The $F_{420}H_2$:heterodisulfide oxidoreductase system from *Methanosarcina* species

2-Hydroxyphenazine mediates electron transfer from $F_{420}H_2$ dehydrogenase to heterodisulfide reductase

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Abstract $F_{420}H_2$-dependent CoB-S-S-CoM reduction as catalyzed by the $F_{420}H_2$:heterodisulfide oxidoreductase from *Methanosarcina* strains was observed in a defined system containing purified $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* Gö1, 2-hydroxyphenazine and purified heterodisulfide reductase from *Methanosarcina thermophila*. The process could be divided into two partial reactions: (1) reducing equivalents from $F_{420}H_2$ were transferred to 2-hydroxyphenazine by the $F_{420}H_2$ dehydrogenase with a V_{max} value of 12 U/mg protein; (2) reduced 2-hydroxyphenazine acted as electron donor for CoB-S-S-CoM reduction as catalyzed by the heterodisulfide reductase. The specific activity was 14–16 U/mg protein at 37°C and 60–70 U/mg protein at 60°C. The partial reactions could be combined in the presence of both enzymes. Under these conditions reduced 2-hydroxyphenazine was rapidly oxidized by the heterodisulfide reductase thereby producing the electron acceptor for the $F_{420}H_2$ dehydrogenase. Above a concentration of 50 μM of 2-hydroxyphenazine, the specific activity of the latter enzyme reached the V_{max} value. When other phenazines or quinone derivatives were used as electron carriers, the activity of $F_{420}H_2$-dependent CoB-S-S-CoM reduction was much lower than the rate obtained with 2-hydroxyphenazine. Thus, this water-soluble analogue of methanophenazine best mimics the natural electron acceptor methanophenazine in aqueous systems.

Key words: Methanogenesis; Electron transport; Energy conservation; Cytochrome; Quinone; Phenazine

1. Introduction

During methanogenesis, from 4 mol of methanol, 1 mol of the methyl groups is oxidized to CO_2 and reducing equivalents are transferred to the central electron carrier F_{420}. In the reductive branch of the pathway, three out of four methyl groups are transferred to coenzyme M (CoM-SH). Methyl-S-CoM is reductively cleaved by methyl-S-CoM reductase, which uses coenzyme B (CoB-SH) as reducing agent to form CH_4 and a mixed disulfide (CoB-S-S-CoM) [1]. Thus, $F_{420}H_2$ and CoB-S-S-CoM are generated as electron donor and acceptor for the electron transport chain of the membrane-bound $F_{420}H_2$:heterodisulfide oxidoreductase system [2]. Reduced F_{420} is oxidized by $F_{420}H_2$ dehydrogenase and CoB-S-S-CoM is reduced by heterodisulfide reductase. Membrane-bound electron transfer from $F_{420}H_2$ to CoB-S-S-CoM gives rise to an electrochemical proton gradient that drives ATP formation from ADP+P_i in *Methanosarcina mazei* Gö1 [3]. An important unresolved question is what factor mediates electron transfer between $F_{420}H_2$ dehydrogenase and heterodisulfide reductase.

In our efforts to define the electron acceptor for $F_{420}H_2$ dehydrogenase, a hydrophobic, redox-active component with a molecular mass of 538 Da was recently isolated from membranes of *Ms. mazei* Gö1 [4]. The component was named methanophenazine and represents a 2-hydroxyphenazine derivative that is connected via an ether bridge to an oligoisoprenoid side chain. Since the cofactor is insoluble in aqueous buffers, the analogue 2-hydroxyphenazine was used for further experiments. It was found that the purified $F_{420}H_2$ dehydrogenase from *Ms. mazei* Gö1 catalyzed $F_{420}H_2$-dependent 2-hydroxyphenazine reduction. Furthermore, the membrane-bound heterodisulfide reductase was able to use reduced 2-hydroxyphenazine for CoB-S-S-CoM reduction. In this publication, it is shown that 2-hydroxyphenazine mediates electron transfer from $F_{420}H_2$ to heterodisulfide in the presence of purified $F_{420}H_2$ dehydrogenase and heterodisulfide reductase.

