with a thermal conductivity detector (Carlo Erba GC series 6000) and a ShinCarbon ST micropacked column (82/100 mesh, 2.0 mm by 2 m; Restek GmbH, Germany). The oven and injection port temperatures were set at 100°C; the detector was set at 150°C. N₂ was used as the carrier gas, and the flow rate was 34 ml min^{-1} . The amount of H₂ was calculated according to the standard curve correlated with peak areas.

RNA isolation and cotranscription analysis by RT-PCR. Total RNA was isolated from *M. thermoacetica* cells grown exponentially on glucose plus CO₂ and harvested at an OD₆₆₀ of 2.0. For RNA extraction, TRIzol reagent (Invitrogen, Darmstadt, Germany) was used according to the manufacturer's protocol. After treatment with RNase-free DNase I (Fermentas, St. Leon-Rot, Germany), samples of 3 μg RNA were analyzed by electrophoresis in a 1.0% agarose gel to assess the integrity of the RNA. Cotranscription of the *hydABC* genes and their neighboring genes was analyzed by using reverse transcription-PCR (RT-PCR) to amplify the intergenic regions, using the ProtoScript M-MuLV Taq RT-PCR kit (New England BioLabs, Frankfurt, Germany) as recommended by the manufacturer. The random primer provided in the kit was used for reverse transcription. The resulting cDNA was used as a template to amplify the intergenic region of every pair of neighboring genes. The specific primers used for amplification are listed in Table S1 in the supplemental material. Genomic DNA and total RNA were used as positive and negative controls, respectively.

Determination of the transcription start site. The transcription start site of the *hydCBAX* operon was determined by 5' random amplification of cDNA ends (RACE), using a 5'/3' RACE kit (second generation) from Roche (Mannheim, Germany), as described in the manual. First-strand cDNA was synthesized by reverse transcription with *hydC*-specific primer 1. After poly(A) tailing of the first-strand cDNA, the product was amplified with *hydC*-specific primer 2 and an oligo(dT) anchor primer provided in the kit. Nested PCR was then performed with *hydC*-specific primer 3 and the PCR anchor primer provided in the kit. After two rounds of amplification, a PCR product band was visible after electrophoresis in a 1.0% agarose gel. The final products were cloned into a pCR-Blunt vector (Invitrogen, Darmstadt, Germany), which was subsequently introduced into competent TOP10 cells by transformation. After amplification, the inserted PCR product was sequenced to identify the transcriptional start site. The three specific primers used here are listed in Table S1 in the supplemental material.

Nucleotide sequence accession number. The *M. thermoacetica* genome GenBank accession number is CP000232.1.

RESULTS

We previously showed that cell extracts of *M. thermoacetica* grown on glucose and harvested at the beginning of the stationary phase catalyze the NAD⁺-dependent reduction of ferredoxin (Fd) with H₂ with a specific activity of 0.1 U/mg and the reduction of methyl viologen with H₂ with a specific activity of 0.4 U/mg. Both activities were associated with the cytoplasmic cell fraction (10). In the meantime, we found that the specific activity of the ferredoxin-

TABLE 1 Purification (43-fold) of electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase from *M. thermoacetica* grown on glucose under 100% CO₂^a

Purification step	Total protein (mg)	Hydrogenase activity			
		MV reduction		NAD ⁺ -dependent Fd reduction	
		U _{total}	U/mg	U _{total}	U/mg
Cell extract (150,000 × g)	560	2,420	4.3	735	1.3
DEAE Sepharose	96	1,610	16.8	547	5.7
Q Sepharose	23	1,260	55	435	19.0
Phenyl Sepharose	2.7	440	163	143	53.0
HiTrap Q HP	2.1	380	181	122	55.8

^a The purification yield was 16%. MV, methyl viologen; Fd, ferredoxin from *C. pasteurianum*. Activities were measured spectrophotometrically in 100 mM Tris-HCl (pH 7.5) at 45°C with 100% H₂ at 1.2 × 10⁵ Pa as the gas phase. One unit equals 2 μmol electrons transferred per min.

