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

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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 (F0/V0/A0), which synthesizes and/or hydrolizes ATP, and the hydrophobic domain (F1/V1/A1), 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 hydrolase 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 inferred molecularly and biochemically (to V. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Holger W. Jannasch, a pioneer of deep sea microbiology.

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**Experimental Procedures**

**Materials**—All chemicals were reagent grade and were purchased from Merck AG (Darmstadt, Germany). N-ethylmaleimide, diethylstilbestrol, and DCCD were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). [14C]DCCD was from NEN, Dreieich, Germany, and [15N]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 with 600 ml of medium as described (10) except that 1 g of yeast extract/liter was added. Strict anaerobic techniques (11) were applied.

**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 at 37 °C for 30 min. 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 added at 4 °C for 24 h. The sample was analyzed by SDS-PAGE and 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, 40 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 as catalyzed by washed membranes of M. jannaschii was maximal 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 sulfhydryl-reactive compound N-ethylmaleimide (I₅₀, 3.4 µmol/mg protein). Low concentrations (0.1 mM) of the F₃ₕ-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 carboxyl group of the proteolipid, subjected to 12.5% (w/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 polypeptides (Fig. 2), of which the N
termini were determined. The N terminus of the high molecular mass protein (MDIVSAIVPLIEMT) is identical with MtrD, a subunit of the $N^5$-methyltetrahydrofolate: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 (MVDPLILGAVGAGLA and VDPLILGAVGAGLA) 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 atpI (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% T, 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 dependence 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 \(M. \text{jannaschii}\) is a triplication of the 8-kDa proteolipid found in other archaea.

**DISCUSSION**

The data presented here demonstrate that the proteolipid-encoding gene of \(M. \text{jannaschii}\) is indeed approximately 3 times the size of all other archaeal proteolipid-encoding genes known so far. The gene can be divided into three parts, \(atpK1\) (bp 1–240), \(atpK2\) (bp 241–423), and \(atpK3\) (bp 424–660), which are very similar to each other (\(atpK1:atpK2, 65\%\) identity; \(atpK2:atpK3, 68\%\) identity; \(atpK1:atpK3, 61\%\) identity). This is clear evidence that \(atpK\) arose by triplication and fusion of an ancestral gene. Not only the gene but also the mature product 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\(_n\), ATPases. This rotation is transmitted via a shaft to the hydrophilic domain. The lack of the proton-translocating carboxyl group as observed in the \(M. \text{jannaschii}\) ATPase, and \(V_1V_0\) 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 \(V_1V_0\) and \(A_1A_0\) ATPases. The capability to synthesize ATP is directly dependent on the number of protons translocated per ATP synthesized. According to \(\Delta G_p = -nF\Delta \phi\), 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 (\(\Delta \phi\)). 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 archaeal 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 \(V_1V_0\) 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 \(M. \text{jannaschii}\), 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 \(M. \text{jannaschii}\) is clearly an ATP synthase (see above). In this context, it would be interesting to determine the threshold values for ATP synthesis in \(M. \text{jannaschii}\). On the other hand, it is conceivable that
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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 A₁A₀ ATPases.

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