Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea

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In methanogenic archaean growing on H₂ and CO₂ the first step in methanogenesis is the ferredoxin-dependent endergonic reduction of CO₂ with H₂ to formylmethanofuran and the last step is the exergonic reduction of the heterodisulfide CoM-S-S-CoB with H₂ to coenzyme M (CoM-SH) and coenzyme B (CoB-SH). We recently proposed that in hydrogenotrophic methanogens the two reactions are energetically coupled via the cytoplasmic MvhADG/HdrABC complex. It is reported here that the purified complex from *Methanothermobacter marburgensis* catalyzes the CoM-S-S-CoB-dependent reduction of ferredoxin with H₂. Per mole CoM-S-S-CoB added, 1 mol of ferredoxin (Fd) was reduced, indicating an electron bifurcation coupling mechanism: 2H₂ + Fdred2 → CoM-S-S-CoB→Fdox2 + CoM-SH + CoB-SH + 2H⁺. This stoichiometry of coupling is consistent with an ATP gain per mole methane from 4 H₂ and CO₂ of near 0.5 deduced from an H₂-threshold concentration of 8 Pa and a growth yield of up to 3 g/mol methane.

flavin-based electron bifurcation | metronidazole | *Methanosarcina barkeri* | hydrogenase | heterodisulfide reductase

Methanogenesis from H₂ and CO₂ is an important reaction in the global methane cycle (1). It is mediated by hydrogenotrophic methanogenic archaea (2). These archaea appear to have a mainly anabolic function (6, 7). The authors declare no conflict of interest.

The free energy change ΔG associated with reaction 1 is more positive and that associated with reaction 2 is less negative at physiological concentrations of substrates and products (2). The remaining question is, How are the two cytoplasmic reactions coupled energetically?

The reduction of CO₂ with H₂ to formyl-MFR (reaction 1) is composed of the two partial reactions 3a and 3b that are dependent on ferredoxin (Fd) as electron carrier. Reaction 3b is catalyzed by a cytoplasmic formyl-MFR dehydrogenase that uses a polyferredoxin as electron carrier. The redox potential (E°r) of the polyferredoxin is not known but can be predicted to lie between that of the 2 H⁺/H₂ couple (−414 mV) and that of the CO₂ + MFR/formyl-MFR couple (−520 mV) (4, 5). The enzyme system catalyzing reaction 3a has not yet been identified. Hydrogenotrophic methanogens do contain at least one membrane-associated energy-converting [NiFe]-hydrogenase catalyzing the H⁺ or Na⁺ motive force-driven reduction of Fd with H₂. However, these enzymes appear to have a mainly anabolic function (6–9):

\[
\text{H}_2 + \text{Fd}_{\text{ox}} \rightarrow \text{Fd}_{\text{red}} - 2 + 2\text{H}^+ \quad [3a]
\]

\[
\text{Fd}_{\text{red}} - 2 + 2\text{H}^+ + \text{CO}_2 + \text{MFR} \rightarrow \text{Fd}_{\text{ox}} + \text{formyl-MFR} + \text{H}_2\text{O}. \quad [3b]
\]

The authors declare no conflict of interest.

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\[
\text{H}_2 + \text{CO}_2 + \text{MFR} \rightarrow \text{formyl-MFR} + \text{H}_2\text{O} \quad \Delta G^o = +20\text{kJ/mol} \quad [1]
\]

\[
\text{H}_2 + \text{CoM-S-S-CoB} \rightarrow \text{CoM-SH} + \text{CoB-SH} \quad \Delta G^o = -55\text{kJ/mol}. \quad [2]
\]

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Results

In MvhADG/HdrABC activity assays Fd with two [4Fe4S] clusters from Clostridium pasteurianum (Eₜ = −400 mV; n = 2) (22) and an absorption maximum at 390 nm (ε = 30,000 M⁻¹·cm⁻¹) (23) was used rather than one of the several ferredoxins from M. marburgensis (9), trusting the experience that even ferredoxins from very distantly related organisms are functionally inter-changeable (24). The reduction of Fd was followed by measuring the decrease in absorbance at 390 nm (Δε = 11,000 M⁻¹·cm⁻¹) (25). When indicated, MTZ was used as an artificial electron acceptor, its reduction being followed by measuring the decrease in absorbance at 320 nm (ε = Δε = 9,300 M⁻¹·cm⁻¹) (13, 26). MTZ is rapidly reduced by reduced Fd and Fd-like proteins in a four-electron consuming spontaneous reaction (n = 4) that proceeds irreversibly (27).

The heterodisulfide used in the assays was a 2:1:1 mixture of CoM-S-S-CoB, CoM-S-S-CoM, and CoB-S-S-CoB (Fig. S2). The mixture rather than pure CoM-S-S-CoB was used because of the property of CoM-S-S-CoB to spontaneously react to CoM-S-CoM and CoB-S-S-CoB until equilibrium is reached even under acidic conditions during freezing (concentrating) as a prerequisite for lyophilization. Interestingly, the disproportionation of CoM-S-S-CoB appears also to take place in vivo as evidenced by the finding that Methanothermobacter species contain a NADP⁺-dependent CoM-S-CoM reductase (28). We convinced ourselves with pure CoM-S-CoM and CoB-S-CoB that under the assay conditions the two homodisulfides did not serve as substrates for the MvhADG/HdrABC complex when present alone. When present together, because of their synproportionation to CoM-S-CoB, some activity was observed that was, however, negligible (<2 nmol/min in the 0.75-mL assay) at low concentrations of the two homodisulfides (<1 mM). In the experiments it was additionally ascertained that CoM-SH and CoB-SH (alone or together) could not substitute for CoM-S-CoB.

