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Biochemistry

Reduced coenzyme F_{420}: Heterodisulfide oxidoreductase, a proton-translocating redox system in methanogenic bacteria

(methanogenesis/heterodisulfide reductase/energy coupling/electron transport phosphorylation)

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ABSTRACT **Washed everted vesicles of the methanogenic bacterium strain Göl were found to couple the $F_{420}H_2$-dependent heterodisulfide reduction with the transfer of protons across the membrane into the lumen of the everted vesicles. The transmembrane electrochemical potential of protons thereby generated was shown to be competent in driving ATP synthesis from ADP + P_i, exhibiting a stoichiometry of 2 H^+ translocated or 0.4 ATP synthesized per $F_{420}H_2$ oxidized. This enzyme system exhibits the phenomenon of coupling and uncoupling and represents a different kind of electron transport chain with the heterodisulfide of 2-mercaptoethanesulfonate and 7-mercaptoheptanoylthreonine phosphate as terminal electron acceptor. The heterodisulfide and methane are formed in the methyl coenzyme M reductase reaction. The reducing equivalents are derived from reduced coenzyme F_{420}, which represents an analogue of NADH + H^+ in other respiratory chains. It is assumed that the proton-translocating oxidoreductase discovered in strain Göl is of principal importance to all methanogenic bacteria not utilizing H_2.**

With the discovery of methanogens and the elucidation of their specialized, strictly anaerobic metabolism, the question was posed whether this group of organisms gains ATP by substrate-level phosphorylation, such as clostridia or streptococci, or whether the process of methanogenesis is somehow coupled with the generation of a transmembrane gradient of protons (1). The pathways involved in methane formation from the few substrates utilized [CO_2 + H_2, formate, methanol, methylamines, and acetate (2)] have been worked out in recent years and led to the discovery of a whole series of coenzymes and cofactors such as coenzyme F_{420}, tetrahydromethanopterin, methanofuran, coenzyme M (2-mercaptoethanesulfonate; HS-CoM), 7-mercaptoheptanoylthreonine phosphate (HTP-SH), and factor F_{430} (3). The knowledge of these pathways did not allow any conclusion about the mode of ATP generation in methanogenic bacteria. However, it was demonstrated in whole cells of *Methanosarcina barkeri* that the reduction of methanol with H_2 to methane (reaction **1**) is coupled with the primary translocation of protons across the cytoplasmic membrane (4).

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad [1]$$

The transmembrane protonic potential ($\Delta\mu_{H^+}$) thereby established drives the synthesis of ATP by means of an ATP synthase (5). The conversion given by reaction **1** consists of two reactions [**2** (6, 7) and **3** (8)]:

$$CH_3OH + CoM\text{-}SH \rightarrow CH_3\text{-}S\text{-}CoM + H_2O \quad [2]$$

$$CH_3\text{-}S\text{-}CoM + H_2 \rightarrow CH_4 + CoM\text{-}SH \quad [3]$$

The reaction underlying **3** was recently shown to drive $\Delta\mu_{H^+}$-mediated ATP synthesis in crude vesicle preparations of the methanogenic bacterium strain Göl (9). After elucidation of the chemical structure of the so-called factor B (HS-HTP) (10), which is required for CH_3-S-CoM reduction, it became clear that this reduction proceeds in two steps [reactions **4** (11, 12) and **5** (13)]:

$$CH_3\text{-}S\text{-}CoM + HTP\text{-}SH \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP \quad [4]$$

$$CoM\text{-}S\text{-}S\text{-}HTP + H_2 \rightarrow CoM\text{-}SH + HTP\text{-}SH \quad [5]$$

Experimental evidence was provided that the reduction of the heterodisulfide (CoM-S-S-HTP) (reaction **5**) might be the actual energy-conserving reaction (14).

F_{420} serves as an important electron carrier in methanogens and may be reduced by H_2 or in conjunction with the oxidation of methanol or methylamines (15). Everted vesicles of strain Göl were recently shown to contain in addition to an H_2-dependent enzyme an $F_{420}H_2$ (reduced F_{420})-dependent heterodisulfide reductase (16) [reaction **6**]:

$$CoM\text{-}S\text{-}S\text{-}HTP + F_{420}H_2 \rightarrow CoM\text{-}SH + HTP\text{-}SH + F_{420} \quad [6]$$

Here we report that the electron transfer from $F_{420}H_2$ to the heterodisulfide as catalyzed by washed vesicles of the methanogenic strain Göl gives rise to a $\Delta\mu_{H^+}$, which drives ATP formation from ADP and inorganic phosphate. These results demonstrate a largely coupled electron transport-driven phosphorylation of ADP in a methanogen at the subcellular level.

