Identification and analysis of proton-translocating pyrophosphatases in the methanogenic archaeon *Methanosarcina mazei*

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Received June 12, 2001; accepted August 14, 2001; published online September 21, 2001

**Summary**  Analysis of genome sequence data from the methanogenic archaeon *Methanosarcina mazei* Gö1 revealed the existence of two open reading frames encoding proton-translocating pyrophosphatases (PPases). These open reading frames are linked by a 750-bp intergenic region containing TC-rich stretches and are transcribed in opposite directions. The corresponding polypeptides are referred to as Mvp1 and Mvp2 and consist of 671 and 676 amino acids, respectively. Both enzymes represent extremely hydrophobic, integral membrane proteins with 15 predicted transmembrane segments and an overall amino acid sequence similarity of 50.1%. Multiple sequence alignments revealed that Mvp1 is closely related to eukaryotic PPases, whereas Mvp2 shows highest homologies to bacterial PPases. Northern blot experiments with RNA from methanol-grown cells harvested in the mid-log growth phase indicated that only Mvp2 was produced under these conditions. Analysis of washed membranes showed that Mvp2 had a specific activity of 0.34 U mg (protein)–1. Proton translocation experiments with inverted membrane vesicles prepared from methanol-grown cells showed that hydrolysis of 1 mol of pyrophosphate was coupled to the translocation of about 1 mol of protons across the cytoplasmic membrane. Appropriate conditions for *mvp1* expression could not be determined yet. The pyrophosphatases of *M. mazei* Gö1 represent the first examples of this enzyme class in methanogenic archaea and may be part of their energy-conserving system.

**Keywords:** energy conservation, inorganic pyrophosphate, methanogenesis, proton pump, pyrophosphatase.

Abbreviations: DCCD, *N,N′*-dicyclohexylcarbodiimide; PPase, inorganic pyrophosphatase; PPI, inorganic pyrophosphate; Δp, proton motive force.

**Introduction**

Inorganic pyrophosphate (PPI) is formed in several enzymatic reactions of various metabolic pathways (e.g., deoxyribonucleic acid and ribonucleic acid polymerization and amino acid and fatty acid activation). It is supposed that subsequent hydrolysis of PPI by pyrophosphatases shifts the overall reaction equilibrium toward product formation. However, according to Rea and Sanders (1987), this assumption may be too restrictive because a considerable amount of metabolic energy would be lost as heat. Alternatively, it is possible that pyrophosphatases utilize a portion of the PPI anhydride bond energy for the generation of a transmembrane proton gradient that could then be used to drive endergonic reactions.

Two families of pyrophosphatases have been identified in the three domains of life. The first class encompasses a wide variety of soluble cytoplasmic enzymes that are not involved in energy conservation (Silvula et al. 1999). The second class comprises tightly membrane-bound pyrophosphatases that were first isolated from vacuolar membranes of higher plants and algae (Maeshima 2000). These enzymes translocate protons across the vacuolar membrane, thereby maintaining the acidic vacuolar interior milieu. Proton-translocating pyrophosphatases have also been found in the cytoplasmic membrane of several bacteria such as *Rhodospirillum rubrum* (Baltscheffsky et al. 1998), *Thermotoga maritima* (Nelson et al. 1999), *Streptomyces coelicolor* (Redenbach et al. 1996), *Synthrophus gentianae* (Schöcke and Schink 1998) and in the hyperthermophilic archaeon *Pyrobaculum aerophilum* (Drozdowicz et al. 1999, Drozdowicz and Rea 2001).

In this report we show that proton-translocating pyrophosphatases are also present in the methanogenic archaeon *Methanosarcina mazei* Gö1. This organism derives its metabolic energy from the conversion of H₂ plus CO₂, acetate, methanol or methylamines to methane. Redox reactions of methanogenesis are partly catalyzed by membrane-bound enzymes that generate or use electrochemical ion gradients (Deppenmeier et al. 1996). The H₂-heterodisulfide oxidoreductase and the F₄₃₀H₂-heterodisulfide oxidoreductase are novel electron transport systems that are able to generate a proton motive force by redox-driven proton translocation (Deppenmeier et al. 1999). A membrane-bound A₆A₇p-ATP synthase utilizes the electrochemical proton gradient for ATP synthesis (Müller et al. 1999). The pyrophosphatases found in *M. mazei* Gö1 represent another class of proton-translocating
enzymes in methanogens and might be involved in a new energy-conserving system.