2. Materials and methods

2.1. Strains and growth of organisms

Ms. mazei Gö1 (DSM 3647) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany) and grown in a 100-l fermenter as described [5]. *Ms. thermophila* strain TM-1 was cultured on acetate by slightly modifying the earlier growth conditions as described [6,7].

2.2. Purification of proteins

$F_{420}H_2$ dehydrogenase from *Ms. mazei* Gö1 and heterodisulfide reductase from *Ms. thermophila* were purified according to Abken and Deppenmeijer [5] and Simianu et al. [6], respectively.

2.3. Purification and synthesis of cofactors

F_{420} was isolated from *Ms. barkeri* or *Ms. mazei* and reduced to $F_{420}H_2$ with $NaBH_4$ as described [2]. CoB-S-S-CoM was synthesized by the method of Noll et al. [8] and Kamlage and Blaut [9]. 2-Hydroxyphenazine was prepared according to Abken et al. [4]. The re-

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Abbreviations: CoB-SH, 7-mercaptoheptanoylthreonine phosphate; CoM-SH, 2-mercaptoethanesulfonate; 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}; Mph, methanophenazine; MpH_2, reduced methanophenazine

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duction of the electron carriers (Table 1) was performed in N_2-gassed ethanol containing a few crystals of platinum(IV) oxide under molecular hydrogen overnight. When the reduction was complete, the solution was centrifuged under anaerobic conditions and the supernatant was used for experiments.

2.4. Assay conditions

The kinetics of $F_{420}H_2$-dependent CoB-S-S-CoM reduction by 2-hydroxyphenazine (Fig. 1) was monitored as follows. The amounts of F_{420} and reduced 2-hydroxyphenazine were calculated from redox difference spectra at the time points indicated. The spectra were recorded with a Kontron spectrophotometer in double-beam mode at 37°C. The sample cuvette contained 0.75 ml 25 mM MOPS, pH 7, 5 mM dithioerythritol, 1 mg/l resazurine (buffer A), 10 μM $F_{420}H_2$ and 0.07 μg purified $F_{420}H_2$ dehydrogenase. The reaction was started by adding 53 μM 2-hydroxyphenazine (from a 20 mM ethanolic stock solution) and was followed by repeated wavelength scans (600–400 nm; 20 nm/cm; 500 nm/min). When the oxidation of $F_{420}H_2$ was complete, 0.25 μg of heterodisulfide reductase and 100 μM CoB-S-S-CoM (final concentration) were added. The reference cuvette contained cofactors only, i.e. proteins were omitted. The experiments shown in Table 1 were performed in 0.75 ml buffer A containing 20 μM $F_{420}H_2$, 1.7 μg heterodisulfide reductase, 0.2 μg $F_{420}H_2$ dehydrogenase and 53 μM of the reduced electron carrier as indicated. The reaction was started by adding 100 μM CoB-S-S-CoM (final concentration) and monitored photometrically at 420 nm. One unit (U) per mg protein is equivalent to 1 μmol $F_{420}H_2$ oxidized per min per mg $F_{420}H_2$ dehydrogenase. For the simultaneous determination of $F_{420}H_2$ oxidation and 2-hydroxyphenazine formation as shown in Fig. 2, the above-mentioned photometer was used and the absorbance at 420 and 475 nm was measured in the double-beam wavelength mode.