MATERIALS AND METHODS

Growth of Cells and Preparation of Washed Vesicles from Strain Göl. The methanogenic bacterium strain Göl was isolated from the sewage plant in Göttingen by F. Widdel (Munich) and was obtained from the Deutsche Sammlung von Mikroorganismen (Brunswick, F.R.G.). This organism has not yet been classified. It is able to grow with H_2 + CO_2 as well as with methanol and had the advantage of being surrounded by a proteinaceous cell envelope. This allowed a convenient preparation of protoplasts (17). Cells of the methanogenic strain Göl were grown in 2-liter glass bottles or, for mass culturing, in 20-liter carboys on the media described (18). All preparation steps were done under strictly anaerobic conditions. Cells of the late logarithmic growth phase were harvested by centrifugation, washed once with 40 mM potassium phosphate (pH 7) containing 20 mM $MgSO_4$, 0.5 M sucrose, 10 mM dithioerythritol, and 1 mg of resazurin per

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Abbreviations: $\Delta\mu_{H^+}$, transmembrane electrochemical gradient of H^+; CoM-SH, 2-mercaptoethanesulfonate; HTP-SH, 7-mercaptoheptanoylthreonine phosphate; DCCD, *N,N'*-dicyclohexylcarbodiimide.

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liter. The cell suspension was treated with Pronase (2.5 mg per liter of cell culture) at 37°C. After 10 min, the reaction was stopped by addition of phenylmethylsulfonyl fluoride. The resulting protoplasts were spun down at 27,000 × *g* for 10 min and the pellet was suspended in the same buffer supplemented with a few crystals of DNase. The protoplasts were passed through a French pressure cell at 65 MPa, resulting in a vesicle preparation in which 90% have an inside-out orientation (19). To remove unbroken protoplasts and cell debris, the resulting crude vesicle preparation was centrifuged twice at 6000 × *g* for 15 min. Vesicle structures were concentrated by ultracentrifugation at 120,000 × *g* for 1 hr. The sedimented material in the lower quarter (≈2 ml) was diluted with 30 ml of potassium phosphate buffer and centrifuged at 38,000 × *g* for 15 min. The sediment was resuspended in the same volume and the centrifugation step was repeated. The resulting pellet was resuspended in the same buffer to a final protein concentration of 1–5 mg/ml. Cytoplasmic fractions were prepared according to Deppenmeier *et al.* (20). F_{420} was isolated from *M. barkeri* and reduced to $F_{420}H_2$ with $NaBH_4$ as described (16). CoM-S-S-HTP was synthesized according to ref. 21.