Materials and methods

Growth of cells

*Methanosarcina mazei* Gö1 (DSM 3647) was grown in 1-l glass bottles or, for mass culturing, in 20-l carboys on 150 mM methanol in a medium described previously (Hippe et al. 1979), supplemented with 1 g l⁻¹ sodium acetate.

Preparation of washed vesicles and washed membranes

Washed inverted vesicles of strain Gö1 were prepared as described by Ide et al. (1999), except that the final protein concentration was 10–15 mg ml⁻¹. Washed membranes from *M. mazei* were prepared according to the method of Abken and Deppenmeier (1997).

Measurement of proton translocation

Proton translocation was monitored as described previously (Ide et al. 1999). Briefly, 3 ml of 40 mM KSCN solution containing 0.5 M sucrose, 1 mg ml⁻¹ resazurin and 10 mM dithioerythritol was added to a nitrogen-flushed, 11-ml reaction vessel, followed by addition of 50–80 µl of washed inverted vesicles (1–1.4 mg protein per assay). Proton uptake coupled to pyrophosphate cleavage was assessed by monitoring the pH of the solution with a pH electrode connected to a chart recorder during the hydrolysis of 5 to 40 nmol Na pyrophosphate (20 mM aqueous stock solution). The uncoupler 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile was added to a final concentration of 12 nmol mg⁻¹ membrane protein where indicated (Figure 4). Changes in pH were calibrated with standard solutions of HCl or NaOH.

Determination of the cytoplasmic phosphate concentration

Five-ml cultures of *M. mazei* were harvested in the exponential growth phase and washed three times with 25 mM MOPS plus 0.5 M sucrose to remove phosphate contaminants. After cell sonication, protein was precipitated by heating at 100 °C for 5 min and removed by centrifugation. The supernatant was used for determination of the cytoplasmic phosphate concentration.

Enzyme activity assays

Pyrophosphatase activity was determined in 1.8-ml glass vessels containing 25 mM MOPS buffer pH 7 and washed membranes (about 1 mg membrane protein). Sodium pyrophosphate and MgCl₂ were added from 0.1 M aqueous stock solutions to final concentrations of 4 and 3 mM, respectively. Membranes were preincubated with MgCl₂ at 37 °C and the reaction was started by the addition of pyrophosphate. To examine PPi hydrolysis, 50-µl samples were withdrawn at intervals and added to 10 µl concentrated HClO₄ to stop enzyme activity. Precipitated protein was removed by centrifugation. The release of free phosphate due to pyrophosphate hydrolysis was determined as described by Saheki et al. (1985) with the following specifications. Each 25-µl sample prepared as described above was added to 750 µl of a 15 mM ammonium molybdate solution with 70 mM zinc acetate (pH 5.0 HCl) and reduced with 250 µl freshly prepared 10% sorbic acid solution (pH 5.0 NaOH). After incubation for 20 min, absorbance of samples at 850 nm was measured against a phosphate blank sample. Concentrations were calculated from a standard curve of 0–250 µM KH₂PO₄.

Construction of ³²P-labeled probes and northern blot analysis

For the construction of specific DNA probes for studies of pyrophosphatase gene expression in *M. mazei* Gö1, non-homologous sequence regions of *mvp*₁ (Probe 1: bp 743–1089) and *mvp*₂ (Probe 2: bp 710–1035) were amplified by polymerase chain reaction (PCR) using chromosomal DNA as template. The DNA fragments were isolated from a 0.8% agarose gel and purified with a gel extraction kit (Qiagen, Hilden, Germany). Probes were radiolabeled with [α-³²P]dATP (Hartmann, Braunschweig, Germany) with a random primer labeling kit (Boehringer, Mannheim, Germany), and purified by gel chromatography (NAP 10, Pharmacia, Uppsala, Sweden). Total cellular RNA was prepared from methanol-grown *M. mazei* Gö1 (harvested in the mid-log phase) with an RNA isolation kit (RNeasy, Qiagen) according to the manufacturer’s instructions. Northern blot analysis was performed as described previously (Deppenmeier et al. 1995).

