The proteolipid of the A1A0 ATP Synthase from *Methanococcus jannaschii* Has Six Predicted Transmembrane Helices but Only Two Proton-translocating Carboxyl Groups*  

Claudia Ruppert‡, Holger Kavermann‡, Sönke Wimmers‡, Roland Schmid§, Joseph Kellermann‡, Friedrich Lottspeich‡, Harald Huber†, Karl O. Stetter‡, and Volker Müller‡**

From the ‡Lehrstuhl für Mikrobiologie der Ludwig-Maximilians-Universität München, Maria-Ward-Strasse 1a, 80638 München, Germany, §FB5, AG Mikrobiologie, Universität Osnabrück, Barbarastrasse 11, 49069 Osnabrück, Germany, ¶Max-Planck-Institut für Biochemie, Abteilung Proteinchemie, Am Klopferspitz 18a, 82152 Martinsried, Germany, and †Lehrstuhl für Mikrobiologie, Universitätsstrasse 31, Universität Regensburg, 93053 Regensburg, Germany

(Received for publication, April 6, 1999, and in revised form, June 21, 1999)

The proteolipid, a hydrophobic ATPase subunit essential for ion translocation, was purified from membranes of *Methanococcus jannaschii* by chloroform/methanol extraction and gel chromatography and was studied using molecular and biochemical techniques. Its apparent molecular mass as determined in SDS-polyacrylamide gel electrophoresis varied considerably with the conditions applied. The N-terminal sequence analysis made it possible to define the open reading frame and revealed that the gene is a triplication of the gene present in bacteria. In some of the proteolipids, the N-terminal methionine is excised. Consequently, two forms with molecular masses of 21,316 and 21,183 Da were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The molecular and biochemical data gave clear evidence that the mature proteolipid from *M. jannaschii* is a triplication of the 8-kDa proteolipid present in bacterial F1F0 ATPases and most archaeal A1A0 ATPases. Moreover, the triplicated form lacks a proton-translocating carboxyl group in the first of three pairs of transmembrane helices. This finding puts in question the current view of the evolution of H+-ATPases and has important mechanistic consequences for the structure and function of H+-ATPases in general.

Proton-pumping ATPases are found in all organisms with an overall applicable bipartite structure consisting of the membrane-extrinsic moiety (F1/V1/A1), which synthesizes and/or hydrolyzes ATP, and the hydrophobic domain (F0/V0/A0), which translocates ions across the membrane. Based on subunit composition and primary structures of the subunits, the archaeal A1A0 ATPases and the eucaryal V1V0 ATPases are closely related (1–5). However, they differ with respect to function. The V1V0 ATPase exhibits only ATP hydrolysis activity and serves to energize the membranes of certain organelles and cells. Methanogenic archaea are not fermentative but are strictly chemiosmotic, and the presence of an ATP synthase has been known for a long time (6, 7). Interestingly, the ATPases isolated from membranes of various methanogens were all classified as V1V0-like enzymes (now called A1A0 ATPases), whereas F1F0-like enzymes have never been isolated (5). Inhibitor studies revealed that the A1A0 ATPase from *Methanosarcina mazei* Go1 is a ΔμH+-driven ATP synthase (8), which gave experimental evidence that methanogens synthesize ATP by means of the A1A0 ATPase. Moreover, the genome of *Methanococcus jannaschii* harbors only genes encoding the A1A0 ATPase but not the F1F0 ATPase (9), which is clear evidence that this hyperthermophile also engages the A1A0 ATPase for ATP synthesis. It was postulated that the diversion of the A1A0 ATPases took place by a duplication and subsequent fusion of the genes encoding the proteolipid, a very hydrophobic, membrane-integral subunit known to participate in transmembrane H+ transport, which in all hitherto known A1A0 ATPases is 8 kDa but is 16 kDa in all V1V0 ATPases (2). The apparent inability of the V1V0 ATPases to synthesize ATP was thought to be the result of this gene duplication. The elucidation of the genomic sequence of *M. jannaschii* revealed only one proteolipid-encoding gene, embedded in the A1A0 ATPase operon, but surprisingly this gene is triple the size of the proteolipid-encoding gene found in every other archaeon investigated so far. This finding stands in sharp contrast to the current view of evolution of structure and function of V1V0/A1A0 ATPases. The proteolipid-encoding gene of *M. jannaschii* is not only triplicated, but in addition, the first of the three predicted transmembrane hairpins lacks the proton-translocating carboxyl group.