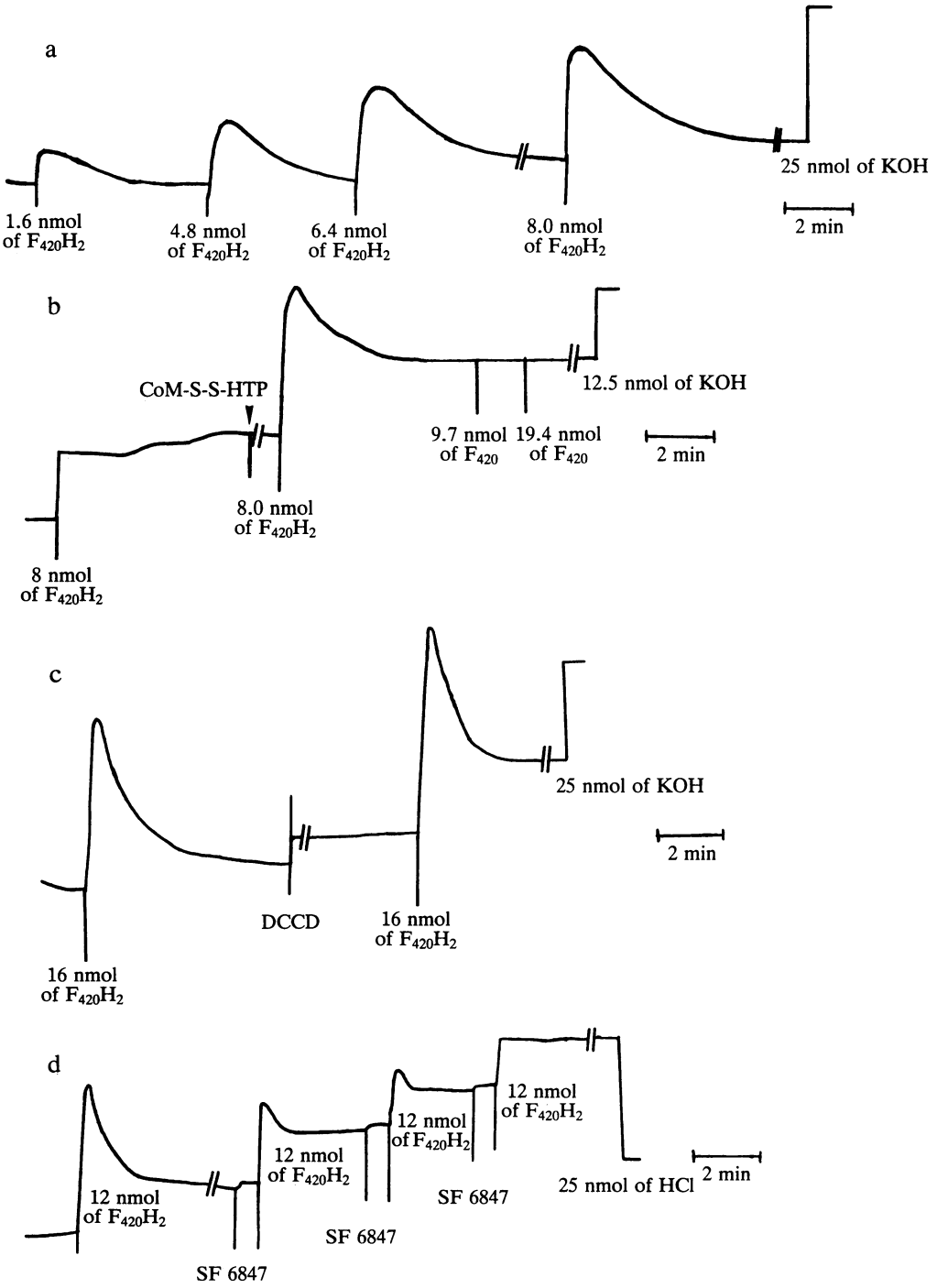

FIG. 1. Proton uptake by washed everted vesicles. Proton translocation was followed as described. Washed vesicles were preincubated with 360 nmol of CoM-S-S-HTP in traces a, c, and d. Where indicated, the following agents were added: DCCD, 150 nmol per mg of protein; SF 6847, 3.8 nmol per mg of protein; CoM-S-S-HTP, 360 nmol per assay. The amount of H^+ translocated was calculated from the difference between the maximum of alkalinization and the final baseline after reacidification. The difference between the starting baseline and the final baseline was due to alkalinization by the additions made. These additions resulted in a change of the pH value in the weakly buffered reaction mixture.

Proton translocation was followed at room temperature in a glass vessel (11 ml) filled with 2.6 ml of 40 mM KSCN/0.5 M sucrose/resazurin (1 mg/liter)/10 mM dithioerythritol. A pH electrode (model 8103 Ross; Orion Research, Küsnacht, Switzerland) was inserted into the vessel from the top through a rubber stopper. The electrode was connected with a model EA 920 (Orion) pH meter and a chart recorder. The vessel was subsequently gassed for 10 min with N_2 by means of two needles inserted from the side through a rubber stopper. After reduction of the medium with 1 μl of titanium(III) citrate, 100 μl of washed vesicles (0.35–0.5 mg of protein per assay) was added, resulting in a final potassium phosphate concentration of 1.5 mM in the reaction mixture. After addition of 360 nmol of CoM-S-S-HTP, the medium was continuously stirred and the pH was adjusted to 6.8–6.9. Additions were made with a microliter syringe from the side arm. The pH changes were calibrated with standard solutions of HCl or KOH.

