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

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In methanogenic archaea growing on H_2 and CO_2 the first step in methanogenesis is the ferredoxin-dependent endergonic reduction of CO_2 with H_2 to formylmethanofuran and the last step is the exergonic reduction of the heterodisulfide CoM-S-S-CoB with H_2 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 H_2. Per mole CoM-S-S-CoB added, 1 mol of ferredoxin (Fd) was reduced, indicating an electron bifurcation coupling mechanism: $2H_2 + Fd_{ox} + CoM\text{-}S\text{-}S\text{-}CoB \rightarrow Fd_{red}^{2-} + CoM\text{-}SH + CoB\text{-}SH + 2H^+$. This stoichiometry of coupling is consistent with an ATP gain per mole methane from 4 H_2 and CO_2 of near 0.5 deduced from an H_2-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 H_2 and CO_2 is an important reaction in the global methane cycle (1). It is mediated by hydrogenotrophic methanogenic archaea (2). These archaea appear to mainly involve only two membrane-associated enzymes in their energy metabolism, a methyltransferase (MtrA-H) and an A_1A_o-ATP synthase (AhaA-IK). The methyltransferase reaction is associated with the buildup of an electrochemical sodium ion potential, which is used via the ATP synthase to drive the synthesis of ATP (3). All of the other enzymes required for CO_2 reduction with H_2 to methane are found in the cytoplasmic cell fraction. The cytoplasmic enzymes include the enzymes catalyzing the first step and the last step in methanogenesis, namely the endergonic reduction of CO_2 and methanofuran (MFR) with H_2 to formyl-MFR (reaction **1**) and the exergonic reduction of the heterodisulfide CoM-S-S-CoB with H_2 to coenzyme M (CoM-SH) and coenzyme B (CoB-SH) (reaction **2**):

$$H_2 + CO_2 + MFR \rightarrow \text{formyl-MFR} + H_2O \quad \Delta G^{o'} = +20\,\text{kJ/mol} \qquad \textbf{[1]}$$

$$H_2 + \text{CoM-S-S-CoB} \rightarrow \text{CoM-SH} + \text{CoB-SH} \quad \Delta G^{o'} = -55\,\text{kJ/mol}. \qquad \textbf{[2]}$$

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 CO_2 with H_2 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_o') of the polyferredoxin is not known but can be predicted to lie between that of the 2 H^+/H_2 couple (−414 mV) and that of the CO_2 + 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 H_2. However, these enzymes appear to have a mainly anabolic function (6–9):

$$H_2 + Fd_{ox} \rightarrow Fd_{red}^{2-} + 2H^+ \qquad \textbf{[3a]}$$

$$Fd_{red}^{2-} + 2H^+ + CO_2 + MFR \rightarrow Fd_{ox} + \text{formyl-MFR} + H_2O. \qquad \textbf{[3b]}$$

Reaction **2** has been proposed to be catalyzed by the cytoplasmic MvhADG/HdrABC complex composed of the [NiFe]-hydrogenase MvhADG and the heterodisulfide reductase HdrABC, which is an iron–sulfur flavoprotein (Fig. S1). The two enzymes are electrically connected via the [2Fe2S] protein MvhD (9, 10). The problem was that the purified complex catalyzed reaction **2** at significant rates only in the presence of viologen dyes that were reduced with H_2 and reoxidized with CoM-S-S-CoB during catalysis (11).

Over 20 y ago Bobik and Wolfe found that cell extracts of *Methanothermobacter thermautotrophicus* (a hydrogenotrophic methanogen) catalyze the reduction of CO_2 to formyl-MFR with H_2 (12) and the reduction of metronidazole (MTZ) with H_2 (13) only in the presence of CoM-S-S-CoB. MTZ reduction (14, 15) and CO_2 reduction to formyl-MFR (16, 17) are now known to be Fd-dependent reactions. With this knowledge the findings by Bobik and Wolfe (12, 13) can be interpreted to indicate that in cell extracts of *M. thermautotrophicus* the reduction of Fd with H_2 and the reduction of CoM-S-S-CoB (E_o' = −140 mV) with H_2 are coupled via the recently discovered mechanism of flavin-based electron bifurcation (18–21). Via this novel mechanism exergonic and endergonic redox reactions with acceptor/donor potentials between 0 and −500 mV can be coupled without involving membrane proteins. We therefore proposed in 2008 (2) that the MvhADG/HdrABC complex couples the endergonic reaction **3a** to the exergonic reaction **2**. The experimental evidence is now presented for the complex purified from the hydrogenotrophic methanogen *Methanothermobacter marburgensis.*