However, because of the presence of three potential translational start codons, it is impossible to predict unambiguously from the genomic sequence the molecular mass of the proteolipid. Apart from the first start codon 103 bp downstream of the stop of *ahaI*, which would lead to a 21.3-kDa proteolipid, two additional putative start codons are present, which would give rise to peptides of 13.5 and 10 kDa, respectively (Fig. 1). Furthermore, post-translational modifications such as peptolytic cleavage, which would result in much smaller mature polyepitides, could not be excluded *a priori*. Therefore, it was essential to establish the gene-polypeptide correspondence and to determine the exact molecular mass of the mature proteolipid. We will provide evidence that the proteolipid-encoding gene of *M. jannaschii* arose by triplication with subsequent fusion of the genes. The proteolipid does not undergo peptolytic cleavage; the mature polypeptide is a triplication of the 8-kDa proteolipid found in other archaea and in bacteria. These findings are discussed in view of the evolution and the structure/function relationship of H+-ATPases.
**Experimental Procedures**

**Materials**—All chemicals were reagent grade and were purchased from Merck AG (Darmstadt, Germany). N-ethylmaleimide, diethylstilbestrol, and DCCD1 were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). [14C]DCCD was from NEN, Dreieich, Germany, and methionine was purchased from Hartmann Analytik, Braunschweig, Germany.

**Organisms**—M. jannaschii (DSMZ 2661) was obtained from the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" (DSMZ, Braunschweig, Germany). Cells were grown in 2-liter serum bottles and pressurized with H2/CO2 (80:20) to 0.3 MPa and incubated at 37 °C in the medium described (10) except that 3 g/liter NaHCO3, 1.8 g/liter KH2PO4, 3 g/liter KH2PO4, 40 mM NaHSO3, 5 mM MgCl2, pH 8) was added. Sodium ions did not stimulate ATPase activity. The membrane-bound ATPase was inhibited by the sulphydryl-reactive compound N-ethylmaleimide (I50, 3.4 μmol/mg protein). Low concentrations (0.1 mM) of the Fe3+-directed inhibitor diethylstilbestrol had a stimulating effect, but higher concentrations decreased the ATPase activity (I50, 2.8 μmol/mg protein) with 100% inhibition at 10 mM. DCCD was the most effective inhibitor tested, and half-maximal and maximal inhibition were obtained at 1.1 and 8.7 μmol/mg protein, respectively, corresponding to 0.5 and 4 mM DCCD. To determine the apparent molecular mass of the proteolipid and the potential cleavage products, membranes of M. jannaschii were labeled with [14C]DCCD, which is known to bind covalently to the proton-translocating carboxylate and the carboxylate-substituting glutamine residue in hairpin one are indicated. The open bars represent potential transmembrane helices. Sequence data are from Ref. 9.

**Preparation of Membranes and Labeling with [14C]DCCD**—M. jannaschii cells (2 mg protein/ml) were lysed by osmotic shock during incubation for 5 min at 37 °C in 25 mM TES buffer, pH 6.8, containing DNase. After cell debris was removed by centrifugation (10,000 × g, 20 min, 4 °C), the membranes were pelleted by ultracentrifugation at 100,000 × g for 60 min at 4 °C. The membranes were resuspended in 100 mM HEPES, 5 mM MgCl2, 10% glycerol (v/v), pH 7, to a protein concentration of 0.02–0.1 mg/ml. These membranes were used for labeling as well as activity measurements.

To solubilize the proteolipid, 0.5% CHAPS was added, and the suspension (20 mg protein/ml) was incubated with 0.5% CHAPS at 50 °C for 45 min, 4 °C. After centrifugation (100,000 × g, 60 min, 4 °C), [14C]DCCD (54 mCi/mmol) was added to the supernatant to a final concentration of 0.36 mM. This solution was incubated at 4 °C for 24 h. The sample was analyzed by SDS-PAGE and by autoradiography as described (12, 13). T denotes the total percentage concentration of acrylamide and bisacrylamide, and C denotes the percentage of the cross-linker relative to the total concentration T (14).

**Determination of ATPase Activity**—To determine ATPase activity, 10–20 μl of the membrane suspension was added to 975 μl of ATPase buffer (100 mM MES, 100 mM Tris, 50 mM NaHSO3, 5 mM MgCl2, pH 8). After preincubation at 37 °C for 5 min, the incubation temperature was increased to 80 °C, and the reaction was started by adding Na2ATP (final concentration, 2.5 mM). Samples were taken at 0, 2, 4, and 6 min. The reactions were stopped by the addition of 40 μl of trichloroacetic acid. Activity was measured as the release of inorganic phosphate as described (15). Membranes were preincubated with inhibitors at room temperature for 30 min. Inhibitors were dissolved in water or ethanol. Controls contained the solvent only.

