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Isolation of a complete A_1A_O ATP synthase comprising nine subunits from the hyperthermophile *Methanococcus jannaschii*

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Abstract Archaeal A_1A_O ATP synthase/ATPase operons are highly conserved among species and comprise at least nine genes encoding structural proteins. However, all A_1A_O ATPase preparations reported to date contained only three to six subunits and, therefore, the study of this unique class of secondary energy converters is still in its infancy. To improve the quality of A_1A_O ATPase preparations, we chose the hyperthermophilic, methanogenic archaeon *Methanococcus jannaschii* as a model organism. Individual subunits of the A_1A_O ATPase from *M. jannaschii* were produced in *E. coli*, purified, and antibodies were raised. The antibodies enabled the development of a protocol ensuring purification of the entire nine-subunit A_1A_O ATPase. The ATPase was solubilized from membranes of *M. jannaschii* by Triton X-100 and purified to apparent homogeneity by sucrose density gradient centrifugation, ion exchange chromatography, and gel filtration. Electron micrographs revealed the A_1 and A_O domains and the central stalk, but also additional masses which could represent a second stalk. Inhibitor studies were used to demonstrate that the A_1 and A_O domains are functionally coupled. This is the first description of an A_1A_O ATPase preparation in which the two domains (A_1 and A_O) are fully conserved and functionally coupled.

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Introduction

Membrane-bound, multisubunit, ion-translocating ATP synthases/ATPases are present in every domain of life and arose from a common ancestor (Gogarten and Taiz 1992). The overall structure of these enzymes is well conserved and consists of two domains connected by (at least) two stalks (Boekema et al. 1997; Böttcher and Gräber 2000; Wilkens and Capaldi 1998). The hydrophilic, cytoplasmic domain catalyzes ATP hydrolysis (Forgac 2000; Futai et al. 1995; Schäfer et al. 1999; Senior et al. 2000), while the membrane-bound domain translocates ions, Na^+ or H^+, against their electrochemical gradient (Dimroth 1997; Fillingame et al. 2000; Müller et al. 2001). The ATPase in bacteria, mitochondria, and chloroplasts is of the F_1F_O type; its subunit composition is different in different organisms or organelles but the minimal subunit composition usually found in bacteria is $\alpha_3\beta_3\gamma\delta\epsilon abc_{9-12}$ (Fillingame 2000). A large proportion of the structure of the F_1F_O ATPase has been elucidated (Abrahams et al. 1994; Menz et al. 2001; Stock et al. 2000). F_1F_O ATPases are fully reversible machines and their typical cellular function is to synthesize ATP by means of the electrochemical ion gradient across the cytoplasmic membrane. The V_1V_O ATPase is found in eukaryotes. Its subunit composition also depends on the organism or organelle but the minimal subunit composition as found in yeast is $A_3B_3CDEFGHac_4c'_1c''_1d$ (Forgac 2000). The structure of the V_1V_O ATPase, and the functions and localizations of their subunits within the enzyme complex are less clear. In vivo, the V_1V_O ATPase does not catalyze ATP synthesis, its cellular function is to generate steep ion gradients by means of ATP hydrolysis. Another difference F_1F_O ATPases is the apparent inability of the isolated V_1 domain to catalyze ATP hydrolysis (Forgac 1999; Kane 1995).

It has long been known that members of the third domain of life, *Archaea*, synthesize ATP by means of ion-gradient-driven phosphorylation, and membrane-bound ATP synthases have been demonstrated in membranes of methanogens (Becher and Müller 1994; Mountfort 1978), halobacteria (Michel and Oesterhelt 1980) and thermoacidophiles (Lübben and Schäfer 1989). Enzymes have been purified and characterized (Chien et al. 1993; Hochstein et al. 1987; Ihara et al. 1997; Inatomi 1986; Inatomi et al. 1993; Lübben et al. 1987; Scheel and Schäfer 1990; Steinert and Bickel-Sandkötter 1996; Wilms et al. 1996). Interestingly, the primary sequences of their major subunits A and B were clearly shown to be more related to V_1V_O than to F_1F_O (Müller et al. 1999). Furthermore, the genomic sequences available today show that the overall subunit composition of the A_1A_O ATPase is very similar to the V_1V_O ATPase. For example, like the V_1V_O ATPase, the A_1A_O ATPase contains only two membrane-bound subunits. Like V_1V_O ATPases, archaeal A_1A_O ATPases may contain duplicated and even triplicated proteolipids (Müller et al. 1999; Ruppert et al. 1999, 2001). Therefore, the A_1A_O ATPase can formally be regarded as a chimeric enzyme combining functional features of F_1F_O such as its cellular function as ATP synthase with structural features of the V_1V_O ATPase (Mukohata and Ihara 1990; Müller et al. 1999; Schäfer and Meyering-Vos 1992).