The H₂: CoM-S-CoB oxidoreductase activity of the purified MvhADG/HdrABC complex at 60 °C was reported to be highest in 1.6 M potassium phosphate, pH 7 (11). Therefore, except when otherwise noted, these conditions were used in the following experiments.

CoM-S-CoB-Dependent Fd Reduction with H₂ in Cell Extracts. Cell suspensions of M. marburgensis catalyze the reduction of CO₂ with H₂ at 60 °C to methane at a specific rate of 6 μmol/min (units) and per milligram protein (29). This is therefore the specific activity in cell extracts of M. marburgensis to be expected for enzymes involved in methanogenesis from H₂ and CO₂ such as the MvhADG/HdrABC complex. The finding by Setzke et al. in 1994 (11) that cell extracts catalyzed the reduction of CoMS-CoB with H₂ only with a specific activity of maximally 0.2 unit/mg was thus an indication that upon cell rupture the enzyme system was either partially inactivated or an essential component became diluted. The component turned out to be Fd.

Cell extracts of M. marburgensis catalyzed the reduction of Fd from C. pasteurianum with H₂ at significant rates only in the presence of CoM-S-CoB (Fig. L4). The rate increased linearly with the protein concentration. Directly after preparation of the cell extract the specific activity was 2 units/mg protein (1 unit = 1 μmol Fd reduced by two electrons per minute). After a 5-h incubation of the cell extract at 4 °C with 100% H₂ it increased to 5 units/mg, which is close to the specific activity of methane formation from H₂ and CO₂ catalyzed by cell suspensions of M. marburgensis (see above). [NiFe]-hydrogenases such as MvHADG inhibited by O₂ are known to be slowly reactivated upon incubation with H₂ (30).

The cell extracts also catalyzed the CoM-S-CoB-dependent reduction of MTZ with H₂ (Fig. 1B). The specific activity was 2 units/mg (1 unit = 1 μmol MTZ reduced by four electrons per minute) before and 4 units/mg after incubation of the cell extract at 4 °C with 100% H₂.

When in the reduction assays cuvettes and rubber stoppers were used that had previously been in contact with methyl viologen or benzyl viologen (BV), the cell extracts catalyzed the reduction of Fd or of MTZ with H₂ already in the absence of CoM-S-CoB. At BV concentrations of ~25 μM the reaction became essentially independent of CoM-S-CoB as shown for MTZ reduction in Fig. S3. An interpretation of this result is that the MvhADG/HdrABC complex catalyzes the reduction of viologen dyes with H₂ in the absence of CoM-S-CoB (11) and that the reduced viologen dye then transfers the electrons to ferredoxin and metronidazole in spontaneous reactions.

Purification of the MvhADG/HdrABC Complex. The enzyme system mediating the CoM-S-CoB-dependent reduction of Fd with H₂ was purified 26-fold in a 14% activity yield (Table 1). After incubation for 10 h at 4 °C under 100% H₂, the purified enzyme complex catalyzed the reduction of Fd at a specific rate of 110 units/mg, before incubation at only 40 units/mg. The specific activity with MTZ as electron acceptor (0.15 mM) was 50 units/mg after and 26 units/mg before incubation.

The cell extract catalyzed the reduction of Fd and of MTZ at almost the same specific rates whereas the purified complex showed a twofold higher specific activity with Fd than with MTZ (Table 1). This result can be explained by the fact that the cell extract contains ferredoxins that stimulate MTZ reduction but that are completely or partially removed during the purification procedure.

Purification was performed in the presence of FAD, which was essential for activity recovery. In the absence of FAD the complex rapidly lost its ability to catalyze the CoM-S-CoB-dependent reduction of Fd (or MTZ) with H₂. Contact with O₂ instantaneously abolished the activity.

SDS/PAGE revealed the presence of 6 subunits with apparent molecular masses corresponding to those of the 6 subunits of the MvhADG/HdrABC complex (Fig. S4; for sequence-predicted masses see also Fig. S1). They were also identified by MALDI-TOF mass spectrometry, which revealed in some preparations the presence of the polyferredoxin MvhB that contains 12 [4Fe4S] clusters (48 kDa) (31).

Kinetic Properties. The purified MvhADG/HdrABC complex was most active in catalyzing the CoM-S-CoB-dependent reduction of Fd with H₂ at pH 7.5 (pH optimum), a potassium phosphate
concentration of 1.6 M (concentration optimum), and a temperature of 70 °C (temperature optimum) (Fig. S5 A–C).

