**H₂: 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 H₂-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 F₄₂₀ 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 F₄₂₀H₂-dependent but no H₂-dependent heterodisulfide oxidoreductase activity. Neither of these activities was detectable in membranes of *Methanococcus thermolithothrophicus*.

**Key words:** Archaeabacteria – Methanogenesis – Membranes – Proton translocation – ATP synthesis – Electron transport phosphorylation – Hydrogenase – F₄₂₀ – Heterodisulfide reduction

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**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 Methanobacterium tindarius (DSM 2278) in 2-1 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 (Balch et al. 1979) in 2-1 glass bottles filled with 500 ml medium with shaking at 37°C. 20-1 mass cultures of strain with Na+ were anaerobically harvested by continuous centrifugation. Anaerobic harvest of \textit{Ml. tindarius} and \textit{Ml. thermolithothrophicus} 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 MgSO₄, 0.5 M sucrose, 1 mM dithiothreitol and 1 mg resazurin/l. Vesicles were concentrated by ultracentrifugation at 120000 g for 1 h at 4°C. The sedimented material was diluted in 9 ml K-phosphate buffer and centrifuged at 38000 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 \textit{Ml. tindarius} and \textit{Ml. thermolithothrophicus} 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 O₂-free N₂ and containing 20 mM MgSO₄, 0.5 M sucrose and 1 mg resazurin/l was 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).

**F₄₂₀ nonreactive- and F₄₂₀-dependent**

\F₄₂₀\ nonreactive- and \F₄₂₀\ dependent hydrogenases were assayed in 1.7-ml glass cuvettes gassed with H₂ 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 \F₄₂₀\ (\(e_{420} = 40 \text{ mM}^{-1} \text{cm}^{-1}\)) or 5 mM methylviologen (\(e = 13.9 \text{ mM}^{-1} \text{cm}^{-1}\)) were added as electron acceptors. The benzylviologen-dependent heterodisulfide reductase was determined at room temperature in 1 ml 50 mM Tris/HCl buffer, pH 7.5, pre-gassed with N₂. After addition of 1.5 mM methylviologen (1 M), 5 µl 50 mM Na-dithionite and 1—5 µl of the membrane preparation, the reaction was started by addition of CoM-S-S-HTP to a final concentration of 90 µM and followed at 575 nm (\(e_{575\text{nm}} = 8.9 \text{ mM}^{-1} \text{cm}^{-1}\)) (Hedderich et al. 1989). The \F₄₂₀\H₂ dehydrogenase and the \F₄₂₀H₂:CoM-S-S-HTP oxidoreductase were followed in N₂-gassed 1 ml K-phosphate buffer, pH 7.0 containing 20 mM MgSO₄, 0.5 M sucrose, 10 mM dithiothreitol and 1 mg resazurin/l. 20 µM \F₄₂₀\H₂ was added as electron donor for both enzymes. Methylviologen (MV) (0.3 mM) + metronidazol (MTZ) (0.5 mM) and CoM-S-S-HTP (90 µM) were used as electron acceptor for the \F₄₂₀H₂:dehydrogenase and the \F₄₂₀H₂:CoM-S-S-HTPoxidoreductase, respectively. The production of \F₂₂₀\ was followed at 420 nm (\(e = 40 \text{ mM}^{-1} \text{cm}^{-1}\)). Protein was quantified by the method of Bradford (1976).

**Results**

1) \(H_2\)-dependent CoM-S-S-HTP reduction independent of coenzyme \F₄₂₀\n
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 \F₄₂₀\). 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 twofold. 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 \F₄₂₀\ content of washed everted vesicles was 0.11 nmol/mg protein corresponding to a \F₄₂₀\ 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 \F₄₂₀\-dependent enzymes for \F₄₂₀\ 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 \F₄₂₀\ at concentrations of 3.2 to 32 µM. This showed that the \(H_2\)-dependent system was independent of coenzyme \F₄₂₀\.

The reduction of CoM-S-S-HTP was not observed in the cytoplasmic fraction or when washed vesicles were omitted.

2) \(H_2\)-dependent CoM-S-S-HTP reduction independent of coenzyme \F₄₂₀\n
Washed everted vesicles of the methanogenic bacterium strain G61 transfer protons across the cytoplasmic mem-