Conventional cloning approaches as well as genome sequencing projects have identified at least nine structural A_1A_O ATPase genes in every archaeon analyzed so far (Fig. 1). The genes *atpI* and *atpK* code for subunit I and subunit K (the proteolipid), while the genes *atpHECFABD* code for hydrophilic proteins. In contrast to the high degree of conservation on the genetic level, the number of polypeptides present in A_1A_O ATPase preparations reported in the literature was lower than expected from the genetic data and ranged from three to six (Chien et al. 1993; Hochstein et al. 1987; Ihara et al. 1997; Inatomi 1986; Inatomi et al. 1993; Lübben et al. 1987; Scheel and Schäfer 1990; Steinert and Bickel-Sandkötter 1996; Wilms et al. 1996). Only the two major subunits A and B and the proteolipid were assigned, the other polypeptides of these preparations were neither identified nor assigned to specific functions. In addition, subunit I (the homologue of subunit *a* of V_1V_O ATPases), which should be essential for ion translocation, was never found in any A_1A_O ATPase preparation. Therefore, the A_1A_O ATPase preparations reported to date lack subunits of the hydrophilic and hydrophobic domains and are thus far from being complete. In particular, a complete membrane domain is a prerequisite for identifying the coupling ion in A_1A_O ATPases, one of the still unresolved questions for methanogenic archaea which generate both primary Na^+ and H^+ gradients across their cytoplasmic membrane during methane formation (Deppenmeier et al. 1996; Müller et al. 1999). Because it is widely assumed that multienzyme complexes from hyperthermophiles are much more stable than those from mesophiles, we investigated whether the A_1A_O ATPase from the completely sequenced, hyperthermophilic archaeon *Methanococcus jannaschii* can be solubilized and purified without loss of subunits. *M. jannaschii* is of particular interest because it is the only organism known so far to have a proteolipid three times the size of that of most bacteria and archaea and, in addition, this proteolipid has lost one of the proton-translocating residues (Ruppert et al. 1999). To monitor solubilization and stability of the membrane domain, the encoding genes were amplified, fused to *malE*, the fusions were purified and antibodies were raised against the heterologously produced proteins. We report here a solubilization and purification procedure yielding an apparently homogeneous A_1A_O ATPase