**Purification of the Proteolipid**—Extraction of membranes by chloroform/methanol was performed as described (16) except that the organic phase after incubation with water was washed with 0.5 volumes of chloroform/methanol/water (3:47:48). The extract was subjected to gel filtration on a Sephadex LH-60 (Amersham Pharmacia Biotech). For N-terminal sequencing, the proteins were blotted on a BioTraceTM polyvinylidene difluoride membrane (pore size 0.45 μm, PALLGelman-Sciences, Rossdorf, Germany) according to (17). The protein bands were excised from the membrane and sequenced with a model 473A sequencer from Applied Biosystems using a faster version of the standard cycle.

**MALDI-TOF MS Analysis**—1 μl of the fraction obtained after Sephadex LH-60 chromatography (1 μg protein/μl) was mixed with 1 μl of a saturated solution of sinapinic acid in acetonitrile/0.1% trifluoroacetic acid (1:1, v/v). 0.5 μl of this solution was applied to the target surface and dried. The measurements were performed with a VOYAGER-MALDI-TOF (PerSeptive Biosystems, Wiesbaden, Germany). The sample was ionized with a nitrogen laser (337 nm, 3-ns pulse length).

**Results**

**ATPase Activity and Inhibitor Sensitivity**—ATPase activity was catalyzed by washed membranes of M. jannaschii at maximal activity at 80 °C and pH 8 (146 milliunits/mg protein). The temperature optimum reflects the optimum for growth (85 °C), whereas the pH optimum was more alkaline than that observed for growth (10). Sodium ions did not stimulate ATPase activity. The membrane-bound ATPase was inhibited by the sulphydryl-reactive compound N-ethylmaleimide (I50, 3.4 μmol/mg protein). Low concentrations (0.1 mM) of the Fe3+-directed inhibitor diethylstilbestrol had a stimulating effect, but higher concentrations decreased the ATPase activity (I50, 2.8 μmol/mg protein) with 100% inhibition at 10 mM. DCCD was the most effective inhibitor tested, and half-maximal and maximal inhibition were obtained at 1.1 and 8.7 μmol/mg protein, respectively, corresponding to 0.5 and 4 mM DCCD. To determine the apparent molecular mass of the proteolipid and the potential cleavage products, membranes of M. jannaschii were labeled with [14C]DCCD, which is known to bind covalently to the proton-translocating carboxylate group of the proteolipid, subjected to 12.5% (v/v) SDS-PAGE and autoradiography. As seen in Fig. 2, only one polypeptide was labeled; however, the apparent molecular mass of 15 kDa is considerably smaller than that predicted from the DNA sequence of the proteolipid-encoding gene.

**Purification and Characterization of the Proteolipid**—To analyze the mature proteolipid, it was purified from membranes of M. jannaschii. Because the proteolipid is very hydrophobic and therefore soluble in organic solvents, the membranes were extracted with chloroform/methanol. This purification led to the isolation of only two proteins (Fig. 2), of which the N

---

1 The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Δψp, phosphorylation potential; Δψ, proton motive force or electrochemical proton potential; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxy- phenylethyl)amino]ethanesulfonic acid.
termi were determined. The N terminus of the high molecular mass protein (MDIVSAIVPLIEMT) is identical with MtrD, a subunit of the N\(^2\)-methyltetrahydrodihydropterin:coenzyme M methyltransferase, which is a primary sodium ion pump (18–21). The higher molecular mass proteins are aggregates of MtrD, as revealed by N-terminal sequencing. The lower molecular mass protein was identified as the proteolipid. The sequences obtained (MVDPLLIGAVGLA and VDPLLIGAVGAGLA) showed that the N-terminal methionine has been excised from a fraction of the proteolipids. These N-terminal sequences revealed that translation initiates 103 bp downstream of atp1 (cf. Fig. 1). The open reading frame encodes a protein of a deduced molecular mass of 21,318 Da. Different concentrations of acrylamide/bisacrylamide were used to analyze the apparent molecular mass in SDS-PAGE. At 12.5% and 3% C the proteolipid migrated as a 15-kDa protein (Fig. 2), as seen before, but at 16.5% T, 6% C, the apparent molecular mass was 19 kDa, which is close to the predicted value. The same dependerentiation of the migration behavior on the acrylamide concentration was observed with MtrD.