GenBank accession numbers

The nucleotide sequences and corresponding amino acid sequences for genes encoding proton-translocating and soluble PPases from *M. mazei* have been deposited in the GenBank database under Accession Numbers AF312701 (*mvp*₁ and *mvp*₂) and AF312700 (gene encoding a soluble PPase).

Results

Molecular biological analysis

DNA sequence data from the *M. mazei* Gö1 genome project (Göttingen Genomics Laboratory, Göttingen, Germany) revealed two open reading frames encoding hypothetical plant V-type pyrophosphatase homologs. The genes were designated *mvp*₁ and *mvp*₂ (*Methanosarcina* vacuolar-type pyrophosphatase). They are linked by a 750-bp intergenic region containing TC-rich stretches and are transcribed in opposite directions (Figure 1). Typical archaeal promoter sequences of the TATA-box initiator type (Brown et al. 1989) were found upstream of both genes. Bacterial-type ribosomal binding sites immediately preceded the genes.

The deduced polypeptides of *mvp*₁ (N-terminus MERLIFT) and *mvp*₂ (N-terminus MDMLIY) consist of 671 (69.1 kDa) and 676 amino acids (69.2 kDa), respectively. Both enzymes represent extremely hydrophobic, integral membrane proteins with 15 predicted transmembrane segments. The overall amino acid sequence similarity between Mvp1 and Mvp2 was 50.1%. The deduced amino acid sequences of *mvp*₁ and *mvp*₂ were compared with proton-translocating PPases from other organ-
isms. Alignments indicated sequence similarities in the range of 35.5 to 50.6%. For Mvp1, the highest scores were obtained for PPases of the eukaryote Acetabularia mediterrana (50.6%), Nicotiana tabacum (45.1%) and Chara corallina (45.4%). In contrast, Mvp2 showed the highest sequence similarities to PPases from the bacterium Rhodospirillum rubrum (45.6%) and the archaeon Pyrobaculum aerophilum (42.9%).

Analysis of pyrophosphatase gene expression

For analysis of the expression pattern of mvp1 and mvp2, specific DNA probes for both genes were synthesized by PCR using chromosomal DNA as template. Total RNA isolated from methanol-grown cells was used for Northern blot analysis. One major band with a size of 1.8 kb was obtained when RNA from these cells was hybridized with the mvp2-specific probe (Figure 2). No specific signals were found with the mvp1-specific probe. The weak signals at 2.5 and 1.4 kb are probably caused by nonspecific binding to 23S rRNA and 16S rRNA, respectively (Deppenmeier et al. 1995). Because the mvp2 transcript has a length of 1.8 kb, it is evident that only this gene, and not mvp1, is expressed by M. mazei cells grown on methanol. Because the following experiments were performed with methanol-grown cells harvested in the mid-log phase, pyrophosphatase activity was attributable to Mvp2 exclusively and there was no interference from Mvp1.

Biochemical analysis

To determine the cellular localization of Mvp2, cell lysates of M. mazei Gö1 were centrifuged at 120,000 g for 2 h to separate the membraneous fraction from the cytoplasm. Almost 100% of the PPase activity was present in the pellet. The pellet was resuspended and centrifuged at 100,000 g for 2 h. As before, almost 100% of the PPase activity was detected in the membraneous fraction, indicating that the enzyme is located in the cytoplasmic membrane. The activity of the membrane-bound PPase was linearly dependent on the concentration of membrane protein up to 1 mg ml⁻¹ (data not shown). Furthermore, the enzyme was air-stable for several hours. The protein showed maximal activity (0.34 µmol PPi hydrolyzed min⁻¹ mg (membrane protein)⁻¹) when 4 mM PPi and at least 2 mM Mg²⁺ were added to the reaction mixture (Figure 3). Lower concentrations of these ions resulted in a decrease in the reaction rate. As already observed for other PPases (Maeshima and Yoshida 1989), the Mvp2 protein was inhibited by N,N’-dicyclohexylcarbodiimide (DCCD), indicated by IC₅₀ values of 1.5 µmol DCCD per mg membrane protein (not shown).

