Membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase in methanogenic bacterium strain Göl and *Methanolobus tindarius*

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Washed membrane or cytoplasmic fractions of the methanogenic bacterium strain Göl catalyzed the oxidation of coenzyme $F_{420}H_2$ with a variety of electron acceptors. The $F_{420}H_2$-oxidizing activity of the cytoplasmic fraction could be assigned to a $NADP^+$:F_{420} oxidoreductase. The membrane fraction but not the cytoplasmic fraction catalyzed the oxidation of $F_{420}H_2$ with the concomitant reduction of the heterodisulfide of 2-mercaptoethanesulfonate and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) at a rate of 100 nmol/min·mg protein according to the following equation: $F_{420}H_2 + CoM\text{-}S\text{-}S\text{-}HTP \rightarrow F_{420} + CoM + HTP\text{-}SH$. The activity depended linearly on the membrane protein up to a concentration of 60 μg/ml. The physiological electron acceptor CoM-S-S-HTP could not be replaced by the corresponding homodisulfides CoM-S-S-CoM and HTP-S-S-HTP or by $NADP^+$. A membrane-bound $F_{420}H_2$-dependent CoM-S-S-HTP reductase was also detected in *Methanolobus tindarius* exhibiting a specific activity of 75 nmol/min·mg protein. The absence of a F_{420}-dependent hydrogenase in this organism excludes the involvement of this enzyme in electron transfer from $H_{420}H_2$ to CoM-S-S-HTP.

F_{420}; Heterodisulfide reductase; Methane formation; Membrane; Methanogenic bacteria

1. INTRODUCTION

Methanogenic bacteria derive their metabolic energy from the conversion of a number of simple substrates (H_2-CO_2, formate, methanol, methylamines and acetate) to methane [1]. Central intermediate is CH_3-S-CoM (2-(methylthio)ethanesulfonate) which is reductively cleaved to CH_4 and HS-CoM (2-mercaptoethanesulfonate) in two reaction steps, the methyl reductase and the heterodisulfide reductase reactions [2,3].

$$CH_3\text{-}S\text{-}CoM + HTP\text{-}SH \longrightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP \quad (1)$$

$$CoM\text{-}S\text{-}S\text{-}HTP + 2[H] \longrightarrow CoM\text{-}SH + HTP\text{-}SH \quad (2)$$

Methanogenesis from CH_3-S-CoM and H_2 as catalyzed by inside-out vesicles of the methanogenic bacterium strain Göl was shown to generate ATP by a chemiosmotic mechanism [4]. In this context the observation is of interest that membranes from various methanogens stimulate methanogenesis from CH_3-S-CoM and H_2 or formaldehyde as it proceeds in cytoplasmic fractions of strain Göl [5]. Since the actual methyl-CoM reductase (eqn 1) and the enzymes responsible for the oxidation of formaldehyde (via methylene H_4MPT) to CO_2 are found in the cytoplasmic fraction of strain Göl after cell breakage, the membranes must play a role in electron transfer from H_2 or from formaldehyde to CoM-S-S-HTP [5]. The question arose how the electrons from the H_2-oxidizing or methylene-H_4MPT-oxidizing enzymes are channeled to the membranes that catalyze the transfer to CoM-S-S-HTP. The methylene-H_4MPT dehydrogenase from *Methanobacterium thermoautotrophicum* was purified and shown to be F_{420}-dependent [6,7]. This led us to assume that F_{420} might serve as electron carrier in the oxidation of methylene-H_4MPT. Here we report on the presence of a membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase in the methanogenic strain Göl and in *Methanolobus tindarius*.

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Abbreviations: CoM-SH, 2-mercaptoethanesulfonate; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); F_{420}, (*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate; $F_{420}H_2$, reduced F_{420}; MF, methanofuran; H_4MPT, tetrahydromethanopterin; HTP-SH, 7-mercaptoheptanoylthreonine phosphate; Pipes, 1,4-piperazinediethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-*bis*(hydroxymethyl)ethyl]-glycine

2. MATERIALS AND METHODS

2.1. *Growth and harvest of cells*

Cells of the methanogenic strain Göl and of *Methanolobus tindarius* were grown on methanol and harvested as described previously [5].