and NAD⁺-dependent hydrogenase as well as that of the methyl viologen-reducing hydrogenase were 10-fold higher in glucose-grown cells harvested in the exponential growth phase. From such cells, we purified the electron-bifurcating ferredoxin- and NAD-dependent hydrogenase (Table 1). In all of the following experiments, ferredoxin from *C. pasteurianum* was used. This ferredoxin has two [4Fe4S] clusters, both of which are reduced by 1 electron at a redox potential (*E*₀′) of −400 mV (35), and the redox potential is pH independent from pH 6.4 to 8.7 (25).

Purification of the electron-bifurcating ferredoxin- and NAD-dependent hydrogenase. Purification was achieved by anion-exchange chromatography (DEAE Sepharose, Q Sepharose, and HiTrap Q HP) and hydrophobic chromatography (Phenyl Sepharose), starting with the supernatant obtained by centrifugation at 150,000 × g. During purification, we monitored both the reduction of methyl viologen with H₂ and the NAD⁺-dependent reduction of ferredoxin with H₂ (Table 1). The enzyme was purified 43-fold, with a yield of 16%. The purification factor and yield were independent of whether the hydrogenase activity was measured with methyl viologen or with ferredoxin plus NAD⁺, which indicates that in the cytoplasm of glucose-grown cells, only one type of hydrogenase that catalyzes both methyl viologen reduction and H₂-driven NAD-dependent ferredoxin reduction was present. Consistently, NADP-reducing hydrogenase activity, which is present in cell extracts (supernatant obtained by centrifugation at 115,000 × g) of H₂/CO₂-grown cells and which also catalyzes the reduction of methyl viologen with H₂ (10), was undetectable in the cell extract of glucose-grown cells (Table 2). In the glucose-grown cells, the genes for the membrane-associated Ech-type [NiFe]-hydrogenase (see the introduction) were also apparently not expressed, as indicated by the finding that the pellet of the cell extract obtained by centrifugation at 150,000 × g, after washing of the pellet by resuspension in buffer and recentrifugation, was essentially devoid of methyl viologen-reducing hydrogenase activity. All [NiFe]-hydrogenases known to date catalyze the reduction of methyl viologen with H₂.

The hydrogenase had to be purified under strictly anoxic conditions because the enzyme was inactivated in the presence of even trace amounts of O₂. All buffers used were supplemented with FMN and FAD. When the flavins were omitted, the purification yields were very low.

TABLE 2 Specific activities at 45°C of the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase (HydABC) from *M. thermoacetica*^d

Reaction	Hydrogenase activity (U/mg)		
	Cell extract (150,000 × g) at pH 7.5	Purified enzyme	
		pH 7.5	pH 7.0
H ₂ → MV _{ox}	3.6	195	
H ₂ → NAD ⁺ + MV _{ox}	2.6 ^a	195	
H ₂ → NAD ⁺ + Fd _{ox}	1.1	57.5	49.7
H ₂ → Fd _{ox}	<0.01	<0.01	
H ₂ → NAD ⁺	0.2	<0.01	
H ₂ → NADP ⁺ + Fd _{ox}	<0.01	<0.01	
H ₂ → NADP ⁺	0.01	<0.01	
NADH + Fd _{red} ^b → H ₂	0.1	4.6	7.8
NADH → H ₂	<0.01		<0.01
Fd _{red} ^b → H ₂	0.015		0.2 ^c

^a The cell extracts catalyzed the reduction of NAD⁺ with reduced methyl viologen.
^b Fd_{red}-regenerating system (ferredoxin from *C. pasteurianum*, pyruvate, thiamine pyrophosphate, coenzyme A, and pyruvate:Fd oxidoreductase from *M. thermoacetica*).
^c This activity cannot be explained only by the fact that the ferredoxin preparations from *C. pasteurianum* used were contaminated with trace amounts of monomeric [FeFe]-hydrogenase catalyzing the formation of H₂ with reduced ferredoxin. For that, the activity was much too high.
^d At the growth temperature optimum of *M. thermoacetica* of 55°C, the specific activities were approximately 2-fold higher (see the text). MV, methyl viologen; Fd, ferredoxin from *C. pasteurianum*. The activities were measured in 100 mM potassium phosphate at pH 7.5 or pH 7.0, as indicated. One unit equals 2 μmol electrons transferred per min. Important activities are printed in boldface type.