To determine the apparent molecular mass accurately, the peptides were submitted to MALDI-TOF MS analysis. The removal of lipids from the sample, which is a prerequisite of this analysis led to the generation of a high molecular mass aggregate of MtrD, which did not leave the MALDI matrix. In the MALDI-TOF MS analysis, two forms of the proteolipid with molecular weights of 21,316 and 21,183 were determined (Fig. 3). This finding is in good correlation with the prediction (21,318 and 21,187 for the methionine-free form); the small deviation of the determination from the predicted molecular masses is due to matrix effects. The MALDI-TOF MS analysis is final proof that the proteolipid from \textit{M. jannaschii} is a triplication of the 8-kDa proteolipid found in other archaea.

**FIG. 2.** SDS-PAGE and autoradiography of \(^{14}C\)DCCD-labeled membranes (A) and SDS-PAGE of proteins isolated by chloroform/methanol extraction of membranes (B and C) from \textit{M. jannaschii}. The molecular mass is given in kDa. Arrows with triangular heads correspond to the low molecular weight from Sigma; arrows with barbed heads correspond to the low molecular weight kit from Amersham Pharmacia Biotech. For details of the labeling experiment (A), see “Experimental Procedures.” Acrylamide concentrations used were as follows: A and B, 12.5% T, 3% C; C, 16.5% T, 6% C.

**FIG. 3.** MALDI-TOF MS analysis of the proteolipid from \textit{M. jannaschii}. The proteolipid was purified from \textit{M. jannaschii} membranes and submitted to gel filtration and MALDI-TOF MS analysis as described under “Experimental Procedures.”

is triplicated, as it is evident from the MALDI-TOF MS analysis of the purified protein. Hydropathy analysis suggests that AtpK consists of three hairpins with two transmembrane helices connected by polar loops. Although the hairpins are very similar to each other (AtpK1:AtpK2, 58% identity; AtpK2: AtpK3, 57% identity; AtpK1:AtpK3, 46% identity), the proton-translocating carboxyl group is only conserved in hairpins two and three but not in hairpin one.

The ATPases are rotary enzymes, and it is suggested that the proteolipid oligomer is organized in a ring-like structure (22, 23). Ion flow across the membrane is assumed to be coupled to rotation of the proteolipid ring against a stator, most probably subunit I in A\(_1\)A\(_0\) ATPases. This rotation is transmitted via a shaft to the hydrophilic domain. The lack of the proton-translocating carboxyl group as observed in the \textit{M. jannaschii} ATPase, and V\(_1\)V\(_0\) ATPases is important in the context of the function of the ATPases. Our experiments gave clear evidence that it is not the size of the proteolipid but the capability to synthesize ATP that distinguishes V\(_1\)V\(_0\) and A\(_1\)A\(_0\) ATPases. The capability to synthesize ATP is directly dependent on the number of protons translocated per ATP synthesized. According to \(\Delta G_p = -nF\Delta \varphi\), a phosphorylation potential \(\Delta G_p\) of \(-50\) to \(-70\) kJ/mol is sustained by the use of \(n = 3-4\) H\(^+\)/ATP at physiological electrochemical proton potentials of \(-180\) mV \(\Delta \varphi\). However, if the number of protons is lower, then ATP can no longer be synthesized. It is assumed that the ring-like proteolipid oligomer contains 24 transmembrane helices (23, 24). In the case of the bacterial and archaical 8-kDa proteolipids with two transmembrane helices, 12 monomers and 12 proton-translocating carboxyl groups are present per oligomer. Taking into account three ATP-synthesizing or hydrolyzing centers, this gives a stoichiometry of 4 H\(^+\)/ATP. In contrast, six copies of the 16-kDa proteolipid with four transmembrane helices are required to form the proteolipid oligomer of V\(_1\)V\(_0\) ATPases (25). Because the proton-translocating group is lost in the first pair of transmembrane helices, the stoichiometry is only 2 H\(^+\)/ATP, which is too low to allow ATP synthesis. In \textit{M. jannaschii}, the proton-translocating group is substituted by a glutamine residue in hairpin one (verified repeatedly by cloning and sequencing of the gene in our laboratory), which results in a H\(^+\)/ATP stoichiometry of 2.7. This stoichiometry is apparently sufficient for ATP synthesis because the enzyme from \textit{M. jannaschii} is clearly an ATP synthase (see above). In this context, it would be interesting to determine the threshold values for ATP synthesis in \textit{M. jannaschii}. On the other hand, it is conceivable that