3. Results

As is apparent from Fig. 1A the purified $F_{420}H_2$ dehydrogenase catalyzed the oxidation of $F_{420}H_2$ and the reduction of 2-hydroxyphenazine with an initial specific activity of 12 U/mg protein in a 1:1 stoichiometry according to the equation:

$$F_{420}\ H_2 + \text{2-OH-phenazine} \rightarrow F_{420} + \text{2-OH-phenazine}H_2 \quad (1)$$

After 40 min, the reaction was complete and the purified heterodisulfide reductase from *Ms. thermophila* [6] and CoB-S-S-CoM were added (Fig. 1B). Reduced 2-hydroxyphenazine formed from Eq. 1 was completely oxidized with an initial rate of 14 U/mg protein at 37°C. When the oxidation of reduced 2-hydroxyphenazine and the reduction of heterodisulfide were followed simultaneously, it was evident that this reaction also followed a 1:1 stoichiometry (not shown):

$$\text{CoB-S-S-CoM} + \text{2-OH-phenazine}H_2 \rightarrow \text{CoM-SH} + \textit{CoB-SH} + \textit{2-OH}\text{-phenazine} \quad (2)$$

Table 1
Specificity of 2-hydroxyphenazine as mediator of electron transport from $F_{420}H_2$ to heterodisulfide

Electron carrier	Specific activity (U/mg protein)
2-Hydroxyphenazine (reduced)	10.4
2,3-Dimethyl-1,4-naphthoquinone (reduced)	1.1
Tetramethyl-*p*-benzoquinone (reduced)	0.6
Phenazine (reduced)	1.1
2-Bromophenazine (reduced)	1.7
Methylviologen (reduced)[a)]	1.1

[a]Methylviologen was reduced by titration with Ti(III) citrate; all other electron carriers were reduced as described in Section 2 and added to a final concentration of 53 μM.

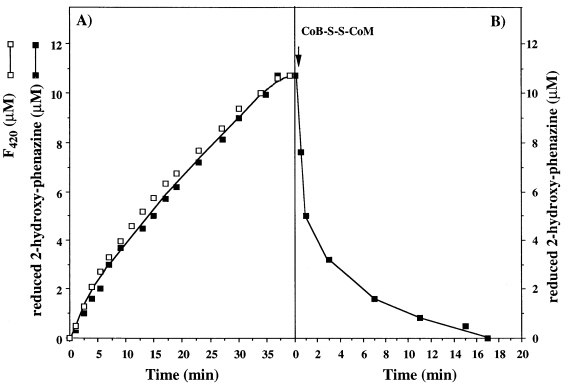

Fig. 1. Kinetics of $F_{420}H_2$-dependent CoB-S-S-CoM reduction mediated by 2-hydroxyphenazine. For assay conditions see Section 2. A redox difference spectrum (600–400 nm) was taken at each time point. A: The oxidation of $F_{420}H_2$ and the reduction of 2-hydroxyphenazine were calculated from the change of absorption at 420 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$) and 475 nm ($\varepsilon = 2.5\ mM^{-1}\ cm^{-1}$) respectively. B: After $F_{420}H_2$ oxidation was complete, heterodisulfide reductase and CoB-S-S-CoM were added. The oxidation of reduced 2-hydroxy-phenazine was estimated from the absorption at 475 nm. The amounts of the products were plotted as a function of time. The calculation for the F_{420} concentration was corrected for the absorbance of 2-hydroxyphenazine at 420 nm ($\varepsilon = 4.4\ mM^{-1}\ cm^{-1}$).

The specific activity of the heterodisulfide reductase from the thermophilic archaeon increased to 60–70 U/mg protein at 70°C. However, under these conditions, 2-hydroxyphenazine had to be reduced chemically since $F_{420}H_2$ dehydrogenase purified from *Ms. mazei* Gö1 was not active at this temperature (not shown).