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Results

In MvhADG/HdrABC activity assays Fd with two [4Fe4S] clusters from *Clostridium pasteurianum* ($E_o' = -400$ mV; $n = 2$) (22) and an absorption maximum at 390 nm ($\varepsilon = 30{,}000\ M^{-1}\cdot cm^{-1}$) (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 interchangeable (24). The reduction of Fd was followed by measuring the decrease in absorbance at 390 nm ($\Delta\varepsilon = 11{,}000\ M^{-1}\cdot cm^{-1}$) (25). Where indicated, MTZ was used as an artificial electron acceptor, its reduction being followed by measuring the decrease in absorbance at 320 nm ($\varepsilon = \Delta\varepsilon = 9{,}300\ M^{-1}\cdot cm^{-1}$) (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-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-S-CoM reductase (28). We convinced ourselves with pure CoM-S-S-CoM and CoB-S-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-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-S-CoB.

The H_2: CoM-S-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-S-CoB-Dependent Fd Reduction with H_2 in Cell Extracts. Cell suspensions of *M. marburgensis* catalyze the reduction of CO_2 with H_2 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 H_2 and CO_2 such as the MvhADG/HdrABC complex. The finding by Setzke et al. in 1994 (11) that cell extracts catalyzed the reduction of CoM-S-S-CoB with H_2 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 H_2 at significant rates only in the presence of CoM-S-S-CoB (Fig. 1*A*). 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% H_2, it increased to 5 units/mg, which is close to the specific activity of methane formation from H_2 and CO_2 catalyzed by cell suspensions of *M. marburgensis* (see above). [NiFe]-hydrogenases such as MvhADG inhibited by O_2 are known to be slowly reactivated upon incubation with H_2 (30).

The cell extracts also catalyzed the CoM-S-S-CoB-dependent reduction of MTZ with H_2 (Fig. 1*B*). 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% H_2.

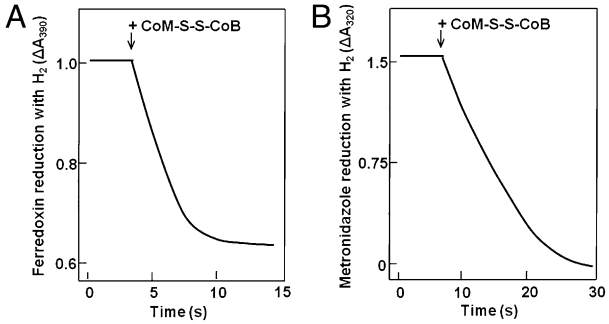

Fig. 1. CoM-S-S-CoB dependence of (*A*) ferredoxin (Fd) reduction and of (*B*) metronidazole (MTZ) reduction with H_2 catalyzed by a cell extract of *M. marburgensis*. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettetes containing 0.75 mL assay mixture and 100% H_2 as the gas phase. The 0.75-mL assay mixture contained 1.6 M potassium phosphate (pH 7), 1 mM CoM-S-S-CoB, ~30 μg (*A*) or ~140 μg (*B*) cell extract, and either 35 μM Fd (*A*) or 150 μM MTZ (*B*). The reduction was started by CoM-S-S-CoB and followed photometrically at 390 nm (*A*) or 320 nm (*B*). After 30 s both Fd and MTZ were reduced to 100%.