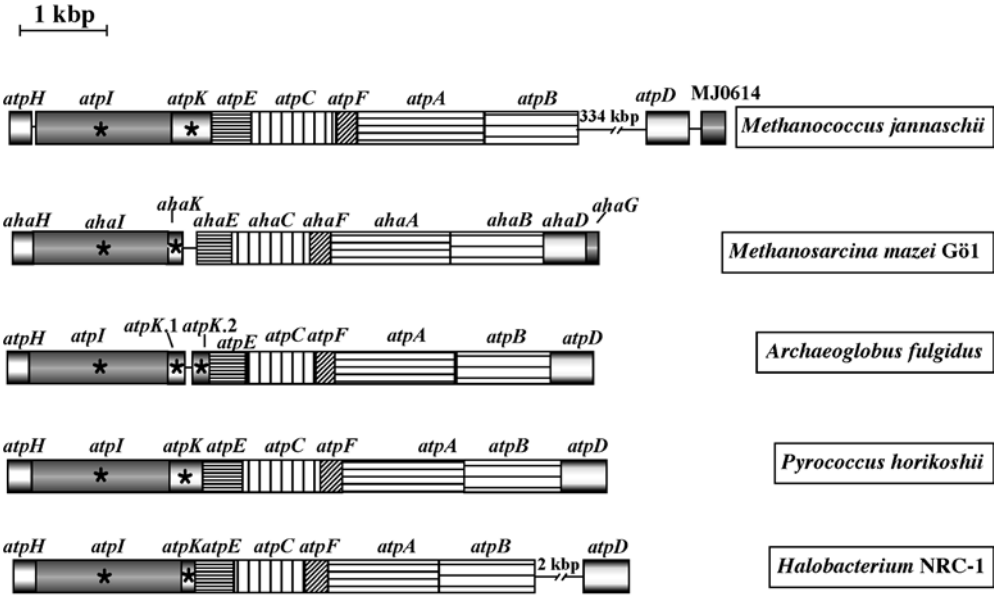

Fig. 1 Genetic organization of the ATPase genes of *Methanococcus jannaschii*, *Methanosarcina mazei* Gö1, *Archaeoglobus fulgidus*, *Pyrococcus horikoshii*, and *Halobacterium NRC-1*. Note that *atpD* is separated from *atpB* by 334 kbp in *M. jannaschii* and 2 kbp in *Halobacterium* NRC-1. Homologous genes are indicated by identical patterns. It is not clear whether *ahaG* is an authentic gene/ATPase gene. MJ0614 could be the homologue of *ahaG* of *M. mazei*, *M. barkeri*, and *M. acetivorans* based on gene arrangements, but the homology is too low for an unequivocal assignment. AhaG homologues were not found in the other archaea

preparation containing nine of the nine subunits deduced from the DNA sequence. The gene–polypeptide correspondence was established by comparing experimentally derived N-terminal sequences of the subunits with sequences deduced from the DNA sequences. Electron microscopy revealed a typical two-domain structure, and inhibitor studies revealed that the A_1 and A_O domains are functionally coupled.

Materials and methods

Materials

All chemicals were reagent grade and were purchased from Merck AG (Darmstadt, Germany). N′,N′-dicyclohexylcarbodiimide (DCCD) and Triton X-100 were from Sigma Chemical (Deisenhofen, Germany).

Organism

M. jannaschii (DSM 2661) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany. For purification of the ATPase, *M. jannaschii* was grown in a 300-l fermentor at 85°C in the medium described at pH 6.0 (Jones et al. 1983) except that 3 g/l $NaHCO_3$, 18 g/l NaCl, 0.5 g/l Na_2S but no cysteine-HCl was added. The fermentor was pressurized to 0.3 MPa with H_2/CO_2 (80:20). The gas flowthrough was adjusted to 1–7 l/min, depending on the growth phase. At an optical density at 600 nm (OD_{600}) of 0.6, the cells were harvested by centrifugation (10,000 *g*; 20 min; 4°C) in a Sorvall Superspeed RC2-B. The pellets were stored at −80°C.

Purification of the A_1A_O ATPase

M. jannaschii cells (36–40 g) were lysed by osmotic shock and homogenized in buffer containing 25 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and DNase. After cell debris had been removed by centrifugation (11,000 *g*; 30 min; 4°C), the membranes were pelleted by ultracentrifugation (100,000 *g*; 2 h; 4°C). The membranes were washed in 100 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 10% glycerol (v/v). The protein concentration was determined as described by Lowry et al. (1951). Membrane proteins were solubilized with Triton X-100 (1 g/g membrane protein) for 30 min at 35°C. After ultracentrifugation (100,000 *g*; 100 min), the supernatant was applied to a 20%–66% sucrose gradient, and centrifuged for 20 h in a vertical rotor (153,000 *g*; 4°C). Samples containing the highest ATPase activity were pooled and applied to DEAE-sepharose, equilibrated with 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10% glycerol (v/v), 0.1% Triton X-100. Elution was performed with a salt gradient (0–1 M NaCl in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10% glycerol (v/v), 0.1% Triton X-100), fractions with ATPase-activity were pooled and applied to a BioPrepSE1000/17 column, equilibrated with 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 10% glycerol (v/v), 0.1% Triton X-100. All steps were performed at 4°C.