2.2. *Preparation of membranes and cytoplasmic fractions from strain Göl or from* Methanolobus tindarius

Membranes of strain Göl were prepared as described previously [5] except that a 100 mM Na-Tricine buffer, pH 8, containing 5 mM dithioerythritol, 3 mM cysteine and 0.1 mg/l resazurin was used for

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the two final wash steps. For measuring the thiol formation from CoM-S-S-HTP, dithioerythritol and cysteine were omitted from the above buffer. Instead, the buffer was reduced by stepwise addition of a few μl of 100 mM Ti(III)-citrate [8] until the resazurin turned colorless. Membranes from *Methanolobus tindarius* 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. The resulting crude extract was centrifuged for 15 min at 38000 × *g*. The sedimented material was composed of two layers: the lower, tightly packed layer consisted of sulfides and cell debris; the upper layer was only loosely sedimented and consisted mainly of large membrane fragments. The latter material was washed with 100 mM Na-Tricine buffer, pH 8, and reduced with dithioerythritol and cysteine as described above. The resulting pellet was resuspended in the same buffer to a final protein concentration of 1–3 mg/ml. The cytoplasmic fraction of strain Göl was prepared as described [5].

2.3. *Purification of F_{420}*

For the preparation of coenzyme F_{420}, 300 g cells of *Methanosarcina barkeri* were suspended under N_2 in 40 mM NH_4-acetate buffer, pH 4.8, containing 10 mM 2-mercaptoethanol. The cells were boiled in a water bath for 30 min under H_2, sedimented by centrifugation at 25000 × *g* for 30 min and resuspended in 800 ml of the same buffer. Boiling and centrifugation was repeated, and the supernatants of both extractions were combined and applied to a DEAE-Sephadex A25 anion exchange column which had been equilibrated with a 40 mM NH_4-acetate buffer, pH 4.8. A linear 1-l gradient (0–1 mM NH_4Cl in NH_4-acetate buffer) was applied. Fractions containing F_{420} were detected by their absorption at 420 nm. They were pooled, diluted 10-fold with 50 mM Tris-HCl buffer, pH 7.5 and loaded onto a second DEAE-Sephadex column (8 × 4 cm). The cofactor was eluted with a 0–1 M NaCl gradient in the same buffer. The green fluorescent solution of F_{420} was lowered in ionic strength by diluting with 50 mM Tris-HCl buffer, pH 7.5, and was placed on a QAE-Sephadex column (2.6 × 7 cm) which had been equilibrated with the same buffer. A 0.5-l gradient of 0–2 M NaCl was passed through the column. Finally, the F_{420}-containing fractions were adjusted to pH 1 with 6 M HCl and rechromatographed on a XAD-4 column (2.6 × 5 cm). Elution occurred upon applying a linear gradient of 0–100% methanol (in a volume of 0.5 l). F_{420} was reduced by addition of a few crystals of $NaBH_4$ at pH 8.0. Excess $NaBH_4$ was removed by acidification (pH < 1.0). Finally the pH of $F_{420}H_2$ was adjusted to 7.0.

2.4. *Synthesis of HTP-S-S-HTP, CoM-S-S-CoM and CoM-S-S-HTP*

HTP-S-S-HTP and CoM-S-S-CoM were prepared as described previously [2,9]. CoM-S-S-HTP was synthesized according to Bobik and Wolfe [10] and purified as described by Ellermann et al. [2].

2.5. *Assays*

$F_{420}H_2$ oxidation was assayed under N_2 in 1 ml 100 mM anaerobic Na-Tricine buffer, pH 8, containing 5 mM dithioerythritol, 3 mM cysteine and 0.1 mg/l resazurin in 1.6 ml cuvettes. After adding 5–50 μl of the membrane fraction and 10 μl $F_{420}H_2$ (final concentration 10 μM), each cuvette was incubated for additional 5 min until a stable baseline was reached. The reaction was started by the addition of 3 μl of electron acceptor and followed photometrically at 420 nm (ϵ = 40 $mM^{-1} \cdot cm^{-1}$). When both the oxidation of $F_{420}H_2$ and the formation of thiol groups (from CoM-S-S-HTP) were to be followed, 3-ml anaerobic cuvettes gassed with N_2 were filled with 2 ml of 100 mM N_2-gassed Na-Tricine buffer, pH 8, containing 0.1 mg/l resazurin. The buffer was reduced by adding a few μl of Ti(III)-citrate solution until the buffer turned colorless. At each time indicated 2 aliquots of 50 μl were withdrawn. One aliquot was transferred into a 1.6-ml anaerobic cuvette containing 1 ml of 100 mM Na-Tricine buffer reduced with 5 mM dithioerythritol, pH 8, to determine the concentration of F_{420} by measuring the absorbance at 420 nm. The other aliquot was used to quantify the thiol groups using DTNB as described by Hedderich et al. [11] except that the absorbance of the complex was not determined at 412 nm but at 450 nm in order to avoid interference by the absorbance of F_{420}.