When in the reduction assays cuvettetes 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 H_2 already in the absence of CoM-S-S-CoB. At BV concentrations of ~25 μM the reaction became essentially independent of CoM-S-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 H_2 in the absence of CoM-S-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-S-CoB-dependent reduction of Fd with H_2 was purified 26-fold in a 14% activity yield (Table 1). After incubation for 10 h at 4 °C under 100% H_2, 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-S-CoB-dependent reduction of Fd (or MTZ) with H_2. Contact with O_2 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-S-CoB-dependent reduction of Fd with H_2 at pH 7.5 (pH optimum), a potassium phosphate

Table 1. Purification of the MvhADG/HdrABC complex from *Methanothermobacter marburgensis*

	Units	Protein, mg	Units/mg	Purification fold	Yield, %
Cell extract	4,025 (3,200)	800	5 (4)	1	100
DEAE-Sepharose	1,800 (1,200)	200	9 (6)	1.8 (1.5)	45 (38)
Q-Sepharose	880 (540)	60	15 (9)	3 (2.3)	22 (17)
Superdex	550 (250)	5	110 (50)	26 (15)	14 (8)

The activity was determined by following the CoM-S-S-CoB-dependent reduction of ferredoxin (Fd) or of metronidazole (MTZ) (numbers in parentheses) with H_2. Before measurement of activity the fractions were incubated with 100% H_2 at 4 °C. The assays were performed at 60 °C in 1.5-mL anaerobic cuvettetes containing 0.75 mL assay mixture and 100% H_2 as the gas phase. The 0.75-mL assay mixture contained 1.6 M potassium phosphate (pH 7), 1 mM CoM-S-S-CoB, 1–150 μg protein, and either 35 μM Fd or 150 μM MTZ. The reaction was started with protein and followed photometrically at 390 nm in the case of Fd and 320 nm in the case of MTZ reduction.

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% H_2 in the gas phase (at 1.2 bar the dissolved H_2 concentration ~0.6 mM H_2) 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. S6 *A* and *B*). The apparent K_m for H_2 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. S6*C*). The apparent K_m for H_2 compares with that of 20% H_2 reported for methane formation from H_2 and CO_2 in growing cultures of *M. marburgensis* (32). CoM-S-S-CoB-dependent MTZ reduction showed apparent K_m values near 0.1 mM for MTZ, <0.1 mM for CoM-S-S-CoB, and <5% for H_2 (Fig. S7 *A–C*). The much lower apparent K_m for CoM-S-S-CoB measured with metronidazole than with ferredoxin as electron acceptor could be due to the fact that the reduction of metronidazole with H_2 is strongly exergonic whereas the reduction of ferredoxin with H_2 is an endergonic reaction.

Energetic Coupling. The dependence of Fd reduction with H_2 on the presence of CoM-S-S-CoB was the first indication that the MvhADG/HdrABC complex couples the endergonic reduction of Fd with H_2 to the exergonic reduction of CoM-S-S-CoB with H_2. Final evidence was obtained by showing that the reduction of Fd with H_2 proceeded beyond the equilibrium concentrations expected for the noncoupled reaction. Fig. 2*A* shows the reduction of Fd with 100% H_2 at pH 7.0 and 1 bar ($E_o' = -414$ mV) catalyzed by the [FeFe]-hydrogenase from *C. pasteurianum* (noncoupled reaction) and Fig. 2*B* shows that by the MvhADG/HdrABC complex in the presence of CoM-S-S-CoB (coupled reaction). In the noncoupled reaction the Fd ($E_o' = -400$ mV) (22) was reduced to 55% ($E' = -410$ mV) and in the coupled reaction to almost 100% ($E' \sim -500$ mV) as in the case of Fd reduction with dithionite ($E_o' = -660$ mV) (33). A 100% reduction of Fd with H_2 (100% at 1 bar) is thermodynamically possible only if the reduction of Fd with H_2 was coupled to the exergonic reduction of CoM-S-S-CoB ($E_o' = -140$ mV) with H_2 ($E_o' = -414$ mV). The Fd used in the experiment had a $\Delta A_{390/280}$ of 0.8, indicating that the Fd was pure and not partially denatured (23).