The rate at 60 °C of Fd reduction with 100% H₂ in the gas phase (at 1.2 bar the dissolved H₂ concentration ~0.6 mM H₂) increased hyperbolically with the Fd and CoM-S-S-CoB concentrations, half-maximal rates being observed at a Fd concentration between 10 and 20 μM (1 mM CoM-S-S-CoB) and at a CoM-S-S-CoB concentration between 0.3 and 0.7 mM (33 μM Fd) (Fig. S6A and B). The apparent Kₘ for H₂ was 5–10% in the gas phase at a Fd concentration of 33 μM and a CoM-S-S-CoB concentration of 1 mM (Fig. S6C). The apparent Kₘ for H₂ compares with that of 20% H₂ reported for methane formation from H₂ and CO₂ in growing cultures of M. marburgensis (32). CoM-S-S-CoB-dependent MTZ reduction showed apparent Kₘ values near 0.1 mM for MTZ, <0.1 mM for CoM-S-S-CoB, and <5% for H₂ (Fig. S7 A–C). The much lower apparent Kₘ for Fd-S-S-CoB measured with metronidazole than with ferredoxin as electron acceptor could be due to the fact that the reduction of metronidazole with H₂ is strongly exergonic whereas the reduction of ferredoxin with H₂ is an endergonic reaction.

Energetic Coupling. The dependence of Fd reduction with H₂ on the presence of CoM-S-S-CoB was the first indication that the MvhADG/HdrABC complex couples the endergonic reduction of Fd with H₂ to the exergonic reduction of CoM-S-S-CoB with H₂. Final evidence was obtained by showing that the reduction of Fd with H₂ proceeded beyond the equilibrium concentrations expected for the noncoupled reaction. Fig. 2A shows the reduction of Fd with 100% H₂ at pH 7.0 and 1 bar (Eₐ’ = −414 mV) catalyzed by the [FeFe]-hydrogenase from C. pasteurianum (noncoupled reaction) and Fig. 2B shows that by the MvhADG/HdrABC complex in the presence of CoM-S-S-CoB (coupled reaction). In the noncoupled reaction the Fd (Eₐ’ = −400 mV) (22) was reduced to 55% (E’ = −410 mV) and in the coupled reaction to almost 100% (E’ ~ −500 mV) as in the case of Fd reduction with dithionite (Eₐ’ = −690 mV) (33). A 100% reduction of Fd with H₂ (100% at 1 bar) is thermodynamically possible only if the reduction of Fd with H₂ was coupled to the exergonic reduction of CoM-S-S-CoB (Eₐ’ = −140 mV) with H₂ (Eₐ’ = −414 mV). The Fd used in the experiment had a ΔA₃₉₀₋₂₈₀ of 0.8, indicating that the Fd was pure and not partially denatured (23).

Stoichiometry of Coupling. After having shown that Fd reduction with H₂ is energetically coupled to the reduction of CoM-S-S-CoB, the question of the stoichiometry of coupling was addressed. For this the molar amounts of Fd or of MTZ reduced with H₂ per mole CoM-S-S-CoB were determined with CoM-S-S-CoB being present in limiting amounts.

The time course of Fd reduction with H₂ (100% in the gas phase) in the presence of three limiting CoM-S-S-CoB concentrations (12, 24, and 36 nmol) is shown in Fig. 3A. Fd reduction leveled off after a few seconds followed by a phase of slow reoxidation. HPLC analysis revealed that after completion of Fd reduction all of the CoM-S-S-CoB had been consumed. Why the reduced Fd was reoxidized again is presently not understood but could be due to contaminations of the MvhADG/HdrABC complex with minute amounts of Fd-dependent hydrogenases present in M. marburgensis (EhaA-T and EthA-Q) (8) and/or to contaminations of the C. pasteurianum Fd with minute amounts of clostridial hydrogenase.

The experiment shown in Fig. 3 and many others of this type suggest that per mole CoM-S-S-CoB added 1 mol of Fd is reduced (Fig. 3B) by 2 H₂. However, with respect to the exact stoichiometry there remains an uncertainty. Because of the high extinction coefficient of oxidized and reduced Fd (Fig. 2), the stoichiometry had to be determined at relatively low concentrations of Fd and therefore also at low CoM-S-S-CoB concentrations. At these low concentrations, the concentrations of CoM-SH and of CoB-SH are difficult to determine reliably. It was therefore not possible to ascertain via HPLC that the CoM-S-S-CoB was completely reduced.

MTZ reduction with H₂ in the presence of limiting CoM-S-S-CoB concentrations was difficult to evaluate because MTZ reduction continued slowly after the expected 0.5 mol MTZ per mole CoM-S-
The reduction was started by enzyme protein and followed photometrically at 320 nm. The reduction was started by enzyme protein and followed photometrically at 390 nm.

MvhADG/HdrABC complex (0.75 mL assay mixture with 1.6 M potassium phosphate (pH 7), MvhADG/HdrABC complex (0.1 unit), Fd from C. pasteurianum, and CoM-S-CoB in the amounts indicated. The reduction was started by enzyme protein and followed photometrically at 390 nm.

S-CoB added was reduced (Fig. 4A). This reduction was probably due to the CoM-SH and CoB-SH formed in the reaction, which reoxidized nonenzymatically to CoM-S-CoB with MTZ. Also the CoM-SH, DT, and FAD present in the enzyme stock solution might contribute to the apparently CoM-S-CoB-independent MTZ reduction, which we tried to correct for by extrapolation (Fig. 4A). With this correction a stoichiometry of 0.6 mol MTZ reduced per mole of CoM-S-CoB added was found (Fig. 4B). At CoM-S-CoB concentrations 0.25 μM the stoichiometry approached 0.5 and at concentrations 75 μM it approached 0.7. Assuming a four-electron reduction of MTZ and a two-electron reduction of CoM-S-CoB, this finding indicates that per mole of CoM-S-CoB reduced, between 2 and 2.4 mol of H₂ were oxidized.