**DISCUSSION**

The data presented here demonstrate that the proteolipid-encoding gene of \textit{M. jannaschii} is indeed approximately 3 times the size of all other archaical proteolipid-encoding genes known so far. The gene can be divided into three parts, \textit{atpK} (bp 1–240), \textit{atpK2} (bp 241–423), and \textit{atpK3} (bp 424–660), which are very similar to each other (\textit{atpK1}:\textit{atpK2}, 65% identity; \textit{atpK2}:\textit{atpK3}, 68% identity; \textit{atpK1}::\textit{atpK3}, 61% identity). This is clear evidence that \textit{atpK} arose by triplication and fusion of an ancestral gene. Not only the gene but also the mature product
not four but six copies are present in the oligomer with, for example, the first hairpin oriented into the center of the ring. In this way, a $H^+/ATP$ stoichiometry of four could be achieved. In any case, the proteolipid from *M. jannaschii* is a rather unique polypeptide offering new insights into the structure and function of $A_1A_0$ ATPases.

REFERENCES

1. Nelson, N., and Taiz, L. (1989) *Trends Biochem. Sci.* 14, 113–116
2. Gogarten, J. P., and Taiz, L. (1992) *Photosynth. Res.* 33, 137–146
3. Wilms, R., Freiberg, C., Wegerle, E., Meier, I., Mayer, F., and Müller, V. (1996) *J. Biol. Chem.* 271, 18843–18852
4. Ruppert, C., Wimmers, S., Lemker, T., and Müller, V. (1998) *J. Bacteriol.* 180, 3448–3452
5. Müller, V., Ruppert, C., and Lemker, T. (1999) *J. Bioenerg. Biomembr.* 31, 15–27
6. Müller, V., Blaut, M., and Gottschalk, G. (1993) in *Methanogenesis* (Ferry, J. G., ed) pp. 360–406, Chapman and Hall, New York
7. Deppenmeier, U., Müller, V., and Gottschalk, G. (1996) *Arch. Microbiol.* 165, 149–163
8. Becher, B., and Muller, V. (1994) *J. Bacteriol.* 176, 2543–2550
9. Bult, C. J., White, O., Olsen, G. J., Zhou, L. X., Fleischmann, R. D., Sutton, G. G., Blake, J. A., Fitzgerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Gough, N. S. M., Weidman, J. F., Fuhrmann, J. L., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P. W., Hanna, M. C., Cotton, M. D., Roberts, K. M., Hurst, M. A., Kaine, B. P., Borodovsky, M., Klenk, H.-P., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. G. (1996) *Science* 273, 1058–1073
10. Jones, W. J., Leigh, J. A., Mayer, F., Woese, C. R., and Wolfe, R. S. (1983) *Arch. Microbiol.* 136, 254–261
11. Balch, W. E., and Wolfe, R. S. (1976) *Appl. Environ. Microbiol.* 32, 781–791
12. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 369–379
13. Chamberlain, J. P. (1979) *Anal. Biochem.* 98, 132–135
14. Hertig, S. (1962) *Arch. Biochem. Biophys.* 99, 466–467
15. Heinonen, J. E., and Lahti, R. J. (1991) *Anal. Biochem.* 113, 313–317
16. Beechey, R. B., Linnett, P. E., and Fillingame, R. H. (1979) *Methods Enzymol.* 55, 426–434
17. Gallagher, S., Winston, S. E., Fuller, S. A., and Hurrell, J. G. R. (1993) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Ch. 10.8.1–10.8.21, Wiley and Sons, Inc., New York
18. Müller, V., Winner, C., and Gottschalk, G. (1988) *Eur. J. Biochem.* 178, 519–525
19. Becher, B., Müller, V., and Gottschalk, G. (1992) *FEMS Microbiol. Lett.* 91, 239–244
20. Becher, B., Müller, V., and Gottschalk, G. (1992) *J. Bacteriol.* 174, 7656–7660
21. Lienard, T., and Gottschalk, G. (1998) *FEBS Lett.* 425, 204–208
22. Junge, W., Lill, H., and Engelbrecht, S. (1997) *Trends Biochem. Sci.* 22, 420–423
23. Fillingame, R. H., Jones, P. C., Jiang, W., Valiyaveetil, F. I., and Dmitriev, O. Y. (1998) *Biochim. Biophys. Acta* 1365, 155–142
24. Jones, P. C., and Fillingame, R. H. (1998) *J. Biol. Chem.* 273, 29701–29705
25. Arai, H., Torres, G., Pina, S., and Forgac, M. (1988) *J. Biol. Chem.* 263, 8796–8802