Stoichiometry of Coupling. After having shown that Fd reduction with H_2 is energetically coupled to the reduction of CoM-S-S-CoB, the question of the stoichiometry of coupling was addressed. For this the mole amounts of Fd or of MTZ reduced with H_2 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 H_2 (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. 3*A*. 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 EhbA-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. 3*B*) by 2 H_2. 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 H_2 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-

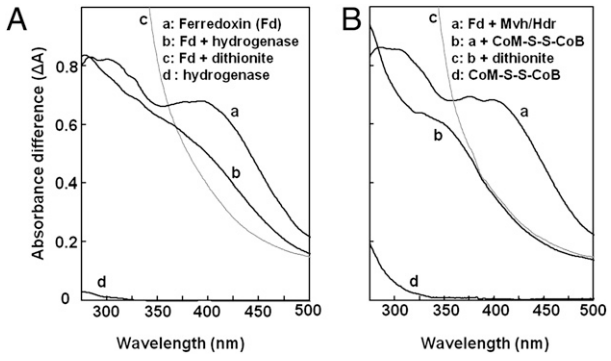

Fig. 2. Ferredoxin (Fd) reduction with 100% H_2 catalyzed by (*A*) [FeFe]-hydrogenase from *Clostridium pasteurianum* and by (*B*) the MvhADG/HdrABC complex from *M. marburgensis* in the presence of CoM-S-S-CoB. The 1.5-mL anaerobic cuvettetes contained 0.75 mL assay mixture containing 1.6 M potassium phosphate (pH 7) and where indicated Fd from *C. pasteurianum* (20 μM), [FeFe]-hydrogenase (~1 unit), MvhADG/HdrABC complex (~0.5 unit), CoM-S-S-CoB (0.5 mM), and/or sodium dithionite (0.5 mM). The gas phase was 100% H_2 at 1.2 bar and the temperature was 40 °C. The reactions were started by the addition of [FeFe]-hydrogenase (*A*, *b*) or dithionite (*A*, *c*) or CoM-S-S-CoB (*B*, *b*) followed by dithionite (*B*, *c*) and were completed within in a few seconds. The absorbance difference between the assay cuvettete and a cuvettete containing only phosphate buffer was recorded. A 0.5-mM dithionite solution in 1.6 M potassium phosphate (pH 7) showed an absorbance maximum near 320 nm with a ΔA_{320} of 1.2; at 380 nm ΔA was 0.

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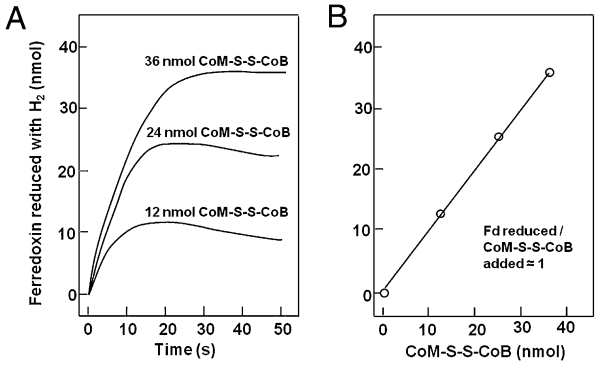

Fig. 3. Ferredoxin (Fd) reduction with 100% H_2 at limiting concentrations of CoM-S-S-CoB catalyzed by the MvhADG/HdrABC complex from *M. marburgensis*. (*A*) Time course with 12 nmol CoM-S-S-CoB (33 nmol Fd), 24 nmol CoM-S-S-CoB (33 nmol Fd), and 36 nmol CoM-S-S-CoB (45 nmol Fd). (*B*) Amounts of Fd reduced versus the amounts of CoM-S-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-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. 4*A*). This reduction was probably due to the CoM-SH and CoB-SH formed in the reaction, which reoxidized nonenzymatically to CoM-S-S-CoB with MTZ. Also the CoM-SH, DTT, and FAD present in the enzyme stock solution might contribute to the apparently CoM-S-S-CoB-independent MTZ reduction, which we tried to correct for by extrapolation (Fig. 4*A*). With this correction a stoichiometry of ~0.6 mol MTZ reduced per mole of CoM-S-S-CoB added was found (Fig. 4*B*). At CoM-S-S-CoB concentrations <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-S-CoB, this finding indicates that per mole of CoM-S-S-CoB reduced, between 2 and 2.4 mol of H_2 were oxidized.