H₂ Thresholds and Growth Yields. In the reduction of CO₂ with H₂ to methane equilibrium ($\Delta G^\circ = 0 \text{kJ/mol}$) is theoretically reached at a H₂ partial pressure (pH₂) of ~0.2 Pa (assuming the partial pressure of CO₂ to be equal to the partial pressure of methane). However, when methanogenesis is coupled with the phosphorylation of ADP to ATP ($\Delta G^\circ = +50 \text{kJ/mol}$ ATP), then the H₂-equilibrium concentration (H₂-threshold concentration) is higher. Thus, when the threshold pH₂ is 30 Pa, then the ATP gain (mol ATP/mol CH₄) is 1. In turn, the ATP gain can be roughly estimated from the H₂-threshold concentration that is therefore an indicator of the stoichiometry of coupling between the exergonic and the endergonic reactions in CO₂ reduction with H₂ to methane.

The H₂-threshold concentration in cell suspensions catalyzing the reduction of CO₂ to methane was determined in three methanogens grown on H₂ and CO₂ and found to be ~8 Pa in the case of M. marburgensis and Methanoarboribacter arboriphilus and 150 Pa in the case of Methanosarcina barkeri, indicating an ATP gain <0 in the two hydrogenotrophic methanogens and an ATP gain >1 in the cytochrome containing methanogen. Consistently, the growth yields (grams cells per mole CH₄) of M. marburgensis and M. arboriphilus on H₂ and CO₂ were found to be much lower (1.5–3 g/mol) than the growth yield of M. barkeri (7 g/mol) (Table S1).

Discussion

At Fdred/Fdox ratios >0.9 the redox potential (E°) of C. pasteurianum Fdox (E° = −400 mV) approaches ~500 mV. The redox potential (E°) of the 2H⁺/H₂ couple is ~414 mV. Therefore, Fd can be almost fully reduced with H₂ (100% at 1 bar) at pH 7 only if the reduction is energy driven. Our finding (Fig. 2B) that the MvhADG/HdrABC complex catalyzes the CoM-S-CoB-dependent complete reduction of Fd with H₂ (100%, at 1 bar) thus unambiguously shows that via the complex the endergonic reduction of Fd with H₂ (reaction 3a) is coupled to the exergonic reduction of CoM-S-CoB with H₂ (reaction 2).

With respect to the stoichiometry of coupling the results suggest that one Fd (two-electron acceptor) and one CoM-S-CoB (two-electron acceptor) are reduced by 2 H₂ (Fig. 3; reaction 4). The reduction of MTZ (four-electron acceptor) at limiting concentrations of CoM-S-CoB concentrations yielded MTZ to CoM-S-CoB ratios between 0.5 to 1 and 0.7 to 1. One explanation for this result could be that the artificial electron acceptor MTZ has a much more positive redox potential (E°) than Fd (E° = −400 mV) and that therefore MTZ can also accept electrons from thiols and from the reduced MvhADG/HdrABC complex at redox potential too positive for Fd reduction:

$$2\text{H}_2 + \text{Fd}^{\text{ox}} + \text{CoM-S-CoB} \rightarrow \text{Fd}^{\text{red}} + \text{CoM-SH} + \text{CoB-SH}$$

$$+ 2\text{H}^+ \Delta \text{AG}^{\circ} = −55 \text{kJ/mol}.$$  

Our finding that the MvhADG/HdrABC complex from hydrogenotrophic methanogens couples the reduction of Fd with H₂ to the reduction of CoM-S-CoB with H₂ can explain the effect first described in 1977 by Robert P. Gunsalus (RPG effect) (34). It was observed that cell extracts of M. thermotrophicus catalyzed the reduction of CO₂ with H₂ to methane only after addition of catalytic amounts of methyl-coenzyme M, fumarate, or serine (12, 34). The three additives have in common that they generate CoM-S-CoB. Methyl-coenzyme M generates CoM-S-CoB via methyl-coenzyme M reductase; fumarate via coenzyme M- and coenzyme B-dependent fumarate reductase (35); and serine via serine hydroxymethyltransferase (36), methylenetetrahydrodihydropterin reductase, methylenetetrahydrodi- hydropterin:coenzyme M methyltransferase, and methylenetetrahydropterin reductase. The CoM-S-CoB generated thus triggers the reduction of Fd with H₂ that is required as an electron donor in the initial step of methanogenesis from CO₂.

Three cytoplasmic enzyme complexes mediating electron bifurcation have been published to date, the Bcd/EtfAB complex from Clostridium kluwyeri (19), the NfnAB complex from C. kluwyeri.

Fig. 3. Ferredoxin (Fd) reduction with 100% H₂ at limiting concentrations of CoM-S-CoB catalyzed by the MvhADG/HdrABC complex from M. marburgensis. (A) Time course with 12 nmol CoM-S-CoB (33 nmol Fd), 24 nmol CoM-S-CoB (33 nmol Fd), and 36 nmol CoM-S-CoB (45 nmol Fd). (B) Amounts of Fd reduced versus the amounts of CoM-S-CoB added. The assays were performed at 40 °C in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture with 1.6 M potassium phosphate (pH 7), MvhADG/HdrABC complex (~0.1 unit), Fd from C. pasteurianum, and CoM-S-CoB in the amounts indicated. The reduction was started by enzyme protein and followed photometrically at 320 nm.