ATPase activity

ATPase activity was measured in an assay mixture containing 100 mM MES [2-(morpholino)ethanesulfonic acid], 100 mM Tris (pH 6.0), 40 mM $NaHSO_3$, 5 mM $MgCl_2$, and enzyme solution. After preincubation for 3 min at room temperature followed by 3 min at 80°C the reaction was started by the addition of Na_2-ATP to a final concentration of 2.5 mM. Activity was measured by the release of inorganic phosphate as described by Heinonen and Lahti (1981). DCCD was dissolved in ethanol and preincubated with the enzyme for at least 20 min at room temperature; controls received the solvent only.

Generation of antibodies and Western blotting

For the expression studies, the transmembrane helices two and three or four and five of *atpK*, and the hydrophilic part of *atpI* were amplified by PCR by introducing restriction sites at the 5′end and the 3′end (primer: OatpKTM23.5′(*Bam*HI): 5′-GGTGCA-*GGATCC*ACAGGAGCA-3′; OatpKTM23.3′(*Pst*I): 5′-GGAAG-G*CTGCAG*AAAACTATTGC-3′; OatpKTM45.5′ (*Bam*HI): 5′-GGTCAG*GGATCC*GCTGCTTC-3′; OatpKTM45.3′(*Pst*I): 5′-GCCAAAA*CTGCAG*CCCT ACCC-3′; atpI.5′(*Nde*I): 5′-GA-GACCCGTA*CATATG*AAGTTA-3′; atpIcD.3′(*Bam*HI): 5′-GAG CAT*GGATCC*CTGT GAGCTACTC-3′). PCR fragments were cloned into pMalc2stop, and transformed in *E. coli* BL21-CodonPlus(DE3)-RIL. Cultures were grown in LB at 37°C, and expression was induced at an OD_{600} of 0.5 by the addition of IPTG (isopropyl-β-D-thiogalactoside) to a final concentration of 0.3 mM. After 2 h of growth, cells were harvested, washed, and disrupted in a French press. Cell debris was removed by centrifugation and the supernatant was applied to an amylose resin to purify the fusion protein. Since there is no MalE in *M. jannaschii* and since a MalE antibody does not crossreact with cell-free extract of *M. jannaschii*, the entire fusion protein was used to immunize rabbits.

Western blotting with SDS polyacrylamide gels was performed as described by Towbin et al. (1979). The nitrocellulose sheets were applied to different antisera and treated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins in a reaction mixture made up of 0.0075% (w/v) Nitro Blue tetrazolium chloride and 0.03% (w/v) 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$, pH 8.8.

Electron microscopy

Samples containing isolated A_1A_O ATPase were negatively stained with 4% (w/v) aqueous uranyl acetate, pH 4.7, as described by Valentine and Chignell (1968), and depicted at calibrated magnifications by conventional transmission microscopy.

N-terminal amino acid sequencing

The proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The proteins were excised and subjected to Edman degradation on a Beckman Proton 3600 sequencer. Identification of the amino acids was performed with Beckman Microbe-HPLC System Gold (Gallagher et al. 1993).

Results

Overproduction of subunits I and K and generation of polyclonal antisera

To monitor the solubilization of the membrane, intrinsic A_O subunits K (synonymous with subunit *c* or proteolipid) and I antibodies were generated. Therefore, the encoding genes were amplified, fused to *malE*, and the constructs were transformed into *E. coli*. Upon induction of gene expression by IPTG, growth of the host cells ceased, but neither a MalE-AtpI nor a MalE-AtpK