The interaction of 2-hydroxyphenazine with both enzymes is also evident from Fig. 2. $F_{420}H_2$ dehydrogenase, heterodisulfide reductase and reduced 2-hydroxyphenazine were combined in the presence of CoB-S-S-CoM and the formation of F_{420} ($\lambda = 420$ nm) and 2-hydroxyphenazine ($\lambda = 475$) were determined simultaneously. In the first phase, reduced 2-hydroxyphenazine was rapidly oxidized by the heterodisulfide reductase with an initial rate of 16.7 U/mg protein. Since the heterodisulfide reductase was added in excess this reaction was almost complete within 10 min. The activity of the $F_{420}H_2$ dehydrogenase increased during the course of the reaction due to increasing amounts of 2-hydroxyphenazine produced by the heterodisulfide reductase. Above a concentration of 50 μM, the specific activity of $F_{420}H_2$-dependent 2-hydroxyphenazine reduction was 10.4 U/mg protein which is in the range of the V_{max} of the $F_{420}H_2$ dehydrogenase [4]. After 30 min, the rate slowly decreased due to the depletion of $F_{420}H_2$.

In addition to 2-hydroxyphenazine, other phenazine derivatives and different quinones were tested for their ability to act as mediators of electron transport from $F_{420}H_2$ to CoB-S-S-CoM in the presence of purified $F_{420}H_2$ dehydrogenase and heterodisulfide reductase. As evident from Table 1, the rate of $F_{420}H_2$-dependent heterodisulfide reduction with reduced 2,3-dimethyl-1,4-naphthoquinone or tetramethyl-*p*-benzoquinone was more than 10-fold lower than that with 2-hydroxyphenazine. Also phenazine and 2-bromophenazine proved to be less effective indicating that among the compounds studied, the methanophenazine analogue 2-hydroxyphenazine best mimics the natural electron acceptor methanophenazine in aqueous systems.

4. Discussion

During methanogenesis from methanol, the intermediates $F_{420}H_2$ and CoB-S-S-CoM are formed. In *Methanosarcina* species, these cofactors are regenerated by the $F_{420}H_2$:heterodisulfide oxidoreductase system (Fig. 3). Reduced F_{420} is oxidized by $F_{420}H_2$ dehydrogenase, an enzyme that has been purified from the archaea *Archaeoglobus fulgidus* [10], *Methanolobus tindarius* [11] and *Ms. mazei* Gö1 [5]. The enzyme from the latter organism has a molecular mass of 115 kDa, consists of five different subunits, and contains FeS clusters and FAD. The heterodisulfide reductase from *Ms. barkeri* which reduces CoB-S-S-CoM was found to be composed of two subunits with molecular masses of 46 and 23 kDa [12]. The large subunit (HdrD) contains two [4Fe-4S] clusters and harbors the active site of heterodisulfide reduction [13]. The small polypeptide (HdrE) represents a *b*-type cytochrome containing two heme groups. The enzyme from *Ms. thermophila* has essentially the same features (unpublished results). $F_{420}H_2$-dependent heterodisulfide reduction has been shown to be competent in driving proton translocation across the cytoplasmic membrane in the methanogenic archeon *Ms. mazei* Gö1 [3]. Electron transport and H^+ transfer are strictly coupled, as indicated by stoichiometries of 3–4 H^+ translocated per heterodisulfide reduced. In this publication, evidence is presented that the $F_{420}H_2$:heterodisulfide oxidoreductase is composed of $F_{420}H_2$ dehydrogenase, heterodisulfide reductase and a phenazine derivative that mediates electron transfer between the protein components (Fig. 3). In vivo the mediator is probably methanophenazine which was recently isolated from the membranes of *Ms. mazei* Gö1 [4]. Thus, the $F_{420}H_2$:heterodisulfide oxidoreductase represents a typical energy-conserving electron transport chain as found in many bacteria and eukarya. However, the components of the system are unique and have not been discovered in organisms others than methanogens or *Archaeoglobus fulgidus*. $F_{420}H_2$ dehydrogenase is functionally homologous to NADH dehydrogenase since both enzymes reveal a complex subunit composition and contain FeS clusters and flavins [14]. Both $F_{420}H_2$ and NADH are reversible hydride donors with similar mid-point potentials. Electrons derived from the oxidation process are transferred to quinones and methanophenazine, respectively, which are similar in possessing isoprenoid side chains that enable them to diffuse into the hydrocarbon phase of the cytoplasmic membrane. The final reaction of methanogenesis is the cleavage of the disulfide bond of CoB-S-S-CoM by heterodisulfide reductase. This reaction resembles polysulfide reduction as performed by extremely thermophilic eubacteria and archaea [15] and by certain mesophilic bacteria including *Desulfuromonas acetoxidans* [16] and *Wolinella succinogenes* [17]. In many organisms, sulfur reduction is coupled to ATP synthesis by a mechanism similar to oxidative phosphorylation [17]. Future experiments will focus on showing if $F_{420}H_2$-dependent 2-hydroxyphenazine reduction or the oxidation of reduced 2-hydroxyphenazine by CoB-S-S-CoM is coupled to the generation of an electrochemical proton gradient in *Ms. mazei* Gö1.