Fig. 4. Metronidazole (MTZ) reduction with 100% H₂ at limiting concentrations of CoM-S-CoB catalyzed by the MvhADG/HdrABC complex from M. marburgensis. (A) Time course with 90 nmol MTZ and 72 nmol CoM-S-CoB. (B) Amounts of MTZ reduced versus the amounts of CoM-S-CoB added. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture with 1.6 M potassium phosphate (pH 7), MvhADG/HdrABC complex (~0.1 unit), MTZ (90 nmol), and CoM-S-CoB as indicated. The reduction was started by enzyme protein and followed photometrically at 320 nm.
(21), and the heterotrimic [FeFe]-hydrogenase from *Thermotoga maritima* (20), catalyzing reactions 5, 6, and 7, respectively. The three complexes have in common with the MvhADG/HdrABC complex (catalyzing reaction 4) that they catalyzeFd-dependent reactions and that they containFAD (in the case of the [FeFe]-hydrogenase from *T. maritima*, FMN) that is only loosely bound when in the reduced form, resulting in a gradual loss ofactivity in the absence of added FAD (FMN). In the Bcd/EtfAB complex FAD is the only prosthetic group found. It is thus reasonable to assume that electron bifurcation is flavin based and that therefore the FAD harboring subunit HdrA is the site of electron bifurcation in the MvhADG/HdrABC complex. Interesting in this respect is that HdrA is one of the most highly conserved proteins in methanogens and that its presence is not restricted to them, suggesting an electron bifurcating function within another context in nonmethanogens (37):

$$2 \text{NADH} + \text{Fd}_{\alpha} + \text{crotonyl-CoA} \rightarrow 2 \text{NAD}^+ + \text{F}d_{\text{red}}^{2-} + \text{butyryl-CoA}$$  \[5\]

$$\text{NADH} + \text{F}d_{\text{red}}^{2-} + 2 \text{NADP}^+ + \text{H}^+ \rightarrow \text{NAD}^+ + \text{Fd}_{\alpha} + 2 \text{NADPH}$$  \[6\]

$$\text{NADH} + \text{F}d_{\text{red}}^{2-} + 3\text{H}^+ \rightarrow 2\text{H}_2 + \text{NAD}^+ + \text{Fd}_{\alpha}.$$  \[7\]

The proposed mechanism of flavin-based electron bifurcation is based on the findings that flavoproteins (FP) can exhibit three different redox potentials, namely an $E_{1/2}$ for the FP/FPH2 couple ($n = 2$), an $E_{1/2}$ for the FP/FDH couple ($n = 1$), and an $E_{1/2}$ for the FPH2-FDH couple ($n = 1$). $E_{1/2}$ (FP/FDH) is generally more positive and $E_{1/2}$ (FP/FPH2) more negative than $E_{1/2}$ (FP/FDH). Oxidation of FPH2 by two one-electron acceptors with different redox potentials thus leads to a bifurcation of the two electrons in FPH2 (21).

The H2-threshold concentration of hydrogenotrophic methanogens was confirmed to be somewhat <10 Pa, indicating that methanogenesis from H2 and CO2 is coupled with the generation of <1 mol ATP per mole methane, which is also indicated by the relatively low growth yields (Table S1). The metabolic scheme shown in Fig. 5 was drawn such that per mole methane 0.5 mol ATP is formed. It assumes that reaction 4 catalyzed by the MvhADG/HdrABC complex is fully coupled (one Fd reduced per mole CoM-S-S-CoB), that methyl group transfer from methyltetrahydrodromethanopterin (CH3-H4MPT) to coenzyme M is associated with the electrogenic translocation of 2 Na+, and that ATP synthesis via the A1Aox-ATP synthase consumes four electrogenic sodium ions (2, 3). Fig. 5 does not take the recent evidence into account that in *Methanococcus maripaludis* the MvhADG/HdrABC complex forms a supercomplex with the formylmethanofuran dehydrogenase complex (38) and that therefore the first and the last step of methanogenesis from H2 and CO2 proceed in close proximity which was already proposed 20 y ago in a review by Rouvier and Wolfe (39).

One can make other assumptions, e.g., if in the scheme shown in Fig. 5 the Na+/ATP stoichiometry was chosen to be 3 to 1 rather than 4 to 1, then the ATP gain would be 0.75 ATP, which would also conform with the determined H2-threshold concentrations. Or, if Fd reduction via the MvhADG/HdrABC complex is partially uncoupled, then Fd has to be additionally reduced with H2 via the energy-converting hydrogenase complexes EhaA-T or EthBA-Q so that CO2 reduction to methane can proceed. In this case the energy required to drive the reverse electron transport from H2 to Fd is no longer available for ATP synthesis with the result that the ATP gain and thus the growth yield decrease. *Methanosarcina* species show a much higher H2-threshold concentration than hydrogenotrophic methanogens (Table S1), indicating that methane formation from CO2 and H2 in these methanogens is coupled with the generation of more than one ATP. In *Methanosarcina* species Fd reduction with H2 is coupled to CoM-S-S-CoB reduction with H2 mainly via a chemiosmotic mechanism (for a scheme see ref. 2).

**Materials and Methods**

*M. marburgensis* (DSM 2133) was obtained from the Deutsche Sammlung von Mikroorganismen (DSMZ). The Archaeon was grown anaerobically at 65 °C on 80% H2/20% CO2/0.1% H2S in a 2-L fermenter containing 1.5 L completely mineral salt medium (32). The methods for the cultivation of the other organisms and for the determination of the H2-threshold concentrations and of growth yields are described in *SI Materials and Methods*.