Molecular properties. SDS-PAGE of the purified hydrogenase revealed three proteins with apparent molecular masses of 67, 63, and 17 kDa (Fig. 1). A scan of the Coomassie blue-stained bands was consistent with a 1:1:1 stoichiometry. The apparent molecular mass of the enzyme determined by gel exclusion chromatography was near 300 kDa, which indicated that the native enzyme is a dimer of the heterotrimer (see Fig. S1 in the supplemental material).

Sequence information obtained by MALDI-TOF MS of the three trypsin-digested proteins indicated that the three proteins were encoded by a gene cluster in *M. thermoacetica* annotated *hydCBA*. The deduced amino acid sequences of the proteins show sequence identity to the sequences of the HydA (43%), HydB

FIG 1 SDS-PAGE of purified electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase (HydABC) from *M. thermoacetica*. The encoding genes were identified by MALDI-TOF MS analysis of the three protein bands after digestion with trypsin. The molecular masses and cofactor binding sites of the three subunits were deduced from the amino acid sequences. [H], [6Fe4S] cluster, the active site of [FeFe]-hydrogenases.

FIG 2 The UV-visible absorption spectra of the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase from *M. thermoacetica*. The sample contained about 2.3 mg of protein per ml 50 mM Tris-HCl (pH 7.6). The dashed line indicates the sample isolated in the anaerobic chamber containing 5% H₂-95% N₂; the solid line indicates that the gas phase of the sample was changed to 100% N₂.

(49%), and HydC (39%) proteins of the hyperthermophilic bacterium *T. maritima*, which form a heterotrimeric electron-bifurcating [FeFe]-hydrogenase (22, 24). The deduced amino acid sequence of HydABC from *M. thermoacetica* shows an even higher identity to that of the electron-bifurcating HydABCD complex of *A. woodii* (13). The fourth subunit, HydD, in the enzyme of *A. woodii*, which lacks a cofactor binding site, shows sequence similarity to the N-terminal part of HydB of *M. thermoacetica* (see Fig. S2 in the supplemental material).

Cofactor content. After washing in flavin-free buffer, the purified hydrogenase was found to contain 211.3 nmol nonheme iron and 5.3 nmol FMN mg protein⁻¹, equivalent to 31 irons and 0.8 FMN per heterotrimer. FAD was not detected. Deduced from the *M. thermoacetica* genome sequence, HydA (63 kDa) is predicted to harbor the H cluster, i.e., the [6Fe4S] hydrogenase active site, three [4Fe4S] clusters, and one [2Fe2S] cluster. HydB (67 kDa) is predicted to harbor three [4Fe4S] clusters, a flavin binding site, and an NAD binding site. HydC (17 kDa) is predicted to harbor one [2Fe2S] cluster (Fig. 1). Altogether, the iron in the clusters adds up to 34 per mol heterotrimer. The UV-Vis spectra of the enzyme isolated under 5% H₂ and of the enzyme after partial oxidation by removal of the H₂ are characteristic of iron-sulfur flavoproteins (Fig. 2). After partial oxidation, the enzyme could be rereduced with H₂. The enzyme from *M. thermoacetica* appears to contain two [2Fe2S] clusters less than the enzyme from *T. maritima* and one [2Fe2S] cluster less than the enzyme from *A. woodii* (see Fig. S2 in the supplemental material). Why this is so is presently not understood.