Assay Conditions. ATP synthesis was followed in 2.7-ml glass vials filled with potassium phosphate buffer to a final vol of 600 μl under an atmosphere of N_2. Membranes and $F_{420}H_2$ were anaerobically transferred by syringe. ADP and CoM-S-S-HTP were added as aerobic aqueous solutions (1–4 μl). The ATP content was determined according to Peinemann *et al.* (9). $F_{420}H_2$ oxidation was followed photometrically at 401 nm in a 1.7-ml glass cuvette under identical assay conditions.

RESULTS

Everted vesicles of the methanogenic strain Göl were concentrated by ultracentrifugation and washed twice. These washed vesicles were suspended in the reaction mixture described in Fig. 1. The addition of $F_{420}H_2$ (pH 6.9) led to alkalinization of the medium as is apparent from the recorded time course of this process (Fig. 1). A short period of alkalinization was followed by a longer period of acidification until a stable baseline (pH) was reached again. This alkalinization is thought to be due to proton movement from the medium into the lumen of the everted vesicles energized by the $F_{420}H_2$-dependent CoM-S-S-HTP reduction. The extent of alkalinization was dependent on the amount of $F_{420}H_2$ added (Fig. 1, trace a). The reversible alkalinization was used to calculate from >60 experiments an average of 1.9 protons translocated per $F_{420}H_2$ oxidized. The observed alkalinization was specifically coupled with the $F_{420}H_2$-dependent CoM-S-S-HTP reduction: No alkalinization was observed if $F_{420}H_2$ or CoM-S-S-HTP was omitted or if $F_{420}H_2$ was replaced by F_{420} (Fig. 1, trace b). It was crucial to exclude the involvement of the proton-translocating ATPase in proton extrusion. This was done by testing the effect of *N,N'*-dicyclohexylcarbodiimide (DCCD). DCCD inhibits ATP synthesis in whole cells of *M. barkeri* (5) and decreases the catalytic activity of the membrane-bound ATPase of this organism (22). It is evident that H^+ translocation was not affected by DCCD at a concentration of 150 nmol per mg of protein (Fig. 1, trace c), which was sufficient to completely inhibit ATP synthesis (see Table 1). Hence the DCCD-sensitive proton-translocating ATPase cannot be responsible for proton translocation. If KCl instead of the membrane-permeable KSCN (23) was added to the reaction mixture, the H^+/F_{420} ratio was reduced to 0.65 (not shown). Increasing concentrations of the protonophore SF 6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile) led to increasing degrees of inhibition of H^+ translocation (Fig. 1, trace d). At SF 6847 concentrations >10 nmol per mg of protein, the proton movement was completely inhibited. The detergent sulfobetain led to a complete inhibition of H^+ translocation.

The washed vesicles were tested for their ability to synthesize ATP from ADP + P_i in response to the reduction of CoM-S-S-HTP with $F_{420}H_2$ under an atmosphere of N_2. It is evident from Fig. 2 that washed vesicles phosphorylated ADP at an initial rate of 72.5 nmol per min per mg of protein. When $F_{420}H_2$ or CoM-S-S-HTP was omitted from the reaction mixture, the rate of ATP synthesis was only 0.4 nmol of ATP per min per mg of protein (not shown). This ATP formation was completely inhibited by a 10-fold excess of AMP, suggesting that the minimal ADP phosphorylation observed in the absence of substrates was due to adenylate kinase activity: 2 ADP ⇌ AMP + ATP. No ATP formation was observed in the cytoplasmic fraction.