**Purification of the MvhADG/HdrABC Complex from M. marburgensis**. Purification was performed under strictly anaerobic conditions at 4 °C except for the chromatographic steps, which were performed at 18 °C in an anaerobic chamber (Coy Laboratory Products). All buffers used contained 2 mM DTT, 2 mM CoM-SH, and 20 mM FAD. Cell extracts were routinely prepared from 15 g (wet mass) of *M. marburgensis* cells that had been freshly harvested under anaerobic conditions. The cells were suspended in ~30 mL 50 mM Tris-HCl, pH 7.6 (buffer A), and passed three times through a French pressure cell at 150 MPa. Cell debris was removed by centrifugation at 160,000 g for 30 min. The supernatant (35 mL), designated cell extract and containing ~800 mg protein, was applied to a DEAE-Sepharose fast flow column (2.6 × 16 cm) equilibrated with buffer A. Protein was eluted by a NaCl step gradient in buffer A: 100 mL 0 M NaCl, 100 mL 0.2 M NaCl, 100 mL 0.3 M NaCl, and 100 mL 0.4 M NaCl (flow rate: 5 mL/min). H2-CoM-S-S-CoB oxidoreductase activity (measured as described in the legend to Table 1) eluted in the last peak. The fractions with the highest activities (~40 mL) were applied to a Hi-Load Q-Sepharose column (2.6 × 16 cm), equilibrated with buffer A. Protein...
was eluted by a NaCl step gradient in buffer A: 100 μL 0 M NaCl, 100 μL 0.3 M NaCl, 100 μL 0.4 M NaCl, 50 μL 0.45 M NaCl, and 50 μL 0.54 M NaCl (flow rate: 1 mL/min). H2: CoM-S-S-CoB oxidoreductase activity eluted in the last peak. Again the fractions with the highest activities (15 mL) were combined and concentrated by ultrafiltration in 10-kDa Amicon tubes from Millipore to 2–3 mL, which were then applied to a Superdex 200 column (2.6 × 60 cm) equilibrated with buffer B (buffer A + 150 mM NaCl). Protein was eluted by washing the column with buffer B (flow rate: 1 mL/min) for ~3.5 h. H2: CoM-S-S-CoB oxidoreductase activity eluted after ~2.5 h in the middle of three peaks with a protein concentration of ~1 mg/mL. The enzyme solution was stored under H2 gas at 4 °C.

Ferredoxin and hydrogenase from C. pasteurianum were prepared according Schönheit et al. (23) and Li et al. (19), respectively.

**Determination of Specific Enzyme Activities and Reaction Stoichiometries.**

The assays were performed in anaerobic 1.5-ml SUPRASIL UV cuvettes from Hellma, which were sealed by cooked and autoclaved butyl rubber stoppers from Deutsch & Naumann, containing 0.75 mL assay mixture and H2 at a pressure of 1.2 bar (at 60 °C ~0.6 mM H2 in solution). For composition of assay mixture see the table and figure legends. Components were added anoxically by Hamilton syringes (Bonaduz). Protein was quantified with Bio-Rad dye reagent and BSA as standard.

Supporting Information

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SI Materials and Methods

Metronidazole, benzyl viologen, coenzyme M (2-mercaptoethanol-sulfonate), and FAD were purchased from Sigma-Aldrich. N₂ (99.996%), H₂ (99.993%), and H₂S (>=99%), as well as H₂/CO₂ (80%/20%) and N₂/CO₂ (80%/20%), were from Messer Industriegase. HPLC columns (Luna 5u C8, 100A; 21.2 mm and 4.6 mm × 250 mm) were obtained from Phenomenex. FPLC columns (DEAE-Sepharose, Q-Sepharose, and Superdex gel filtration) were from Pharmacia Biotech or GE Healthcare. The HPLC system was from Sykam and the FPLC system was from Pharmacia Biotech.

Cultivation of Microorganisms. Methanobrevibacter arborphilus (DSM 744) and Methanosarcina barkeri (DSM 804) were grown anaerobically at 37 °C on 80% H₂/20% CO₂/0.1% H₂S in a 2-L fermenter containing 1.5 L complete mineral salt medium. The medium for M. arborphilus contained (per liter deionized water) 0.75 g KH₂PO₄, 0.75 g K₂HPO₄, 1 g NH₄Cl, 4.8 g NaHCO₃, 1 g yeast extract, 0.5 g cystein-hydrochloride, and 500 µL resazurin (0.2%); 10 mL vitamin solution containing (per liter deionized water) 2 mg biotin, 2 mg folic acid, 10 mg pyridoxale-hydrochloride, 5 mg thiamin-hydrochloride, 5 mg riboflavin, 5 mg nicotinic acid, 0.1 mg vitamin B₁₂, 5 mg p-aminobenzoic acid, and 5 mg DL-α-liponate; and 10 mL trace element solution containing (per liter deionized water) 9 g Titrilplex I (pH 6.7 with NaOH), 8 g MgCl₂ × 6 H₂O, 1 g FeCl₃ × 4 H₂O, 20 mg CoCl₂ × 6 H₂O, 20 mg Na₂MoO₄ × 2 H₂O, 0.12 g NiCl₂ × 6 H₂O, and 0.11 g CaCl₂ × 2 H₂O. After autoclaving the solution 5 mL of MgCl₂ solution (636 g MgCl₂ × 6 H₂O/L), 10 mL of Na₂S solution (28 g Na₂S × 9 H₂O/L), and 1 mL penicillin solution (100 µg/mL) were added.