3. RESULTS

3.1. *$F_{420}H_2$-oxidation by membranes and cytoplasmic fraction of the methanogenic strain Göl*

Vesicle preparations of the methanogenic strain Göl were fractionated by centrifugation and tested for $F_{420}H_2$-oxidizing activity using a variety of electron acceptors. It is evident from table 1 that F_{420}-oxidizing activity was found in both the cytoplasmic and the membrane fraction when artificial electron acceptors such as methylviologen or metronidazole were used. Similarly, FAD or FMN were effective electron acceptors with both fractions. $NADP^+$, however, served as substrate for the cytoplasmic fraction but not for the membrane fraction. In contrast, the heterodisulfide (CoM-S-S-HTP) was only reduced by washed membranes. A disulfide reduction was observed with CoM-S-S-HTP, but not with CoM-S-S-CoM, HTP-S-S-HTP or cystine. It is concluded from these results that the $F_{420}H_2$-oxidizing activity in the soluble fraction is due to a F_{420}:$NADP^+$ oxidoreductase. Such an enzyme was previously reported to be present in *Methanobacterium thermoautotrophicum* and *Methanococcus voltae* [12,13]. The membrane-bound $F_{420}H_2$-oxidizing activity described here for the first time will be referred to as $F_{420}H_2$-dependent heterodisulfide reductase.

3.2. *Features of the $F_{420}H_2$-dependent heterodisulfide reductase*

As evident from fig.1, the reduction of CoM-S-S-HTP and the oxidation of $F_{420}H_2$ occurred con-

Table 1

$F_{420}H_2$ oxidation by membranes or by cytoplasmic fractions of strain Göl[a]

Electron acceptor	Concentration (μM)	Activity (nmol F_{420}/min·mg protein)	
		Membranes[b]	Cytoplasm[c]
H_2O[d]		<1.0	<1.0
Methylviologen	150	184.7	45.9
Metronidazol	750	10.0	2.8
$NADP^+$	150	<1.0	11.8
NAD^+	150	<1.0	<1.0
FAD	10	54.9	168.8
FMN	10	97.6	231.4
Methyl-CoM	150	<1.0	<1.0
HTP-S-S-CoM	20	97.1	<1.0
HTP-S-S-HTP	20	<1.0	n.d.
CoM-S-S-CoM	20	<1.0	n.d.
Cystine	20	<1.0	n.d.

[a] The oxidation of $F_{420}H_2$ was followed as described in section 2
[b] Washed membranes of strain Göl; 10 μg protein/assay
[c] Cytoplasmic fraction of strain Göl; 200 μg protein/assay
[d] 3 μl aerobic H_2O were added

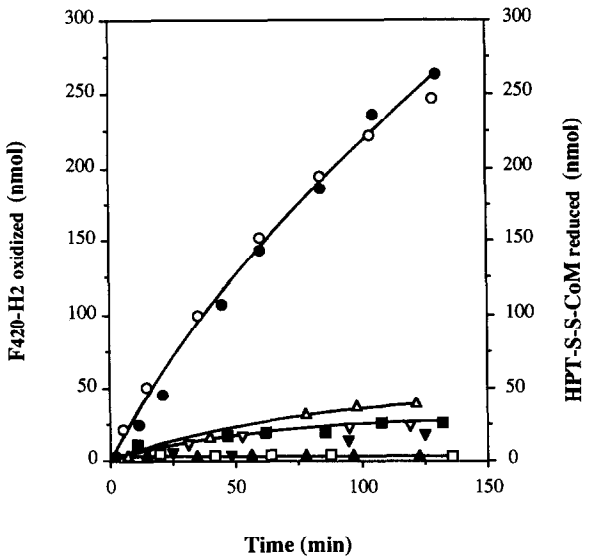

Fig.1. Stoichiometric oxidation of $F_{420}H_2$ and reduction of CoM-S-S-HTP by washed membranes of strain Göl. Washed membranes (80 μg protein) were suspended under N_2 in 2 ml 100 mM Na-Tricine buffer reduced with Ti(III)-citrate, pH 8.0. After addition of 480 nmol $F_{420}H_2$ the reaction was started by addition of 500 nmol CoM-S-S-HTP. F_{420}, HTP-SH and CoM-SH were determined as described in section 2. (Open symbols) Oxidation of $F_{420}H_2$; (closed symbols) reduction of HTP-S-S-CoM. (○,●) Complete assay; (△, ▲) CoM-S-S-HTP omitted; (▽, ▼) $F_{420}H_2$ omitted; (□, ■) membranes omitted.

comitantly and stoichiometrically according to:

$$CoM\text{-}S\text{-}S\text{-}HTP + F_{420}H_2 \longrightarrow CoM\text{-}SH + HTP\text{-}SH + F_{420}$$