H_2 Thresholds and Growth Yields. In the reduction of CO_2 with H_2 to methane equilibrium ($\Delta G = 0$ kJ/mol) is theoretically reached at a H_2 partial pressure (pH_2) of ~0.2 Pa (assuming the partial pressure of CO_2 to be equal to the partial pressure of methane) (2).

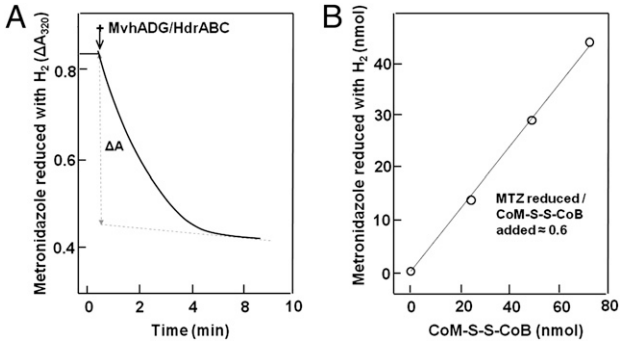

Fig. 4. Metronidazole (MTZ) reduction with 100% H_2 at limiting concentrations of CoM-S-S-CoB catalyzed by the MvhADG/HdrABC complex from *M. marburgensis*. (*A*) Time course with 90 nmol MTZ and 72 nmol CoM-S-S-CoB. (*B*) Amounts of MTZ reduced versus the amounts of CoM-S-S-CoB added. The assays were 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-S-CoB as indicated. The reduction was started by enzyme protein and followed photometrically at 320 nm.

However, when methanogenesis is coupled with the phosphorylation of ADP to ATP ($\Delta G' = +50$ kJ/mol ATP), then the H_2-equilibrium concentration (H_2-threshold concentration) is higher. Thus, when the threshold pH_2 is 30 Pa, then the ATP gain (mol ATP/mol CH_4) is 1. In turn, the ATP gain can be roughly estimated from the H_2-threshold concentration that is therefore an indicator of the stoichiometry of coupling between the exergonic and the endergonic reactions in CO_2 reduction with H_2 to methane.

The H_2-threshold concentration in cell suspensions catalyzing the reduction of CO_2 to methane was determined for three methanogens grown on H_2 and CO_2 and found to be ~8 Pa in the case of *M. marburgensis* and *Methanobrevibacter arboriphilus* and 150 Pa in the case of *Methanosarcina barkeri*, indicating an ATP gain <1 in the two hydrogenotrophic methanogens and an ATP gain >1 in the cytochrome containing methanogen. Consistently, the growth yields (grams cells per mole CH_4) of *M. marburgensis* and *M. arboriphilus* on H_2 and CO_2 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 Fd_{red}^{2-}/Fd_{ox} ratios >100 the redox potential (E') of *C. pasteurianum* Fd ($E_o' = -400$ mV) approaches −500 mV. The redox potential (E_o') of the 2 H^+/H_2 couple is −414 mV. Therefore, Fd can be almost fully reduced with H_2 (100% at 1 bar) at pH 7 only if the reduction is energy driven. Our finding (Fig. 2*B*) that the MvhADG/HdrABC complex catalyzes the CoM-S-S-CoB-dependent complete reduction of Fd with H_2 (100%, at 1 bar) thus unambiguously shows that via the complex the endergonic reduction of Fd with H_2 (reaction **3a**) is coupled to the exergonic reduction of CoM-S-S-CoB with H_2 (reaction **2**).