M. barkeri had first to be adapted from growth on a methanol-containing medium. This medium contained (per liter deionized water) 10 mL methanol, a solution of 1.24 g CaCl₂ × 2 H₂O, 2 g MgCl₂ × 6 H₂O, 2 g KCl, 10 g NaCl, 2.5 g NH₄Cl, 25 mg FeCl₃ × 6 H₂O, and 500 µL resazurin (0.2%), which was slowly added to a solution of 0.39 g NaH₂PO₄ × 2 H₂O, 0.44 g Na₂HPO₄ × 2 H₂O, 0.14 g cysteinhydrochloride, 13.6 g imidazole (pH 6.4 with HCl), 10 mL of vitamin solution (see M. arborphilus) and 10 mL of trace element solution element solution (per liter deionized water) 1.5 g Titrilplex I (pH 6.5 with KOH), 0.1 g FeSO₄ × 7 H₂O, 0.1 g ZnSO₄ × 7 H₂O, 0.1 g CoCl₂ × 6 H₂O, 10 mg Na₂MoO₄ × 2 H₂O, 30 mg NiCl₂ × 6 H₂O, 10 mg CuSO₄ × 7 H₂O, 10 mg AlCl₃ × 6 H₂O, and 15 mg NaHSeO₃). After autoclaving the medium 10 mL of Na₂S solution (28 g Na₂S × 9 H₂O/L) and 1 mL penicillin solution (100 µg/mL) were added. The media for growing M. barkeri on 80% H₂/20% CO₂ contained no methanol but 2 g of yeast extract and 1 g of tryptophan and the pH was set at 7.4 rather than at 6.4.

H₂-Threshold Concentration Determination. H₂-threshold concentrations of M. marburgensis, M. arborphilus, and M. barkeri (grown as described above) were determined with 1-g cells (wet mass) harvested in the logarithmic growth phase and resuspended in 1 mL 50 mM potassium phosphate buffer (pH 7) with 25 mM NaCl, 2 mM DTT; and 2 mM MgCl₂ or medium, respectively. The 2-mL cell suspensions were transferred to 150 mL H₂-free Müller–Krempel flasks (Büchler) sealed by Perbunan rubber stoppers (Deutsch & Naumann) and repeatedly evacuated and filled with 80% N₂/20% CO₂ to remove remaining traces of H₂. Then H₂ was added, up to 30 Pa in the case of M. marburgensis and M. arborphilus and up to 600 Pa in the case of M. barkeri. The flasks were then rapidly shaken at 60 °C (M. marburgensis) or 37 °C (M. arborphilus and M. barkeri). Gas samples were taken with a gas-tight syringe (Hamilton) at first every 10 min and then later every hour. After the H₂ concentration reached constant, H₂ was added again to ascertain that the same H₂ threshold was reached a second time. The samples were analyzed by gas chromatography using a stainless steel separation column (0.5 nm molecular sieve; 80/100 mesh; length = 2 m × 4 mm) kept at 120 °C. High mixing ratios of H₂ [200–5,000 parts per million by volume (ppmv)] were detected with a thermal conductivity detector (GC-8A; Shimadzu) (carrier gas, N₂; flow rate, 40 mL/min) and low mixing ratios of H₂ [0.01–200 ppmv] with an H₂O reduction gas detector (Trace Analytical; Techmation) (carrier gas, H₂-free synthetic air; flow rate, 40 mL/min) (1). The peak heights/areas were proportional to the H₂ concentration. Calibrations were done with calibration gas mixtures (1,000 ppmv and 50 ppmv) from Messer Industriegase.

Growth Yield Determination. From cultures grown as described above culture samples for optical density (OD) determination and gas samples for methane determination were taken every few hours depending on the doubling times of the organisms. OD was measured at 578 nm, which between 0 and 0.3 was proportional to the cell concentration. If necessary, samples had to be diluted. The gas samples for methane determination were injected with a gas-tight syringe (Hamilton) for methane analysis into a SRI 8610C gas chromatograph (SRI Instruments) equipped with a flame ionization detector (GC 8000; CE Instruments) using a stainless steel separation column (0.5-nm molecular sieve; 80/100 mesh, 130 × 2 cm) kept at 120 °C and N₂ as carrier gas (flow rate: 30 mL/min). The peak heights/areas were proportional to the H₂ concentration. Calibrations were done with CH₄ from Messer Industriegase.

Fig. S1. Drawing of the MvhADG/HdrABC complex highlighting the subunits stoichiometry and the subunits prosthetic groups. The six subunits are present in a 1:1:1:1:1:1 stoichiometry (1). MvhA is the [NiFe] center harboring large hydrogenase subunit, MvhG harbors the small hydrogenase subunit with sequence motifs for two [4Fe4S] and one [3Fe4S] clusters, MvhD harbors a subunit with one [2Fe2S] cluster, HdrA harbors an FAD and four [4Fe4S] clusters, HdrB harbors the zinc active site and an unusual [4Fe4S] center, and HdrC is a subunit with two [4Fe4S] clusters (1–6). The genes encoding these proteins are organized in three transcription units, mvhDGAB, hdra, and hdrBC (4, 7, 8). mvhb encodes a polyferredoxin with 12 [4Fe4S] clusters that partially copurifies with the MvhADG/HdrABC complex and that has also been characterized (9, 10). The complex is found in most methanogenic archaea, exceptions being several members of the Methanomicrobiales (11).