fusion was detectable in Coomassie-stained SDS polyacrylamide gels loaded with whole-cell lysates of the transformants. Therefore, parts of the two genes were amplified and fused to *malE*. When the gene fragment encoding the hydrophilic domain of subunit I was fused to *malE* (pHK201), the transformants produced a fusion protein (MalE-AtpI$_{CD}$) of the expected size (data not shown). Subunit K has six predicted transmembrane helices; from the two constructs tested only the transmembrane helices two and three could be produced as MalE fusions (MalE-ATPK$_{TM23}$; data not shown), but the yield was low. The fusion proteins were purified by affinity chromatography on an amylose matrix and used to immunize rabbits. The specificity of the antisera was tested with cell-free extract, cytoplasmic and membrane fractions of *M. jannaschii*. As can be seen in Fig. 2A, the antiserum against the cytoplasmic domain of subunit I reacted with a single protein of an apparent molecular mass of 66 kDa, which corresponds well with the deduced size of subunit I of 72 kDa. This protein was exclusively found in the membrane fraction, underlining the prediction that subunit I is part of the membrane domain. In contrast to subunit I from the mesophilic methanogenic archaeon *Methanosarcina mazei* Gö1, subunit I does not undergo degradation in the time period examined (up to 3 months). The antiserum against subunit K gave one strong signal with a protein of apparent molecular mass 27 kDa (Fig. 2B), which most likely represents a dimer of the proteolipid. Interestingly, the antibody also reacted with a protein of apparent molecular mass 66 kDa, which could represent the K-oligomer (synonymous with *c*-oligomer); SDS-resistant *c*-oligomers have been found previously in the Na^+-F_1F_O-ATPase from *Acetobacterium woodii* (Reidlinger and Müller 1994) and *Propionigenium modestum* (Laubinger and Dimroth 1988). In addition, the monomer (16 kDa) and degradation products of the proteolipid were seen. The immunoblots verified the membrane localization of subunits K and I, and demonstrate that the antisera are suitable for monitoring the fate of the A_O domain during solubilization and purification of the A_1A_O ATPase.

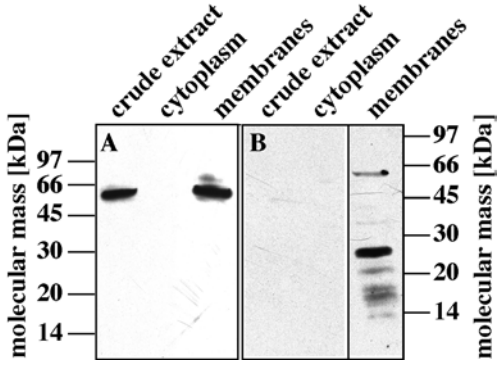

Fig. 2 Cellular localization of subunit I (**A**) and subunit K (**B**). Crude extract (50 μg), the cytoplasm (50 μg) and the cytoplasmic membrane [50 μg (**A**) or 150 μg (**B**)] were applied to SDS-PAGE, transferred to a nitrocellulose membrane and then hybridized against an anti-MalE-AtpI$_{CD}$-antiserum (**A**) or an anti-MalE-AtpK$_{TM23}$-antiserum (**B**)

Solubilization of the A_1A_O ATPase from membranes of *M. jannaschii*

Different detergents were tested under various conditions for their capability to solubilize an ATP-hydrolyzing complex still containing the membrane-bound subunit I. Triton X-100, octylglucosid, or CHAPS (3-3-(cholamidopropyl)dimethylammonio-1-propanesulfonate) were used at a concentration of 1 g/g membrane protein, and incubated with the membranes for 30 min at 35°C. With CHAPS, about 30% of the total activity was solubilized, and 80%–94% of the activity was recovered. With octylglucosid, 32%–46% of the activity was solubilized. Triton X-100 led to a solubilization of 25%–51% of the activity. Higher amounts of detergent, different temperatures or longer solubilization times did not enhance the yield. Subunit I was detected in every solubilisate. For the following experiments we chose Triton X-100 as a detergent for solubilization.

Purification of the A_1A_O ATPase from membranes of *M. jannaschii*

The solubilisate was applied to sucrose density gradient fractionation. The highest ATPase activity was recovered in the last third of the gradient. The enrichment factor was 7.6, and the yield was 21.4%. The ATPase-containing fractions were applied to a DEAE-sepharose column, and eluted with a NaCl gradient from 0 to 1 M. The ATPase eluted at 140–260 mM NaCl as a single peak with a specific activity of 3,420 mU/mg and a yield of 13.8%. Because the preparation still contained contaminating proteins, the ATPase-containing fractions were concentrated by ultrafiltration and subjected to a gel filtration on BioPrep SE1000/17. The ATPase eluted as a single sharp peak with a specific activity of 3,450 mU/mg and an overall yield of 4%. The specific activities of various enzyme preparations ranged from 3.5 to 0.8 U/mg protein. The purification is summarized in Table 1.

