H$_2$: heterodisulfide oxidoreductase, a second energy-conserving system in the methanogenic strain Gö1

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Abstract. Washed everted vesicles of the methanogenic bacterium strain Gö1 catalyzed an H$_2$-dependent reduction of the heterodisulfide of HS-CoM (2-mercaptoethanesulfonate) and HS-HTP (7-mercaptoheptanoylthreonine phosphate) (CoM-S-S-HTP). This process was independent of coenzyme F$_{420}$ and was coupled to proton translocation across the cytoplasmic membrane into the lumen of the everted vesicles. The maximal H$^+$/CoM-S-S-HTP ratio was 2. The transmembrane electrochemical gradient thereby generated was shown to induce ATP synthesis from ADP + Pi, exhibiting a stoichiometry of 1 ATP synthesized per 2 CoM-S-S-HTP reduced (H$^+$/ATP = 4). ATP formation was inhibited by the uncoupler 3,5-di-tert-butyl-4-hydroxybenzylidene-malononitrile (SF 6847) and by the ATP synthase inhibitor N,N'-dicyclohexylcarbodiimide (DCCD). This energy-conserving system showed a stringent coupling. The addition of HS-CoM and HS-HTP at 1 mM each decreased the heterodisulfide reductase activity to 50% of the control. Membranes from Methanolobus tindarius showed F$_{420}$H$_2$-dependent but no H$_2$-dependent heterodisulfide oxidoreductase activity. Neither of these activities was detectable in membranes of Methanococcus thermolithotrophicus.

Key words: Archaeabacteria – Methanogenesis – Membranes – Proton translocation – ATP synthesis – Electron transport phosphorylation – Hydrogenase – F$_{420}$ – Heterodisulfide reduction

Methanogenic bacteria utilize a number of simple substrates (methanol, methylamines, acetate, formate and H$_2$/CO$_2$) as carbon and energy source. The central intermediate in the methanogenic pathway is CH$_3$-S-CoM [2-(methylthio)ethanesulfonate] which is reductively demethylated by the methyl-CoM methylreductase system. Recently the structure of factor B (HS-HTP, 7-mercaptoheptanoylthreonine phosphate), an obligatory cofactor of the methylreductase, was unravelled, and the reduction of CH$_3$-S-CoM could be formulated as the sum of two reactions:

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\begin{align*}
\text{CH}_3\text{-S-CoM} + \text{HTP-SH} & \rightarrow \text{CH}_4 + \text{CoM-S-S-HTP} \quad (1) \\
\text{CoM-S-S-HTP} + 2[\text{H}] & \rightarrow \text{CoM-SH} + \text{HTP-SH} \quad (2)
\end{align*}
\]

Recently it was shown that crude everted vesicle preparations of the methanogenic bacterium strain Gö1 catalyze the H$_2$-dependent reduction of the heterodisulfide of HS-CoM (mercaptoethanesulfonate) and HS-HTP and that this reduction is coupled to ATP synthesis (Peinemann et al. 1990). In subsequent work with washed vesicle preparations a reduced F$_{420}$: heterodisulfide oxidoreductase was discovered (Deppenmeier et al. 1990a) which accomplishes proton translocation and ATP synthesis (Deppenmeier et al. 1990b). The question arose whether the heterodisulfide reduction with H$_2$ as electron donor proceeds via coenzyme F$_{420}$ or whether there are two independent redox systems present in the membrane, a coenzyme F$_{420}$-dependent one and an H$_2$-dependent one functioning in the absence of F$_{420}$. A soluble H$_2$-dependent enzyme was discovered and characterized in Methanobacterium thermoautotrophicum (Hedderich and Thauer 1988).

Materials and methods

Growth and harvest of cells

Strain Gö1 (DSM 3647) was grown in 20-l carboys on the medium described by Hippe et al. (1979), but additionally supplemented...
with 1 g/l sodium acetate. For cultivation of *Methanolobus tindarius* (DSM 2278) in 2-l bottles the medium described by König and Stetter (1982) was used. *Methanococcus thermolithotrophicus* (DSM 2095) was grown autotrophically in a medium described for growth of *Methanococcus voltae* (Baich et al. 1979) in 2-l glass bottles filled with 500 ml medium with shaking at 37 °C. 20-l mass cultures of strain with *N*. were anaerobically harvested by continuous centrifugation. Anaerobic harvest of *Ml. tindarius* and *Mc. thermolithotrophicus* was done in 400-ml air-tight centrifuge bottles at 24000 g.