Fig. S3. Rate of metronidazole (MTZ) reduction with H₂ in the absence (△) and presence (○) of CoM-S-S-CoB versus the benzyl viologen concentration catalyzed by a cell extract of *M. marburgensis*. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture and 100% H₂ as gas phase. The mixture contained 1.6 M potassium phosphate (pH 7), 0.5 mM CoM-S-S-CoB, 150 μM MTZ, and 50–100 μg cell extract. The reduction was started by the cell extract and followed photometrically at 320 nm.

Fig. S4. Purification of the MvhADG/HdrABC complex from *M. marburgensis* as followed by SDS/PAGE. Lane A, molecular mass standards; lane B, ∼20 μg cell extract protein; lane C, ∼20 μg protein after DEAE-Sepharose; lane D, ∼20 μg protein after Q-Sepharose; and lane E, ∼100 μg protein after Superdex gel filtration. Protein was denatured in SDS at 95 °C for 5 min and separated in 12% slab gels (7 × 10 cm) that were subsequently stained with Coomassie blue G250 (1).


Fig. S5. (A) pH, (B) temperature, and (C) potassium phosphate concentration optima of the purified MvhADG/HdrABC complex catalyzing the CoM-S-S-CoB-dependent reduction of ferredoxin with H₂. The assays were performed in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture and 100% H₂ as gas phase. The 0.75-mL assay mixture contained 1 mM CoM-S-S-CoB, ∼1–10 μg purified MvhADG/HdrABC complex from *M. marburgensis*, and 33 μM ferredoxin. The reaction was started by protein and followed photometrically at 390 nm. The pH was 7, the temperature 60 °C, and the potassium phosphate concentration 1.6 M or as indicated. Almost identical profiles were obtained when measuring the CoM-S-S-CoB-dependent reduction of metronidazole with H₂.
Fig. S6. Lineweaver–Burk plots of the rates of the CoM-S-S-CoB-dependent ferredoxin (Fd) reduction with $\text{H}_2$ versus the concentration of (A) Fd, (B) CoM-S-S-CoB, and (C) $\text{H}_2$. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture with 1.6 M potassium phosphate (pH 7) and $\sim 1–10 \mu\text{g}$ protein. The reaction was started by protein and followed photometrically at 390 nm. The concentration of Fd was 33 $\mu$M, that of CoM-S-S-CoB was 1 mM, and that of $\text{H}_2$ was 100% in the gas phase or as indicated. The data are from two independent experiments in which each point was measured twice.

Fig. S7. Lineweaver–Burk plots of the rates of the CoM-S-S-CoB-dependent metronidazole (MTZ) reduction with $\text{H}_2$ versus the concentration of (A) MTZ, (B) CoM-S-S-CoB, and (C) $\text{H}_2$. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettes containing 0.75 mL assay mixture with 1.6 M potassium phosphate (pH 7) and $\sim 1–10 \mu\text{g}$ protein. The reaction was started by protein and followed photometrically at 320 nm. The concentration of MTZ was 0.15 mM, that of CoM-S-S-CoB was 1 mM, and that of $\text{H}_2$ was 100% in the gas phase or as indicated. The data are from two independent experiments in which each point was measured twice.
Table S1. H₂-threshold concentrations and growth yields of *M. marburgensis* in comparison with those of other hydrogenotrophic and cytochrome-containing methanogens

<table>
<thead>
<tr>
<th>Methanotrophic methanogens</th>
<th>H₂ threshold, Pa (references)</th>
<th>Y(CH₄), g/mol (references)</th>
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<tbody>
<tr>
<td><em>Methanothermobacter marburgensis</em></td>
<td>8 (this work)</td>
<td>Up to 3 (this work and refs. 1 and 2)</td>
</tr>
<tr>
<td><em>Methanobrevibacter arborophilus</em></td>
<td>8 (this work and ref. 3)</td>
<td>Up to 2.5 (this work and refs. 4 and 5)</td>
</tr>
<tr>
<td><em>Methanobacterium bryantii</em></td>
<td>7 (6)</td>
<td></td>
</tr>
<tr>
<td><em>Methanobacterium formicicum</em></td>
<td>7 (3, 6)</td>
<td>3.5 (9)</td>
</tr>
<tr>
<td><em>Methanobrevibacter smithii</em></td>
<td>10 (3)</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>Methanococcus vannielli</em></td>
<td>7.5 (3)</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>Methanospirillum hungatei</em></td>
<td>9.5 (3, 6)</td>
<td>n.d.</td>
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<th>Cytochrome containing methanogens</th>
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<tbody>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>150 (this work)</td>
<td>Up to 7.5 (this work and refs. 1 and 10)</td>
</tr>
<tr>
<td><em>Methanosarcina sp. strain 227</em></td>
<td>n.d.</td>
<td>8.7 (11)</td>
</tr>
</tbody>
</table>

The H₂-threshold concentrations were determined with cell suspensions and the growth yields with exponentially growing cultures. For details see Material and Methods. n.d., not determined.