Catalytic properties. The purified hydrogenase catalyzed the coupled reduction of NAD (1 mM) and ferredoxin (30 μM) with H₂ (100% in the gas phase) at 45°C at pH 7.5 with a specific activity of 55 U/mg. At pH 7.0, the specific activity was 50 U/mg (Table 2). The rate of the reaction was proportional to the protein concentration in the range tested (not shown) and followed Michaelis-Menten kinetics. The rate was hyperbolically dependent on the H₂ and NAD concentrations, with apparent *K_m* values for H₂ of about 6% in the gas phase and for NAD⁺ of about 0.2 mM NAD⁺ (see

Fig. S3 in the supplemental material). At the growth temperature optimum of *M. thermoacetica* of 55°C, the specific activity was 100 U/mg at pH 7.5, which is twice as high as that at 45°C, the temperature at which, for practical reasons, the activity was routinely measured (see Materials and Methods).

The purified enzyme catalyzed the reduction of methyl viologen with H₂ at pH 7.5 at 45°C with a specific activity of almost 200 U/mg (Table 2). At a methyl viologen concentration of either 10 mM or 0.1 mM, the reduction of the viologen dye was independent of the presence of NAD, indicating that the viologen dye as one electron donor/acceptor uncouples the electron bifurcation mechanism (29).

The purified enzyme catalyzed the reverse reaction—the formation of H₂ from NADH and reduced ferredoxin—at a specific rate of 4.6 μmol min⁻¹ mg protein⁻¹ at pH 7.5 and at 7.8 μmol min⁻¹ mg protein⁻¹ at pH 7.0 at 45°C (Table 2). At 55°C, the specific rate at pH 7.5 was 10 μmol min⁻¹ mg⁻¹.

The specific activity of ferredoxin and NAD reduction with H₂ decreased when the pH was lowered from 7.5 to 6.5, whereas that of the reverse reaction increased. This can be explained by the fact that the reduction of ferredoxin and NAD⁺ with H₂ is associated with the formation of protons, and the reverse reaction is associated with the consumption of protons (see reaction 1 below).

The assay mixtures always contained FMN (10 μM), which stimulated the activity up to 2-fold depending on how thoroughly the enzyme preparation was washed free of flavins. FAD (10 μM) also stimulated the activity albeit to a lesser extent than FMN. Even at higher concentrations, FAD stimulated the activities less than FMN, indicating that the stimulatory effect of FAD was not the result of the FAD preparation containing small amounts of FMN formed by FAD hydrolysis. As indicated above, the purified enzyme contained only FMN, although it was purified in the presence of both FMN and FAD. The results thus indicated that the hydrogenase can use both FMN and FAD as cofactors, with a strong preference for FMN.

Stoichiometry. We determined the stoichiometry of ferredoxin and NAD⁺ reduction with H₂ by monitoring both the reduction of NAD⁺ at 340 nm and the reduction of ferredoxin at 430 nm. This is possible because upon reduction, *C. pasteurianum* ferredoxin does not change its absorbance significantly at 340 nm, and NADH does not absorb at 430 nm. Per mol NAD⁺ reduced, 0.84 mol ferredoxin was reduced (Fig. 3A). The same stoichiometry was found when the mole amounts of ferredoxin reduced with H₂ per mol NAD⁺ added was determined (not shown). The stoichiometry of H₂ formation from reduced ferredoxin and NADH was determined by measuring the mole amounts of H₂ generated from fully reduced ferredoxin per mol of NADH added. In the experiment, ferredoxin was kept fully reduced by a regenerating system composed of pyruvate, pyruvate:ferredoxin oxidoreductase, and coenzyme A. Per mol NADH, about 2 mol H₂ was formed (Fig. 3B). The results indicated that HydABC catalyzes the following reversible reaction:

Energetic coupling. The coupling of the endergonic reduction of ferredoxin with H₂ to the exergonic reduction of NAD⁺ with H₂ was demonstrated by showing that at pH 7.0, ferredoxin (*E*₀' = -400 mV) was reduced to more than 95% by H₂ (100% in the gas phase; *E*₀' = -414 mV) in the presence of HydABC from *M. thermoacetica* and NAD⁺ but only to about 55% in the presence