Subunit composition and gene polypeptide correspondence

The ATPase preparation contained 11 polypeptides of apparent molecular masses 76, 70, 66, 54, 45, 28, 27, 25, 16, 13, and 12 kDa. The N-termini of the polypeptides were used to identify the gene products. The 76-, 70-, and 66-kDa polypeptides were only resolved by 10% SDS-PAGE (see Fig. 3). The sequence of the 66-kDa polypeptide matches exactly the sequence deduced from *atpI*; furthermore, the polypeptide cross reacts with the

Table 1 Purification of the A_1A_O ATPase of *M. jannaschii*

Purification step	Protein (mg)	ATPase activity (mU)	Specific activity (mU/mg)	Enrichment (fold)	Yield (%)
Membranes	600	127,800	213	1	100
TX-100 supernatant	56	31,500	560	2.6	24.6
Sucrose gradient	17	27,300	1,630	7.6	21.4
DEAE column	5	17,640	3,420	14.8	13.8
Ultrafiltration	2	7,400	3,360	15.7	5.7
Gel filtration	1.5	5,175	3,450	16.2	4

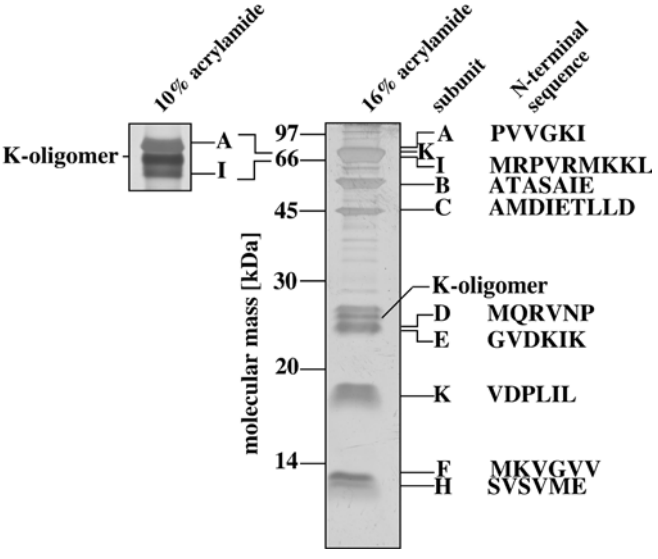

Fig. 3 Subunit composition of the A_1A_O ATPase of *M. jannaschii*. The purified enzyme (100 μg) was subjected to SDS-PAGE on 10% and 16% gels. Proteins were silver-stained, and the N-terminal sequences were obtained by Edman degradation

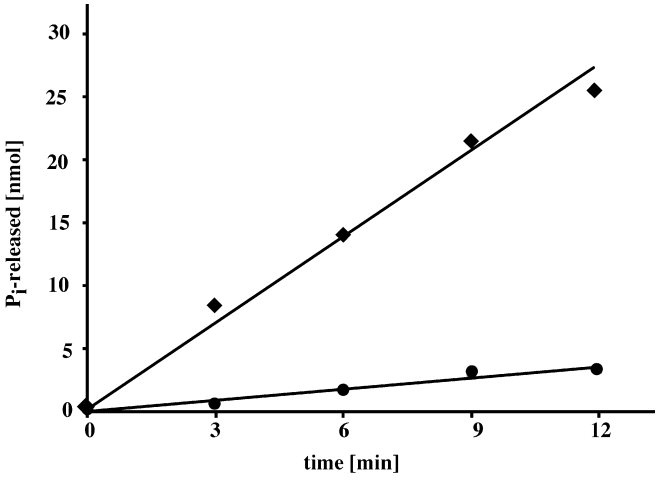

Fig. 4 Kinetics of ATP hydrolysis. After preincubation for 20 min at room temperature and 3 min at 80°C, the reaction was started by addition of Na_2-ATP to a final concentration of 2.5 mM. The buffer contained 100 mM MES, 100 mM Tris, 5 mM $MgCl_2$, and 40 mM $NaHSO_3$, pH 6.0, and 16.5 μg of the purified A_1A_O ATPase of *M. jannaschii* (*diamonds*). For the thermal hydrolysis of ATP (*circles*), no enzyme was added