The reaction proceeded with a linear rate for 60 min. After 120 min, about 60% of both substrates was reduced and oxidized, respectively. The reverse reaction, i.e. the reduction of F_{420} with the concomitant formation of CoM-S-S-HTP from CoM-SH and HTP-SH could not be observed. If CoM-S-S-HTP was omitted from the reaction mixture, $F_{420}H_2$ was oxidized at a low rate. This CoM-S-S-HTP-independent oxidation of $F_{420}H_2$ could either be due to a hydrogenase-mediated $F_{420}H_2$ oxidation followed by H_2 evolution or to the presence of small amounts of oxygen in the assay. We checked for the former possibility, but were unable to detect any molecular hydrogen. Furthermore, the presence of 5 mM dithioerythritol prevented the $F_{420}H_2$ oxidation in the absence of CoM-S-S-HTP completely (not shown). This excludes the possibility that $F_{420}H_2$ was oxidized by a hydrogenase. It is more likely that contaminating amounts of oxygen led to the low rate of $F_{420}H_2$ oxidation observed in the absence of CoM-S-S-HTP under assay conditions (no DTE present).

There was also a low background activity of CoM-S-S-HTP reduction occurring in the absence of $F_{420}H_2$. It may be attributed to a chemical reduction of this compound by traces of Ti(III)-citrate present in the buffer or by reduced electron carriers in the membranes.

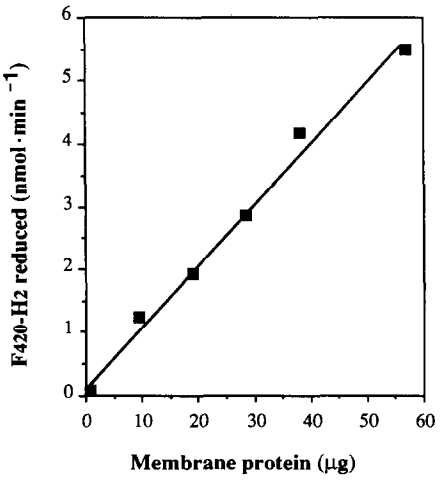

Fig.2. Effect of the protein concentration on the $F_{420}H_2$-dependent heterodisulfide reductase. The formation of F_{420} was followed as described in section 2. The substrate concentrations were adjusted to 60 μM CoM-S-S-HTP and 100 μM $F_{420}H_2$, respectively.

The activity depended linearly on the concentration of membrane protein up to 60 μg/ml (fig.2). The rate was 99 nmol $F_{420}H_2$ oxidized/min·mg protein. No $F_{420}H_2$ oxidation was observed when membranes were omitted.

3.3. *The presence of a membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase in* Methanolobus tindarius

It was important to exclude the theoretical possibility that the $F_{420}H_2$-dependent heterodisulfide reductase in strain Göl was due to the combined action of a F_{420}-dependent hydrogenase and a H_2-dependent heterodisulfide reductase. The former enzyme would oxidize $F_{420}H_2$; the H_2 thereby produced would be taken up by a F_{420}-independent hydrogenase and the electrons channeled to the heterodisulfide reductase. Since Göl contains both methylviologen-dependent hydrogenase and F_{420}-dependent hydrogenase we tested *Methanolobus tindarius* for its ability to reduce CoM-

Table 2

Comparison of hydrogenase and $F_{420}H_2$-dependent heterodisulfide reductase activity in membranes of *Methanolobus tindarius*

Enzyme[a]	Electron acceptor	Conc. (μM)	Electron donor	Specific activity (nmol/min·mg protein)
$F_{420}H_2$-heterodisulfide reductase	HTP-S-S-CoM	60	$F_{420}H_2$[b]	75.0
F_{420}-hydrogenase	F_{420}	12	H_2	<0.1
Methylviologen-hydrogenase	methylviologen	5000	H_2	13.2

[a] Protein concentration: 67.5 μg/ml assay
[b] Concentration of $F_{420}H_2$, 30 μM