FIG 3 Stoichiometry of the reactions catalyzed by electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase from *M. thermoacetica*. (A) NAD^+ and *C. pasteurianum* ferredoxin (Fd) reduction with H_2 . The reaction took place in anoxic cuvettes and was monitored simultaneously at 340 nm (for NAD^+ reduction) and 430 nm (for ferredoxin reduction). The gas phase was H_2 at 1.2×10^5 Pa. The absorbance change at 340 nm caused by ferredoxin reduction was very small and could therefore be ignored. The amount of NADH and reduced ferredoxin was calculated based on their molar extinction coefficients ($\epsilon_{340\text{ nm}} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ for NAD^+ ; $\epsilon_{430\text{ nm}} = 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$ for ferredoxin). (B) NADH-dependent H_2 formation in the presence of an excess amount of reduced ferredoxin. The reaction took place in 6.5-ml anoxic serum bottles sealed with rubber stoppers and containing 0.8 ml of the reaction mixture. The gas phase was N_2 at 1.2×10^5 Pa. The reduced ferredoxin was supplied by a regenerating system containing 20 μM ferredoxin, 10 mM pyruvate, 0.1 mM thiamine pyrophosphate, 1 mM coenzyme A, and about 0.3 U of pyruvate:ferredoxin oxidoreductase purified from *M. thermoacetica*. The amount of H_2 was quantified via gas chromatography.

of the monomeric [FeFe]-hydrogenase from *C. pasteurianum* (Fig. 4). The *C. pasteurianum* hydrogenase, which is not electron bifurcating, catalyzes the reversible reduction of ferredoxin with H_2 in the absence of NAD^+ . Under the experimental conditions used, equilibrium is achieved in the presence of this [FeFe]-hydrogenase when 55% of the ferredoxin is reduced. Any further reduction must therefore be energy driven.

Encoding genes. As indicated above, HydABC from *M. thermoacetica* is encoded by the gene cluster *hydCBA* (Fig. 5). The open reading frame upstream of the cluster (MoTh_1720 [MoTh for *M. thermoacetica*]) is predicted to encode an *O*-methyltransferase, and the open reading frame downstream of the cluster (MoTh_1716) is predicted to encode a hydrolase. RT-PCR analysis suggests that the genes *hydC*, *hydB*, *hydA*, and *orf1716* (the hydrolase gene, now designated *hydX*) form a transcriptional unit (Fig. 5). The function of the hydrolase is unknown. A homolog of *hydX* is not found in the genomes of *A. woodii* and *T. maritima*, which synthesize a closely related electron-bifurcating hydrogenase. Studies to unravel the function of HydX were not performed.

In the region between the stop codon of the *O*-methyltransferase gene and the start codon of *hydC*, a transcriptional terminator, putative -35 and -10 promoter sequences, the transcription start site, and the ribosome binding site (RBS) were identified or predicted from the DNA sequence (Fig. 6). We determined the transcription start site using the 5' RACE method. In the region between the stop codon of *hydA* and the start codon of the hydrolase gene *hydX*, a transcriptional terminator, a promoter region, and a ribosome binding site were predicted by Softberry software (Softberry, Inc., New York, NY) (not shown). HydABC activity is found in both glucose- and H_2/CO_2 -grown cells (10).

As indicated in the introduction, the genome of *M. thermoacetica* harbors two gene clusters encoding multimeric [FeFe]-hydrogenases,

FIG 4 Fd reduction with H_2 catalyzed by the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase (HydABC) from *M. thermoacetica*. As controls, Fd reduction with 100% H_2 catalyzed by the monomeric Fd-dependent [FeFe]-hydrogenase (Hyd) from *C. pasteurianum* as well as the spontaneous reduction of Fd with sodium dithionite were used. The reactions took place in 1.5-ml anoxic cuvettes filled with 0.8 ml 100 mM morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.0) containing 30 μM Fd from *C. pasteurianum* and, where indicated, *C. pasteurianum* monomeric [FeFe]-hydrogenase (~ 0.02 units), *M. thermoacetica* HydABC (~ 0.04 units), or 0.5 mM sodium dithionite. The gas phase was 100% H_2 at 1.2×10^5 Pa. The temperature was 45°C . The reactions were started by the addition of enzyme or dithionite. With dithionite, 100% of the Fd was reduced, and with H_2 plus *C. pasteurianum* Hyd, about 55% of the Fd was reduced.