antiserum against subunit I which is unequivocal evidence that it is subunit I. The 76-, 54-, and 45-kDa proteins were identified as subunits A, B, and C, respectively, by their N-terminal sequences. The 25-kDa protein was identified by the N-terminal sequence as a mixture of subunits D and E. The 16-kDa protein represents the monomeric form of the proteolipid, and the 13- and 12-kDa proteins are subunits F and H, as evident from their N-terminal sequences. These analyses demonstrate that every subunit deduced from the DNA sequence was indeed present in the preparation. However, it should be mentioned that additional polypeptides also appeared in the SDS polyacrylamide gel upon storage of the enzyme. The 70-kDa protein which could only be resolved from subunits A and I in 10% acrylamide reacted with the antibody against the proteolipid, indicating that it represents an SDS-resistant oligomer of the proteolipid; the same is true for the 27-kDa protein (data not shown). SDS-resistant oligomers of proteolipids have been discovered previously in the Na^+-F_1F_O-ATPases from *Acetobacterium woodii* (Reidlinger and Müller 1994), *Ilyobacter tartaricus* (Neumann et al. 1998), and *Propionigenium modestum* (Laubinger and Dimroth 1988). The 28-kDa protein was not identified, but could probably be a degradation/aggregation product of any other subunit. These experiments revealed that the preparation contained all the subunits deduced from the DNA sequence.

Catalytic properties of the purified A_1A_O ATPase

The ATPase hydrolyzed ATP with a constant rate over a period of 12 min (Fig. 4), thereafter the rate declined. Therefore, only initial rates of phosphate release were determined in the following measurements, and the phosphate released from ATP in the absence of enzyme was subtracted from the values used for rate calculations. The ATPase activity was optimal at pH 6.0 (Fig. 5). While pH values lower than 6.0 decreased the activity very strongly, at values higher than 6.0 the decrease was less pronounced (92% activity at pH 7.0). The enzyme was active at temperatures of 60° (21% activity), 70° (28% activity), 90° (89% activity), and 100°C (85% activity); optimal temperature was 80°C. In contrast, when the enzyme was preincubated for 30 min in the absence of ATP at 90° or 100°C, activity was lost completely. Preincubation temperatures ranging from room temperature to 80°C had no effect on ATPase activity (measured at 80°C). Apart from ATP, the

enzyme hydrolyzed GTP (86% activity) and even UTP (54% activity) at rather high rates, but CTP (9% activity) at low rates. This result indicates that the ATPase of *M. jannaschii* is a good GTPase, which was also shown for the *Methanococcus voltae* ATPase (Chen and Konisky 1993). $MnCl_2$ was superior to $MgCl_2$ (109%), but this is also true for other A_1A_O ATPases (Inatomi 1986; Inatomi et al. 1993; Steinert et al. 1997). Zn^{2+} could replace Mg^{2+} to some extent (64%) but Ca^{2+}, Cu^{2+}, Fe^{2+}, and Ni^{2+} were less effective. The K_M value for Mg-ATP was determined to be 1.2 ± 0.2 mM.

Ultrastructure of the A_1A_O ATPase from *M. jannaschii* as revealed by electron microscopy

In electron micrographs the A_1A_O ATPase appeared to be composed of a base, a stalk and a head part (Fig. 6). Often several A_1A_O ATPases were aggregated in opposite orientations, brought about by interaction of their A_O parts (see large arrow in Fig. 6b). The group of arrows in Fig. 6b points to elongated masses, arranged in a similar way to the slices of an orange. These elongated protein masses are interpreted to represent subunits A and B of the heads of the ATPase complexes seen in side-on views. The arrowhead (Fig. 6b) points to a stalk structure which is interpreted to represent the primary stalk connecting the head with the membrane domain. Paired structures, located between the surface of the cytoplasmic membrane and the enzyme head (Fig. 6c), are structurally distinct from the primary stalk. These are interpreted to represent components of the second stalk, which is part of the stator of the rotatory machine. The A_1A_O ATPase has an overall length of ~23 nm, with an A_1 domain of ~11 nm in width and ~10 nm in length. The length of the A_O part is about the width of the A_1 particle and the height is ~6.0 nm. The electron micrographs clearly demonstrate the two-domain structure of the enzyme isolated.