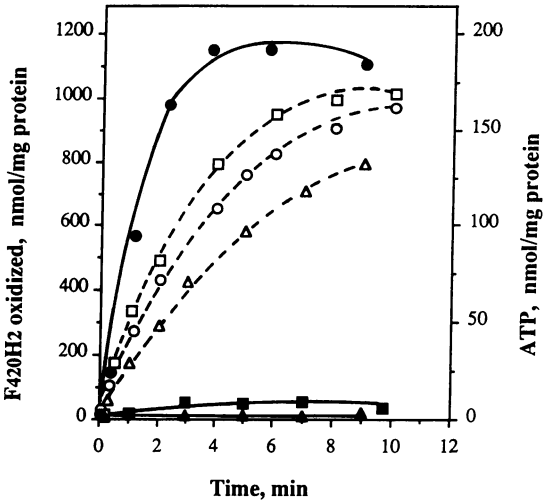

FIG. 2. ATP synthesis and $F_{420}H_2$ oxidation as catalyzed by washed everted vesicles. In parallel experiments, washed vesicles (27 μg of protein) were preincubated under N_2 in a final vol of 0.6 ml of potassium phosphate buffer (pH 7.0) with 30 nmol of $F_{420}H_2$ and 10 nmol of ADP at room temperature. The reaction was started by the addition of 100 nmol of CoM-S-S-HTP. The oxidation of $F_{420}H_2$ was followed in 1.7-ml glass cuvettes at 401 nm ($\varepsilon = 27\ mM^{-1} \cdot cm^{-1}$). ATP synthesis was determined in 2.7-ml glass vials according to Peinemann *et al.* (9) under identical conditions. The ATP concentration was corrected for the ATP present in the added ADP solution. Open symbols, oxidation of $F_{420}H_2$; solid symbols, ATP concentration; circles, no further additions; squares, preincubation with 10 nmol of SF 6847 per mg of protein; triangles, ADP omitted.

F_{420} was formed at an initial rate of 222 nmol per min per mg of protein (within the first 2 min). After 10 min, 90% of the $F_{420}H_2$ was oxidized and no further consumption was observed. Within the first 2 min of the reaction, $F_{420}H_2$ oxidation and ATP formation occurred at a ratio of 1 mol of ATP/2.4 mol of F_{420}. It is apparent that ATP synthesis occurred only when ADP was added. The initial rate of F_{420}

Table 1. Effect of DCCD on rates of $F_{420}H_2$ oxidation and ATP synthesis as catalyzed by washed vesicles of the methanogenic strain Göl

DCCD, nmol per mg of protein	$F_{420}H_2$ oxidation		ATP synthesis	
	Rate	%	Rate	%
0	107.7	100.0	31.5	100.0
64	88.5	82.2	15.7	49.8
96	85.0	78.9	10.5	33.3
128	69.2	64.3	0.2	0.6
256	63.5	59.0	0.1	0.3
256 + SF	101.9	94.6	0.2	0.6

Rates are expressed as nmol per min per mg of protein. Washed vesicles (34.0 μg of protein) were preincubated for 10 min with DCCD at the concentration indicated in a final vol of 0.6 ml of potassium phosphate buffer (pH 6.9) under an atmosphere of N_2. After addition of 34 nmol of $F_{420}H_2$ and 10 nmol of ADP, the reaction was started with 100 nmol of CoM-S-S-HTP. Where indicated, SF 6847 was added at a final concentration of 9.4 nmol per mg of protein.

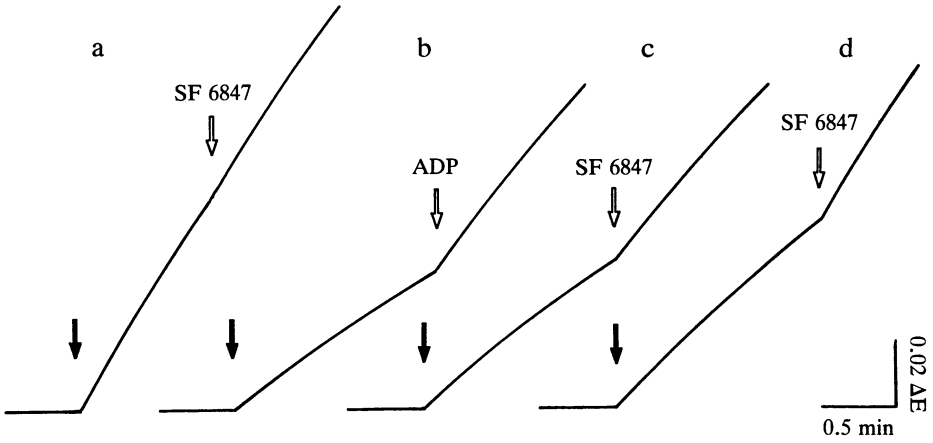

FIG. 3. Effect of ADP, DCCD, and SF 6847 on F_{420} formation from $F_{420}H_2$ as catalyzed by washed everted vesicles. The oxidation of $F_{420}H_2$ was followed photometrically at 401 nm in 1.7-ml glass cuvettes under N_2. Washed vesicles (12.9 μg of protein) were preincubated in 0.5 ml of potassium phosphate buffer (pH 7.0) with 34 nmol of $F_{420}H_2$ and with the agents indicated. The reaction was started by addition of 35 nmol of CoM-S-S-HTP (solid arrows). At the times indicated (open arrows), 28 nmol of SF 6847 per mg of protein or 20 μM ADP was added. Trace a, preincubation with 20 μM ADP; traces b and c, preincubation without ADP; trace d, preincubation with 20 μM ADP and 390 nmol of DCCD per mg of protein. The rates of $F_{420}H_2$ oxidation were calculated from the initial slope of the curves before and after addition of SF 6847 and ADP, respectively.