namely, MoTh_1717 to MoTh_1719 and MoTh_1883 to MoTh_1888. Since MoTh_1717 to MoTh_1719 has been shown to code for the electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase, the second cluster must code for the NADP-reducing hydrogenase synthesized only when the cells grow on H_2 and CO_2 (10).

DISCUSSION

The purified HydABC complex from *M. thermoacetica* was found to catalyze the reversible coupled reduction of NAD^+ and ferredoxin with 2H_2 (reaction 1) and the reduction of methyl viologen with H_2 . Under the experimental conditions used, the reduction of ferredoxin to more than 95% with H_2 ($10^5 \text{ Pa} = 100\% \text{ H}_2$ in the gas phase) was endergonic and there-

FIG 5 Cotranscription of *hydABC* in *M. thermoacetica* shown by RT-PCR analysis. After reverse transcription, cDNA (third lane) was used as the template to amplify the intergenic regions. Genomic DNA (first lane) and total RNA (second lane) were used as positive and negative controls, respectively. MoTh_1720, MoTh_1716, and MoTh_1715 are annotated in GenBank as genes encoding an *O*-methyltransferase, a hydrolase, and a transcriptional regulator belonging to the XRE family, respectively.

O-methyltransferase gene
 CTTTGTGATATCAATGAATACCGGCTGGAGATCCGCCAACGCCGAGTAAATTCATTCTCTGTAGGGGTGGGCTAAAACCTAAAAAGA
 L L I S
 ATGCTAAATCGGGCTGAGGGGGTAAACTCCGGGGCCGATTGTTTGTATCGGGAGCAGATTCAAGAGAACGAAAAATCAAAATTTA
 TGAACAGTACTGTACAAAAAATGAATGGCTAATAAGAATTTTGTGAGACC-35 TGGCAGGAGAAATTTATTTTATGCGAA-10 TAAGCTTT
 CTATACGAACTATCACAAGGTGACTTAACAATAATAGTGAATAGCCACACTATCACCCTGCAATTACAGGCCGATAAACCCGGGCC
 +1
 TGCTTTAGAAAAAGAGGGATTATCCCTCCAGGCCGGCTCTATTTCCCGGTACGGCTCGAGGCGGTTTCGGGCAGACGGCTTATT
 GCAGGATGTATACCCCTGAGTGTACCATGGCTACTCTTGAATGAGGAGTTGTTCTAATGGAACCTGTCAATGC hydC gene
 RBS M E T C Q C

FIG 6 Mapping of the transcription start site of *hydCBAX* from *M. thermoacetica* using 5' RACE. The transcription start site (+1), putative ribosome binding site (RBS), and extended -10 and -35 promoter sequences are indicated. The stop codon and the putative transcription terminator of the upstream O-methyltransferase gene are shaded gray. The putative transcription terminator was predicted by using the software provided by the ARNold finding terminators at the IGM-Web server (<http://rna.igmors.u-psud.fr/toolbox/arnold/index.php>) as well as the PEPPER Transcription Terminator Prediction toolbox of the University of Groningen, the Netherlands (<http://pepper.molgenrug.nl/>).

fore possible only if energetically coupled to the exergonic reduction of NAD^+ with H_2 (Fig. 4).