With respect to the stoichiometry of coupling the results suggest that one Fd (two-electron acceptor) and one CoM-S-S-CoB (two-electron acceptor) are reduced by 2 H_2 (Fig. 3) (reaction **4**). The reduction of MTZ (four-electron acceptor) at limiting CoM-S-S-CoB concentrations yielded MTZ to CoM-S-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 ($E_o' > 0$ V) than Fd ($E_o' = -400$ 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:

$$2H_2 + Fd_{ox} + \text{CoM-S-S-CoB} \rightarrow Fd_{red}^{2-} + \text{CoM-SH} + \text{CoB-SH} + 2H^+ \quad \Delta G^{o'} = -55 \text{ kJ/mol}. \qquad [4]$$

Our finding that the MvhADG/HdrABC complex from hydrogenotrophic methanogens couples the reduction of Fd with H_2 to the reduction of CoM-S-S-CoB with H_2 can explain the effect first described in 1977 by Robert P. Gunsalus (RPG effect) (34). It was observed that cell extracts of *M. thermautotrophicus* catalyzed the reduction of CO_2 with H_2 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-S-CoB. Methyl-coenzyme M generates CoM-S-S-CoB via methyl-coenzyme M reductase; fumarate via coenzyme M- and coenzyme B-dependent fumarate reductase (35); and serine via serine hydroxymethyltransferase (36), methylenetetrahydromethanopterin reductase, methyltetrahydromethanopterin:coenzyme M methyltransferase, and methyl-coenzyme M reductase. The CoM-S-S-CoB generated thus triggers the reduction of Fd with H_2 that is required as an electron donor in the initial step of methanogenesis from CO_2.

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

(21), and the heterotrimeric [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 catalyze Fd-dependent reactions and that they contain FAD (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 of activity 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):

$$2\ \mathrm{NADH} + \mathrm{Fd_{ox}} + \text{crotonyl-CoA} \rightarrow 2\ \mathrm{NAD^+} + \mathrm{Fd_{red}^{2-}} + \text{butyryl-CoA} \quad [5]$$

$$\mathrm{NADH} + \mathrm{Fd_{red}^{2-}} + 2\ \mathrm{NADP^+} + \mathrm{H^+} \rightarrow \mathrm{NAD^+} + \mathrm{Fd_{ox}} + 2\ \mathrm{NADPH} \quad [6]$$

$$\mathrm{NADH} + \mathrm{Fd_{red}^{2-}} + 3\mathrm{H^+} \rightarrow 2\mathrm{H_2} + \mathrm{NAD^+} + \mathrm{Fd_{ox}}. \quad [7]$$

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_o' for the FP/FPH$_2$ couple ($n = 2$), an E_o' for the FP/FPH couple ($n = 1$), and an E_o' for the FPH/FPH$_2$ couple ($n = 1$). E_o' (FP/FPH) is generally more positive and E_o' (FPH/FPH$_2$) more negative than E_o' (FP/FPH$_2$). Oxidation of FPH$_2$ by two one-electron acceptors with different redox potentials thus leads to a bifurcation of the two electrons in FPH$_2$ (21).

The H_2-threshold concentration of hydrogenotrophic methanogens was confirmed to be somewhat <10 Pa, indicating that methanogenesis from H_2 and CO_2 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 methyltetrahydromethanopterin (CH_3-H_4MPT) to coenzyme M is associated with the electrogenic translocation of 2 Na^+, and that ATP synthesis via the A_1A_o-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 H_2 and CO_2 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 H_2-threshold concentrations. Or, if Fd reduction via the MvhADG/HdrABC complex is partially uncoupled, then Fd has to be additionally reduced with H_2 via the energy-converting hydrogenase complexes EhaA-T or EhbA-Q so that CO_2 reduction to methane can proceed. In this case the energy required to drive the reverse electron transport from H_2 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 H_2-threshold concentration than hydrogenotrophic methanogens (Table S1), indicating that methane formation from CO_2 and H_2 in these methanogens is coupled with the generation of more than one ATP. In *Methanosarcina* species Fd reduction with H_2 is coupled to CoM-S-S-CoB reduction with H_2 mainly via a chemiosmotic mechanism (for a scheme see ref. 2).