In addition to mvp1 and mvp2, genome sequencing of M. mazei revealed the presence of a gene encoding a soluble pyrophosphatase (GenBank Accession No. AF312700). However, the cytoplasmic fraction did not contain PPase activity. Thus, it appears that the soluble PPI-hydrolyzing enzyme was not produced when cells were grown on methanol and harvested in the mid-log phase.

Multi-alignments and biochemical analysis indicated that Mvp2 is a membrane-bound proton-translocating pyrophosphatase. To verify this hypothesis, washed inverted vesicles from M. mazei Gö1 were tested for their ability to couple PPI hydrolysis to the transfer of protons across the cytoplasmic membrane. Concentrated vesicles were diluted with a sucrose/thiocyanate solution under an atmosphere of molecular nitrogen and pulsed with PPI as shown in Figure 4. When the substrate was added, there was a short period of alkalinization, as a result of rapid proton movement into the lumen of the inverted vesicles. In the second phase, reacidification was observed until a stable pH value was reached. It is thought that this reacidification was caused by decay of the generated
chemical potential (Δμ\textsubscript{H+}) by passive diffusion of protons from vesicle lumena back to the medium. The addition of the protonophore SF 6847 led to complete inhibition of reversible alkalinization (Figure 4), indicating that protons function as coupling ions. After calibration of the system, an average of 0.47 ± 0.12 protons translocated per PPi hydrolyzed was calculated from more than 30 experiments. Taking into account that about 50% of the membrane structures present in the vesicle preparations are unable to establish a proton gradient (Deppenmeier et al. 1999), the hydrolysis of 1 mol PPi is coupled to the translocation of about 1 mol of protons.

Discussion

Energetic considerations

Methanogenesis in \textit{M. mazei} is not coupled to substrate-level phosphorylation. Instead, it has been shown that energy is conserved by a chemiosmotic mechanism (Deppenmeier et al. 1999). The membrane-bound electron transport system of this organism is able to translocate 4 mol of protons in the course of the generation of 1 mol of methane. Thus, it is evident that \textit{M. mazei} has only a limited ability to generate electrochemical ion gradients. Keltjens et al. (1988) reported that methane formation is coupled to PPi synthesis in \textit{Methanobacterium thermoautotrophicum}. However, it was later shown by Ellermann et al. (1989) that these experiments were not reproducible. Accordingly, methanogenesis from methanol plus H\textsubscript{2} catalyzed by washed cell suspensions of \textit{M. mazei} Gö1 was not coupled to PPi synthesis (unpublished results). Irrespective of this controversy, it is evident that several core biosynthetic pathways generate PPi (e.g., DNA and RNA synthesis, amino acid activation, polysaccharide synthesis and formation of fatty acyl-CoA (Maeshima 2000)). Thus, the idea that PPi may function as an energy-rich intermediate for ATP synthesis is reasonable. This hypothesis is supported by the finding that the PPi concentration in the cytoplasm of \textit{M. mazei} Gö1 is low (< 0.2 mM), even though soluble PPase activity is lacking. Thus, the membrane-bound PPases may be the only enzymes responsible for the disposal of cytosolically produced PPi, thereby pulling the biosynthetic reactions to completion. In addition, the enzymes salvage a portion of the free energy of PPi hydrolysis by proton translocation and the formation of a proton gradient. The actual free energy change for PPi hydrolysis in the cytoplasm has been calculated to be 27.3 kJ mol\textsuperscript{-1} at pH 7.3 (Davis et al. 1993). The electrochemical potential
that can be established by a given proton pump depends on the free energy change ($\Delta G$) of the driving reaction and on the number of protons transported per cycle ($\Delta G = nF\Delta p$, where $n =$ number of protons, $F =$ Faraday’s constant, and $\Delta p =$ proton motive force). It has been shown that $\Delta p$ of $M. mazei$ is about $-150$ mV (Peinemann 1989). Thus, for the translocation of 1 mol of protons, a free energy change of at least 14.6 kJ is necessary. Taking the above-mentioned free energy change of 27.3 kJ mol$^{-1}$ into account, PPI hydrolysis coupled to proton transfer is still an exergonic process that can function as a driving force for biosynthetic reactions. The advantage of a membrane-integral PPase is that some of the energy is conserved in the form of a transmembrane electrochemical proton gradient, whereas soluble PPases merely dissipate all free energy thermally.