formation decreased to 65% in the absence of ADP. Preincubation of washed vesicles with the uncoupling agent SF 6847 inhibited the rate of ATP synthesis almost completely (Fig. 2). In contrast, F_{420} formation was even increased to a slight degree. The initial rate of 222 nmol of F_{420} per min per mg of protein was enhanced by SF 6847 to 246 nmol of F_{420} per min per mg of protein, while the final F_{420} concentration was almost identical.

DCCD was tested for its ability to decrease ATP synthesis in the vesicle system investigated here. It is apparent from the data in Table 1 that the inhibition of ATP synthesis correlated with the DCCD concentration in the reaction mixture. It was totally inhibited at concentrations of 128 or 256 nmol of DCCD per mg of protein, which at the same time decreased the $F_{420}H_2$ oxidizing activity to 64% or 59% of the control. The inhibitory effect of DCCD on F_{420} formation was largely reversed by addition of the uncoupling agent SF 6487.

To further characterize the electron transport system and its coupling to ATP synthesis, the effect of ADP, SF 6847, and DCCD on $F_{420}H_2$ oxidation was investigated. This was done by following the initial rate of F_{420} formation at 401 nm (Fig. 3). Reactions were started by the addition of heterodisulfide and after $\approx$1 min SF 6847 or ADP was administered. SF 6847 had only a slight effect on $F_{420}H_2$ oxidation when washed vesicles had been preincubated with ADP (trace a). In contrast, the rate of F_{420} formation was increased by SF 6847 from 71 to 147 nmol of F_{420} per min per mg of protein when ADP had been omitted (trace c). The same effect was observed when ADP was added instead of SF 6847 (stimulation from 64 to 127 nmol of F_{420} per min per mg of protein; trace b). This showed that $F_{420}H_2$ oxidation depended on the presence of ADP but that this dependence could be overcome by the addition of the uncoupling agent SF 6847 (dissipation of the $\Delta\mu_{H^+}$). In accordance with this, incubation with ADP

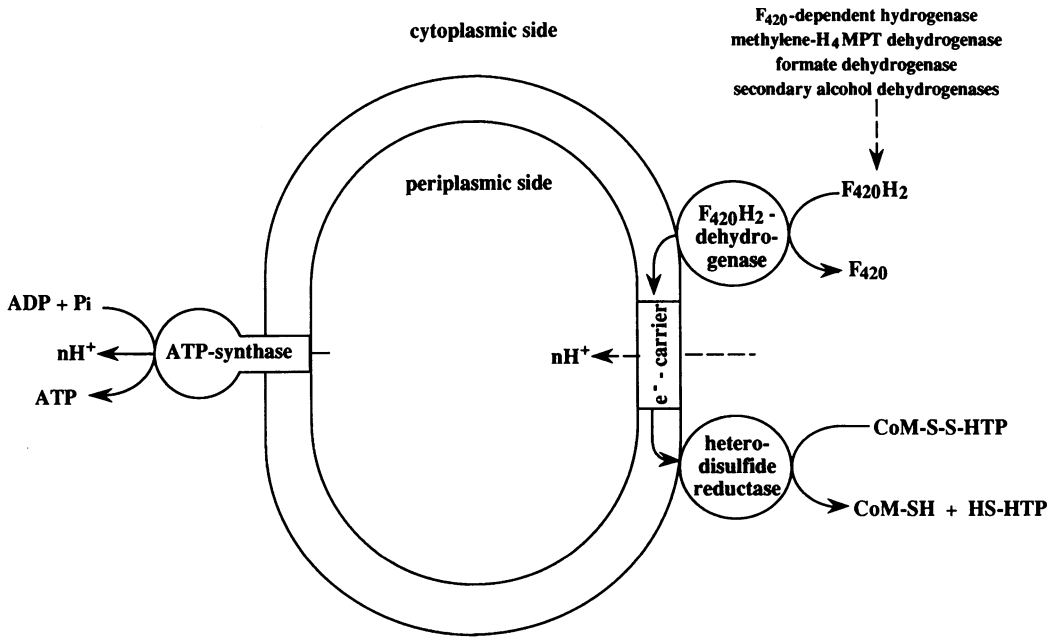

FIG. 4. Scheme of electron transfer from $F_{420}H_2$ to CoM-S-S-HTP coupled with proton translocation and ATP synthesis catalyzed by everted vesicles of the methanogenic strain Gö1.