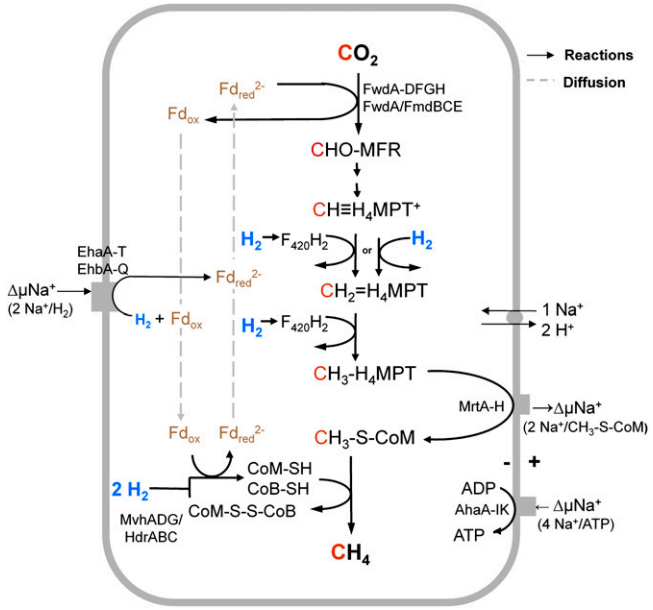

Fig. 5. Reactions, coenzymes, and enzymes involved in CO_2 reduction with 4 H_2 to CH_4 in *M. marburgensis*. Reduced ferredoxin (Fd), which is required for CO_2 reduction to formylmethanofuran (CHO-MFR), is regenerated in the MvhADG/HdrABC catalyzed reaction. If the latter is not completely coupled, then less reduced Fd is regenerated than required. In this case the energy-converting hydrogenases (EhaA-T and/or EhbA-Q) that catalyze the sodium motive force-driven Fd reduction with H_2 are proposed to step in. Eha and Ehb mainly have an anabolic function in providing the reduced Fd required for the reduction of CO_2 to CO and of acetyl-CoA + CO_2 to pyruvate. In *M. marburgensis* there are several ferredoxins that are considered to form a pool into which electrons are fed and from which electrons can be taken out (Discussion) (9). MFR, methanofuran; H_4MPT, tetrahydromethanopterin; CH≡H_4MPT$^+$, methenyl-H_4MPT$^+$; CH_2 = H_4MPT, methylene-H_4MPT; CH_3-H_4MPT, methyl-H_4MPT; MtrA-H, methyl transferase; FwdA-DFGH/FwdAFmdBCE, formyl-methanofuran dehydrogenase; AhaA-IK, A_1A_0 ATP synthase.

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% H_2/20% CO_2/0.1% H_2S 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 H_2-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 μM FAD. Cell extracts were routinely prepared from 15 g (wet mass) of *M. marburgensis* cells that had been freshly harvested under anoxic 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). H_2: 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 mL 0 M NaCl, 100 mL 0.3 M NaCl, 100 mL 0.4 M NaCl, 50 mL 0.45 M NaCl, and 50 mL 0.54 M NaCl (flow rate: 5 mL/min). H_2: 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. H_2: 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 H_2 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 cuvettetes from Hellma, which were sealed by cooked and autoclaved butyl rubber stoppers from Deutsch & Naumann, containing 0.75 mL assay mixture and H_2 at a pressure of 1.2 bar (at 60 °C ~0.6 mM H_2 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.

Synthesis of CoM-S-S-CoB, CoM-S-S-CoM, and CoB-S-S-CoB. CoM-S-S-CoB was synthesized from CoM-SH (excess) and CoB-SH via oxidation with O_2 at pH 9. CoM-SH (2-mercaptoethanesulfonate) was from Sigma-Aldrich. CoB-SH, CoB-S-S-CoB, and CoM-S-S-CoM were synthesized as described previously (40). CoM-S-S-CoB was separated from CoM-S-S-CoM and CoM-SH on SERDOLIT PAD II material (0.1–0.2 mm) from Serva. After the PAD II column (2.5 × 15 cm) CoM-S-S-CoB was separated from remaining CoB-S-S-CoB and dephosphodisulfides by HPLC on a Luna 5u C8, 100A (21.2 × 250-mm) column from Phenomenex, using a H_2O/trifluoroacetic acid (0.05%) (pH 2)/acetonitrile gradient and monitoring the separation at 210 nm. Identity and purity were checked by ^{1}H NMR, ^{13}C NMR, and MALDI-TOF mass spectrometry and by HPLC on an analytical Luna 5u C8, 100A (4.6 × 250 mm) column.

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