**Putative enzyme structure and sequence characteristics**

Sequence comparisons and computer-assisted topological and functional analyses demonstrated that the enzyme encoded by mvp2 belongs to the membrane-associated proton-translocating family of PPases. The gene mvp1 was not expressed in methanol-grown cells, but multiple alignments of the deduced amino acid sequence showed that Mvp1 also belongs to this class of enzymes. The most thoroughly investigated proton-translocating PPases are those found in the vacuolar membranes of plant cells (Maeshima 2000) and the cytoplasmic membrane of the photosynthetic bacterium *Rhodospirillum rubrum* (Baltscheffsky et al. 1998). Structural studies indicate that these enzymes are composed of only one subunit with a molecular mass of 67.5 to 80.8 kDa (Maeshima 2000). Evidence for the presence of proton-translocating PPases has also been found in several other organisms such as the thermophilic bacterium *Thermotoga maritima* (Nelson et al. 1999), the marine alga *Acetabularia mediterranea* (Ikeda et al. 1999), the parasitic protist *Trypanosoma cruzi* (Hill et al. 2000), the malaria parasite *Plasmodium falciparum* (Luo et al. 1999), the syntrophic bacterium *Syntrophus gentianae* (Schöcke and Schink 1998) and the soil bacterium *Streptomyces coelicolor* (Redenbach et al. 1996; for a review see Drozdowicz and Rea 2001). Recently, Drozdowicz et al. (1999) described a vacuolar-type membrane pyrophosphatase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*, the first report of the presence of this category of pump in an archaeon. This discovery indicates that these proteins are distributed among all three domains of life, a conclusion supported by the discovery of PPase orthologs in the methanogenic archaeon *M. mazei*. Interestingly, genes encoding proton-translocating PPases are not present in the genomes of *Methanobacterium thermoautotrophicum* and *Methanococcus jannaschii* (Bult et al. 1996, Smith et al. 1997). Moreover, they are not found in the sulfate-reducing archaeon *Archaeoglobus fulgidus* (Klenk et al. 1997), a close relative of *M. mazei*.

The deduced amino acid sequences of mvp1 and mvp2 possess most of the structural features characteristic of PPases (Figure 5). Because of the evolutionary distance between methanogenic archaea and other organisms containing PPases, the evaluation of highly conserved amino acid sequences may contribute to the identification of sequence motifs likely involved in core catalysis by all V-type PPases.

Sixteen transmembrane helices were predicted for both Mvp1 and Mvp2 with the TopPredII program. Residues 656 to 676 at the C-terminus met the criterion for a transmembrane $\alpha$-helix. However, as pointed out by Drozdowicz et al. (1999), this structure is unlikely because protein fusions of apo-