and DCCD led to a decrease in the $F_{420}H_2$-dependent CoM-S-S-HTP reduction from 135 (control) to 71 nmol per min per mg of protein. Addition of SF 6847 reversed this inhibitory effect almost completely (trace d). These results are analogous to the effects of ADP, dinitrophenol, and oligomycin on mitochondrial electron transport and indicate that the electron transport in these vesicles is under respiratory control (24).

DISCUSSION

This report describes ATP synthesis from ADP and P_i coupled to the oxidation of $F_{420}H_2$ and the concurrent reduction of CoM-S-S-HTP as catalyzed by a washed vesicle system of a methanogenic bacterium. Soluble components had been completely removed from these washed vesicles. This is evident from the absolute ADP requirement for ATP synthesis. A relatively high concentration of ADP is found in the cytoplasmic fraction, rendering its addition superfluous for ATP formation in crude vesicle preparations (9). Furthermore, no F_{420}:$NADP^+$ oxidoreductase activity was detectable in washed vesicle preparations; this enzyme is exclusively located in the soluble fraction of strain Göl (16).

The inhibitor studies presented here indicate that energy is conserved by electron transport phosphorylation. The uncoupler SF 6847 inhibited ATP synthesis, while the heterodisulfide reduction continued at a slightly increased rate. Addition of DCCD, an inhibitor of the ATP synthase (22), prevented ATP formation and decreased the rate of $F_{420}H_2$ oxidation. The coupling between CoM-S-S-HTP reduction and ATP formation was stringent, with 1 mol of ATP being synthesized per 2–3 mol of $F_{420}H_2$ oxidized. It is also apparent from the following results: (*i*) Addition of SF 6847 reversed the inhibition of $F_{420}H_2$ oxidation by DCCD; (*ii*) ADP stimulated $F_{420}H_2$ oxidation; (*iii*) SF 6847 stimulated $F_{420}H_2$ oxidation in the absence of ADP. Further evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria came from measuring proton uptake into everted vesicles induced by $F_{420}H_2$ oxidation. This process was unaffected by DCCD and was not dependent on ADP, while the uncoupling agent SF 6847 led to a complete inhibition.

A model summarizing the results obtained with the everted vesicle preparation is presented in Fig. 4. All enzyme activities involved in this process, such as the $F_{420}H_2$ dehydrogenase, the heterodisulfide reductase, and the ATP synthase are tightly membrane bound. The enzymes are accessible to the substrates because of the everted orientation of these vesicles. The nature and number of the membrane-bound electron carriers, catalyzing the electron transfer from the $F_{420}H_2$ dehydrogenase to the heterodisulfide reductase as well as the exact mechanism of H^+ translocation (redox loop, redox cycle, or conformational proton pump) are still unknown. Possible candidates as electron carriers are Fe-S centers, membrane-integral corrinoids (25), and cytochromes. Recent studies led to the assumption that Fe-S centers in membranes of strain Göl are involved in electron transfer from H_2 to methyl-CoM (20). Fe-S centers were also found in membranes of *M. barkeri* (26) and *Methanobacterium thermoautotrophicum* (27). In this context, the discovery of genes encoding a polyferredoxin is of particular interest (28). Cytochromes have so far only been detected in membranes of methylotrophic methanogens (29). They have been suggested to be involved in the oxidation of methyl groups (30).

F_{420} is a central electron carrier in methanogens and serves as coenzyme in several oxidoreductase reactions. The $F_{420}H_2$ formed in the oxidation of formate, H_2, methylene-H_4MPT, and secondary alcohols (15) may be reoxidized by the $F_{420}H_2$:heterodisulfide oxidoreductase. This oxidoreductase represents a kind of anaerobic respiration and is coupled with proton translocation and ATP synthesis. Similarities with aerobic respiration (using $F_{420}H_2$ instead of NADH) and with S^0-reducing bacteria (using organic -S-S- bridges instead of S^0) are of particular interest in the context of the evolution of respiratory chains.

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