The specific activity of the purified enzyme was about 100 U/mg in the H_2 uptake direction and about 10 U/mg in the H_2 formation direction at the growth temperature optimum of 55°C (Table 2). For comparison, the specific activity of the coupled ferredoxin and NAD reduction with H_2 catalyzed by the enzyme from *A. woodii* was reported previously to be 4 U/mg at its growth temperature optimum of 30°C (13), and the specific activity of H_2 formation from NADH and reduced ferredoxin catalyzed by the enzyme from *T. maritima* was reported previously to be 10 U/mg at its growth temperature optimum of 80°C (22).

The experiments with the enzyme from *M. thermoacetica* were performed with ferredoxin from *C. pasteurianum* ($E_0' = -400$ mV). It was shown 50 years ago that ferredoxins from different organisms are functionally interchangeable *in vitro* (36, 37). With ferredoxin from *C. pasteurianum*, the free energy change, ΔG° , associated with reaction 1 under physiological standard conditions (pH 7.0) is -21 kJ mol^{-1} . Such a free energy change indicates that the HydABC complex should catalyze the oxidation of H_2 at pH 7 with a higher catalytic efficiency (k_{cat}/K_m) than the formation of H_2 . This is predicted by the Haldane equation (38), which relates catalytic efficiencies of enzyme-catalyzed forward and back reactions to the equilibrium constant of the reaction. Although for the HydABC complex, we determined specific activities and apparent K_m only under standard assay conditions, and although the apparent K_m for H_2 with methyl viologen as the electron acceptor is not necessarily the same as the K_m with ferredoxin plus NAD, the results do confirm that at pH 7, the H_2 oxidation reaction is kinetically favored when ferredoxin from *C. pasteurianum* is used as the electron acceptor.

For *M. thermoacetica*, a ferredoxin similar to that of *C. pasteurianum* was shown to be involved in ferredoxin-dependent reactions, such as the CO dehydrogenase reaction (39, 40). The *M. thermoacetica* ferredoxin (Fd-II) also harbors two [4Fe4S] clusters but differs from *C. pasteurianum* ferredoxin in that the two clusters have two different midpoint potentials, -454 mV and -487 mV (39), rather than both having the same midpoint potential of -400 mV , as in the case of the *C. pasteurianum* ferredoxin (35). With ferredoxin from *M. thermoacetica*, reaction 1 under physiological standard conditions (pH 7.0) is exergonic by only -7 kJ mol^{-1} . Unfortunately, Fd-II from *M. thermoacetica* was not available to test these predictions. However, when the crystal structure of the electron-bifurcating [FeFe]-hydrogenase in complex with ferredoxin is determined, Fd-II from *M. thermoacetica* will have to be used.

The pH optimum for growth of *M. thermoacetica* is pH 6.8 (5). The intracellular pH of *M. thermoacetica* is probably somewhat lower because the fermentation product acetic acid is a weak acid ($\text{pK}_a = 4.7$), which in the undissociated form can freely diffuse through the cytoplasmic membrane and lower the cytoplasmic pH (41, 42). At pH <7, the free energy change of reaction 1 with ferredoxin from *M. thermoacetica* approaches $\pm 0 \text{ kJ mol}^{-1}$. For a ΔG° of 0 kJ mol^{-1} , the Haldane equation predicts that the catalytic efficiencies of the HydABC complex in the catalysis of the forward and back reactions are equal.

What is the situation *in vivo*? During growth of *M. thermoacetica* on glucose under an atmosphere of 100% CO_2 , small amounts of H_2 are formed (19, 20), and under these growth conditions, only the HydABC complex was found to be present. Almost 100% of the H_2 :methyl viologen oxidoreductase activity in cell extracts was associated with the HydABC complex (Table 2). Neither the activities of cytoplasmic NADP-reducing hydrogenase nor those of membrane-associated Ech hydrogenase, which both also catalyze methyl viologen reduction with H_2 , could be detected. In glucose-grown cells, HydABC thus most probably is the enzyme responsible for H_2 formation. In previous studies, it was shown by native gel electrophoresis that in glucose-plus- CO_2 -grown cells, there appears to be only one methyl viologen-reducing hydrogenase activity (19, 21).