**Preparation of membranes and washed vesicles**

Crude vesicles of strain G61 with 90% inside-out orientation (Mayer et al. 1987) were prepared as described previously (Deppenmeier et al. 1990b) with the exception that a 40 mM K-phosphate buffer, pH 7.2 was used containing 20 mM MgSO_4_, 0.5 M sucrose, 1 mM dithiothreitol and 1 mg resazurin/l. Vesicles were concentrated by ultracentrifugation at 120 000 g for 1 h at 4°C. The sedimented material was diluted in 9 ml K-phosphate buffer and centrifuged at 38 000 g for 30 min. After resuspending the sediment this centrifugation step was repeated twice. The resulting pellet was suspended in the same buffer to a final protein concentration of 5–10 mg/ml. Membranes of *Ml. tindarius* and *Mc. thermolithotrophicus* were prepared by freezing and thawing of cell suspensions in a 25 mM Na-PIPES-buffer, pH 6.8 reduced with 0.2 mM Ti(III)-citrate. A few crystals of DNase were added to the resulting crude extracts which were centrifuged under identical conditions as described for strain G61. Cytoplasmic fraction of strain G61 was prepared as described previously (Deppenmeier et al. 1989).

**Assay conditions**

The experiments were performed at room temperature under an atmosphere of hydrogen in 2.7-ml glass vials. 600 μl 40 mM K-phosphate-buffer, pH 7.2 gassed with O_2_-free N_2_ and containing 20 mM MgSO_4_, 0.5 M sucrose and 1 mg resazurin/l was reduced by stepwise addition of a few microliters Ti(III)-citrate until resazurin turned colourless. After addition of 10–20 μl washed vesicles (27 μg protein) the reaction was started with 0.6 mM CoM-S-S-HTP. To follow the reduction of CoM-S-S-HTP aliquots of 20 μl were withdrawn and analyzed for thiol groups with Ellman's reagent as described previously (Ellman 1958). The thiol content measured at zero time was subtracted. To determine the ATP concentration 2–5 μl aliquots were withdrawn by syringe and analyzed using the luciferin/luciferase assay (Kimmich et al. 1975). Additions were made as indicated. N,N'-dicyclohexylcarbodiimide (DCCD) and N,N′-dicyclohexylcarbodiimide (DCCD) and 3,5-di-tert-butyI-4-hydroxy-benzylidemalononitrile (SF 6847) were added as ethanolic solutions. The controls received ethanol only. Proton translocation was followed as described by Deppenmeier et al. (1990b).

F_420 nonreactive- and F_420-dependent hydrogenases were assayed in 1.7-ml glass cuvettes gassed with H_2_ and filled with 1 ml anaerobic 100 mM Tricine buffer, pH 8.0 containing 5 mM dithiothreitol, 3 mM cysteine and 1 mg resazurin/l. 20 μM F_420 (ε = 40 mM cm^−1 cm^−1) or 5 mM methylviologen (ε = 13.9 mM cm^−1 cm^−1) were added as electron acceptors. The benzylidemalononitrile (SF 6847) was used as ethanolic solutions. The controls received ethanol only. Proton translocation was followed as described by Deppenmeier et al. (1990b).

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**Results**

1) H_2-dependent CoM-S-S-HTP reduction independent of coenzyme F_420

Washed everted vesicles were tested for their ability to catalyze H_2-dependent CoM-S-S-HTP reduction in the presence or absence of added F_420. The experiments were performed in the presence of the uncoupler SF 6847 in order to compare maximal electron transport rates; SF 6847 has been shown to stimulate the heterodisulfide reduction in this system. As is evident from Fig. 1 the reduction of CoM-S-S-HTP as determined from thiol formation proceeded at an initial rate of 240 nmol/min · mg protein. The F_420 content of washed everted vesicles was 0.11 nmol/mg protein corresponding to a F_420 concentration of 20–30 nM in the reaction mixture after addition of washed vesicles (0.17–0.26 mg protein/ml assay). The K_m values of F_420-dependent enzymes for F_420 are in the order of 10–20 μM (Yamazaki and Tsai 1986; Livingston et al. 1987; Ma and Thauer 1990). The H_2-dependent CoM-S-S-HTP reduction was not stimulated by the addition of coenzyme F_420 at concentrations of 3.2 to 32 μM. This showed that the H_2-dependent system was independent of coenzyme F_420. The reduction of CoM-S-S-HTP was not observed in the cytoplasmic fraction when washed vesicles were omitted.

2) Proton translocation

Washed everted vesicles of the methanogenic bacterium strain G61 transfer protons across the cytoplasmic mem-