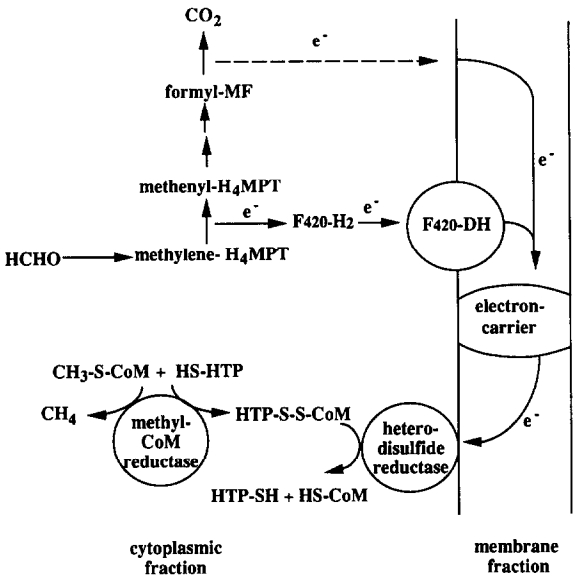

Fig.3. Tentative scheme of electron transfer from methylene-H_4MPT to CH_3-S-CoM. (Broken lines) The electron transfer from the formyl-MF dehydrogenase to the methyl-CoM reductase is unknown. H_4MPT, tetrahydromethanopterin; MF, methanofuran; F_{420}-DH, $F_{420}H_2$-dependent dehydrogenase.

S-S-HTP at the expense of $F_{420}H_2$ oxidation. *Ml. tindarius* is an obligate methylotrophic methanogen, i.e. the organism is unable to grow on H_2-CO_2 or on methanol-H_2 [14]. This situation is reflected by the absence of a F_{420}-dependent hydrogenase activity in membranes of this organism (table 2). Despite this, heterodisulfide reduction with $F_{420}H_2$ was catalyzed by these membranes. The absence of F_{420}-dependent hydrogenase excludes the involvement of such an enzyme in $F_{420}H_2$-dependent CoM-S-S-HTP reduction and rules out the indirect route outlined above.

4. DISCUSSION

An H_2-dependent heterodisulfide reductase was found in *Methanobacterium thermoautotrophicum* by Hedderich and Thauer [11]. This enzyme activity was associated with the cytoplasmic fraction. We report here on a membrane-bound enzyme from the methanogenic bacterium strain Göl and from *Ml. tindarius* that catalyzes the reduction of CoM-S-S-HTP at the expense of coenzyme $F_{420}H_2$ oxidation. The different locations might be due to the isolation procedures employed. Whereas the *Mb. thermoautotrophicum* cell extract was obtained by passage of the cells through a French pressure cell, the procedure employed here proceeded via the formation of protoplasts and gentle disruption in a French cell at a reduced pressure using a buffer with a sucrose concentration of 0.5 M [15–17] or via lysis of cells through freezing and thawing.

The question arises: what is the physiological role of this enzyme activity? Two possibilities can be envisaged. (i) $F_{420}H_2$ is the universal electron donor for the heterodisulfide reductase in all methanogens. In this case, a F_{420}-dependent hydrogenase would reduce F_{420} with electrons derived from H_2. The membrane-bound $F_{420}H_2$-dependent heterodisulfide reductase would then reoxidize $F_{420}H_2$. (ii) The enzyme is found exclusively in methanogens capable of oxidizing methyl group-containing substrates. In that case, the electrons derived from methyl group oxidation would first be transferred to F_{420}. In order to decide which of the two possibilities is realized in methanogens, the distribution of the $F_{420}H_2$-dependent heterodisulfide reductase has to be investigated in both hydrogenotrophic and in methylotrophic methanogens.

For strain Göl we suggest a role of the $F_{420}H_2$-dependent heterodisulfide reductase in methylene-H_4MPT oxidation as depicted in fig.3. A F_{420}-dependent methylene-H_4MPT dehydrogenase as described in *Mb. thermoautotrophicum* oxidizes formaldehyde (via methylene-H_4MPT) and leads to the formation of $F_{420}H_2$ which in turn is reoxidized by the membrane-bound heterodisulfide reductase. The $F_{420}H_2$-dependent heterodisulfide reductase consists of at least two partial activities: (i) a $F_{420}H_2$ dehydrogenase that catalyzes the electron transfer to artificial electron acceptors like methylviologen, metronidazol, FMN or FAD; (ii) the actual membrane-bound heterodisulfide reductase which allows the use of CoM-S-S-HTP as electron acceptor. Previous experiments [5] led us to assume that an additional membrane-bound carrier connects the electron flow in the membrane between the $F_{420}H_2$-oxidizing enzyme and the heterodisulfide-reducing enzyme. HS-HTP finally transfers the electrons to the methyl reductase leading to the formation of CH_4 from CH_3-S-CoM.

A role of heterodisulfide reduction in energy conservation has been suggested [17]. In this respect, the membrane-bound $F_{420}H_2$-dependent enzyme from strain Göl is of particular interest. The activity described here might comprise the minimal system capable of energy conservation. Future experiments will have to show whether this system is able to generate ATP in response to $F_{420}H_2$-dependent heterodisulfide reduction.

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