The NADH and the reduced ferredoxin required for H_2 formation via HydABC are regenerated during glucose oxidation to 2 acetic acid and 2 CO_2 in the NAD-specific glyceraldehyde phosphate dehydrogenase reaction (10, 43) and the pyruvate:ferredoxin oxidoreductase reaction (32, 44). Since most of the NADH and reduced ferredoxin are reoxidized during the reduction of 2 CO_2 to acetic acid (10), the HydABC complex probably has the function of a valve that allows glucose oxidation and thus ADP phosphorylation when CO_2 reduction to acetic acid becomes rate limiting.

During growth of *M. thermoacetica* on H_2 and CO_2 , the situation is different. In cells grown in the presence of H_2 , activities of HydABC and of NADP-reducing hydrogenase are found (10). During growth on H_2 and CO_2 , the reduction of ferredoxin with H_2 via HydABC is most probably the only reduced ferredoxin-regenerating reaction, which is required for the reduction of CO_2 to CO as an intermediate in the total synthesis of acetate from 2 CO_2 . In principle, ferredoxin could also be reduced with NADPH via the NfnAB complex, which is thought, however, to have a function in NADPH regeneration from reduced ferredoxin and NADH rather than in ferredoxin reduction (10). Thus, depending on the growth conditions, the HydABC complex in *M. thermoacetica* catalyzes either the formation of H_2 (during growth on

glucose) or its uptake (during growth on H_2 and CO_2). The only other hydrogenase shown to operate in both directions *in vivo* in one organism is the cytoplasmic F_{420} -reducing hydrogenase in some methanogenic archaea (45). This enzyme catalyzes the uptake of H_2 during growth of these methanogens on H_2 and CO_2 and the formation of H_2 during growth on formate.

For *A. woodii*, which grows on H_2 and CO_2 and forms acetic acid, the finding of an electron-bifurcating hydrogenase complex, HydABCD, allowed the formulation of an energy metabolism in which the oxidation and reduction reactions are balanced and in which CO_2 reduction to acetic acid is coupled with energy conservation (12, 13). Key to the understanding are the presence of NAD-specific methylene-tetrahydrofolate dehydrogenase, NAD-specific methylene-tetrahydrofolate reductase (46, 47), and a membrane-associated energy-conserving reduced ferredoxin: NAD⁺ oxidoreductase (RnfA-G complex) (11). In *M. thermoacetica*, the methylene-tetrahydrofolate dehydrogenase is NADP specific (48, 49). The physiological electron donor of methylene-tetrahydrofolate reductase is not known (10), and an energy-converting RnfA-G complex is lacking (17). In *M. thermoacetica*, it thus remains unknown how the NAD⁺ required in the reaction catalyzed by HydABC during growth of the organism on H_2 and CO_2 is regenerated.

From the genome sequence of *M. thermoacetica* (17), it is predicted that this organism could contain a membrane-associated, energy-converting hydrogenase of the Ech type, which in some organisms catalyzes the proton motive force-driven reduction of ferredoxin with H_2 and in others catalyzes the reduction of protons with reduced ferredoxin to H_2 coupled with the buildup of a proton motive force (45). This Ech-type hydrogenase is the only energy-converting membrane complex predicted from the genome of *M. thermoacetica* that could operate within the redox potential range set by the substrates and intermediates involved in CO_2 reduction with H_2 to acetic acid. The most negative E_0' of -520 mV is of the CO_2/CO couple (50), and the most positive E_0' of -200 mV is of the methylene-tetrahydrofolate/methyl-tetrahydrofolate couple (51). The redox potential of menaquinone found in *M. thermoacetica* (14, 15) is -75 mV and is thus far too positive to be involved in electron transport between CO and methylene-tetrahydrofolate (52). If and how the Ech complex is involved in CO_2 reduction with H_2 to acetic acid in *M. thermoacetica* are important questions that need to be answered before a function of the electron-bifurcating hydrogenase HydABC in *M. thermoacetica* during growth on H_2 and CO_2 can definitely be assigned.

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