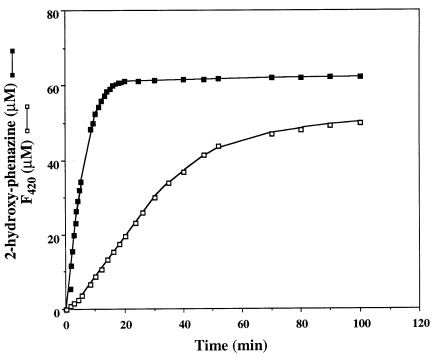

Fig. 2. Determination of the requirements of the $F_{420}H_2$:heterodisulfide oxidoreductase. The reaction was performed in 0.75 ml buffer A containing 54 μM $F_{420}H_2$, 62 μM reduced 2-hydroxyphenazine, 0.12 μg $F_{420}H_2$ dehydrogenase and 0.75 μg heterodisulfide reductase. The reaction was started by adding 150 μM CoB-S-S-CoM. The formation of F_{420} and 2-hydroxyphenazine were followed photometrically at 420 nm and 475 nm, respectively. The calculation of the F_{420} concentration was corrected for the absorbance of 2-hydroxyphenazine at 420 nm ($\varepsilon = 4.4\ mM^{-1}\ cm^{-1}$).

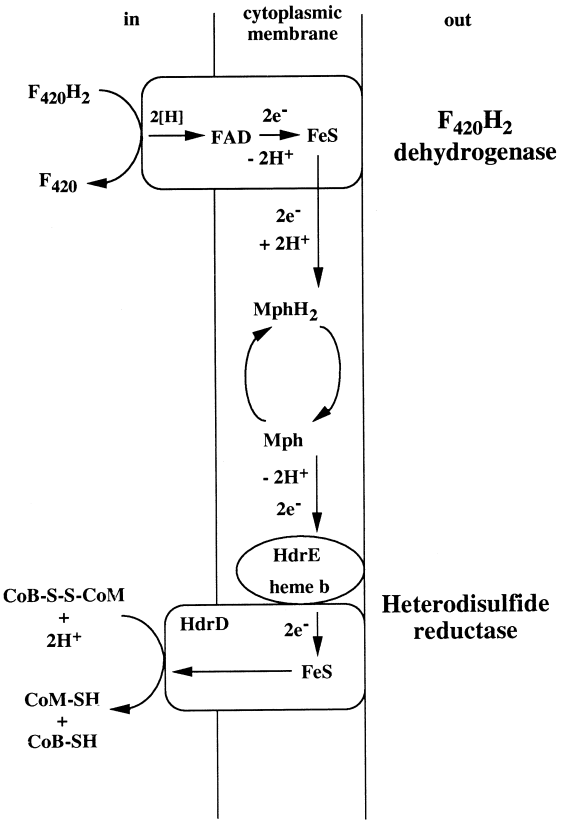

Fig. 3. Tentative model of electron transport from $F_{420}H_2$ to CoB-S-S-CoM as catalyzed by the $F_{420}H_2$:heterodisulfide oxidoreductase.

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