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Figure 5. Predicted topology of Mvp2 and identification of conserved amino acids in the family of proton-translocating PPases. The topology prediction was performed with the program TopPredII. Multi-alignments for the identification of identical and functionally homologous amino acid residues were generated with the program MegAlign. Functionally homologous amino acids were defined as follows: acidic = D, E; basic = H, K, R; hydrophobic = A, F, I, L, M, P, V, W; polar = C, G, N, Q, S, T, Y. The amino acid sequence of proton-translocating PPases from the following species were compared with Mvp2: *Acetabularia mediterrana*, *Arabidopsis thaliana*, Beta vulgaris, *Plasmodium falciparum*, *Pyrobaculum aerophilum*, *Rhodospirillum rubrum*, *Streptomyces coelicolor*, *Thermotoga maritima* and *M. mazei* (Mvp1). Residues found in all sequences are shown in white on a black background. Functionally homologous amino acids are indicated in black on a grey background.
aqequorin and the C-terminus of the vacuolar PPase in transgenic *Arabidopsis* plants indicated that the C-terminus of the enzyme is located in the cytoplasm. Moreover, antibody binding to the C-terminus inhibited PPI hydrolysis. Therefore, it was supposed that the C-terminus is close to the catalytic site of the cytoplasmic loop III (Takasu et al. 1997). Considering the fundamental correspondence of the putative topology of Mvp1 and Mvp2 to that of other V-type PPases, a basic uniformity of secondary structure is most likely (Zhen et al. 1997). Therefore, the C-terminus of the methanogenic proteins may also form a helix-like structure in the cytoplasm. In summary, Mvp2 (Figure 5) is predicted to form 15 transmembrane spans that are connected by large cytoplasmic loops and relatively small outside loops. A multiple alignment of PPase sequences from Eukarya (*Arabidopsis*, Beta, Acetabularia and Plasmodium), Bacteria (*Rhodospirillum*, *Streptomyces* and *Thermotoga*) and Archaea (*Pyrococcus*, *M. mazei* Mvp1 and Mvp2) was performed (not shown) and conserved residues are shown in white on a black background in Figure 5.

The greatest similarities between the Mvp2 sequence and the sequences of other proton-translocating PPases were detected within the hydrophilic loops III and VI with cytoplasmic orientation and the C-terminal tail, all of which are probably part of the substrate-binding and hydrolysis domain or contribute to it (Takasu et al. 1997). Moreover, there are highly conserved Gly residues in Helices 3, 4, 5, 9, 13 and 15 that could be responsible for tight localization of the corresponding helices.

Furthermore, several sequence motifs and residues assumed or demonstrated to be essential for catalysis by plant PPases are also present in the methanogenic enzymes. Comparison of all proton-translocating PPases has revealed one remarkably conserved segment (Maeshima 2000) located in the cytosolic loop III and containing the putative catalytic motif Dx7KxE found in soluble and membrane-associated PPases (Rea et al. 1992). The sequence TKAADVGLVGLKE from Mvp2 corresponds to this region and exactly matches the respective segment in vacuolar PPases of land plants. Is has been proposed that this subdomain participates directly in substrate and Mg\(^{2+}\) binding (Rea et al. 1992). Mutational analysis of the proton-translocating PPase from mung bean (Maeshima 2000, Nakamish et al. 2001) showed that the underlined charged residues are essential for enzymatic activity (D191, K199 and E201 in Mvp2; see Figure 5).

In addition, other residues may be essential for formation of the catalytic site. Substitution of E305 and D504 in the proton-translocating PPase from *Arabidopsis* (Zhen et al. 1997) resulted in loss of hydrolytic activity and proton translocation (E235 and D426 in the methanogenic enzymes). The cytoplasmic loop V of many plant proton-translocating PPases contains the span-loop interface motif TETYTS (TEHYTS in Mvp2), which encompasses a Glu residue also implicated in coupling of PPI hydrolysis to proton translocation (Zhen et al. 1997). A carbodiimide-reactive Glu residue has been identified within the C-terminal part of the enzyme from pumpkin (Rea and Poole 1993). It was proposed that the DCCD-sensi-

**Acknowledgments**

This work was supported by a grant (De 488/2-5) of the Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg), by the DFG priority program 1070 (De 488/6-1) and by a grant of the Ministry of Science and Culture of the state Lower Saxony.

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