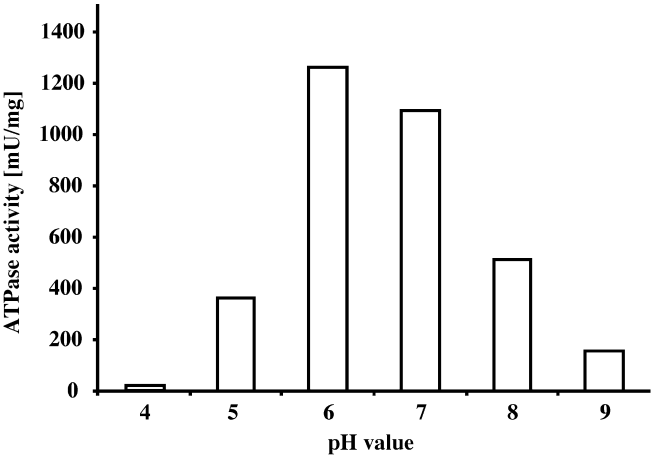

Fig. 5 pH dependence of ATPase activity. After preincubation for 20 min at room temperature and 3 min at 80°C, the reaction was started by addition of Na_2-ATP to a final concentration of 2.5 mM. The buffer contained 100 mM MES, 100 mM Tris, 5 mM $MgCl_2$, and 40 mM $NaHSO_3$, the pH was as indicated in the figure

Inhibition of ATP hydrolysis by DCCD

To test for a functional coupling between the A_1 and A_O domains, DCCD, an inhibitor of the A_1A_O ATPase from methanogens (Becher and Müller 1994), known to block ion flow through the membrane domain, was used. As can be seen from Fig. 7, ATPase activity was inhibited by DCCD. A 50% inhibition was obtained at about 450 μM DCCD. These data demonstrate that most of the A_1 and A_O domains in this preparation are functionally coupled.

Discussion

To open new avenues to the functional and structural analyses of archaeal A_1A_O ATPases, we chose methanogenic archaea as model systems and started two approaches, a molecular and a biochemical. The A_1 ATPase genes from the mesophile *M. mazei* Gö1

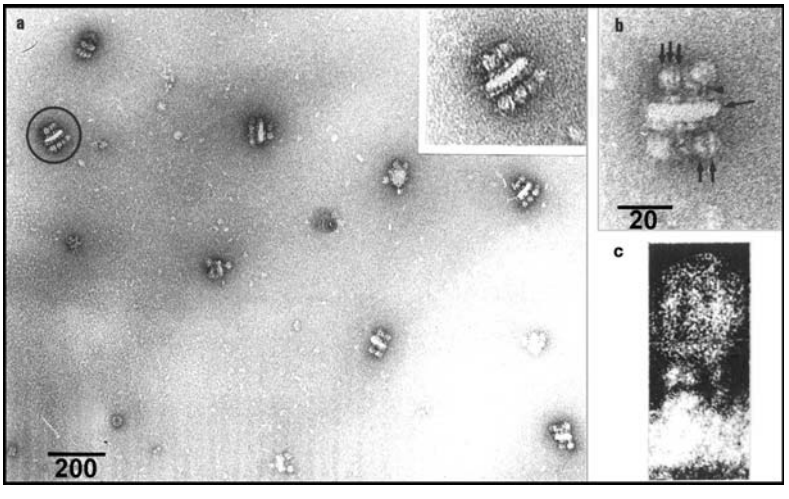

Fig. 6 Electron micrograph of the A_1A_O ATPase of *M. jannaschii*. Dimensions are given in nm. For explanations, see text

could be overexpressed in *E. coli*, and a functional A_1 ATPase subcomplex suitable for biochemical and structural analyses was produced (Coskun et al. 2002; Grüber et al. 2001a, 2001b; Lemker et al. 2001). However, up until now, neither the A_O nor the A_1A_O could be overproduced heterologously. As shown here, single subunits of A_O could not even be produced as MalE fusions; their overproduction led to growth inhibition of the host. It has been shown previously for the proteolipid from *M. mazei* Gö1 that it can be produced in *E. coli* and is targeted to the membrane, but overproduction of the proteolipid apparently has dramatic, growth-inhibiting consequences for the energy status of the host (Ruppert et al. 1998). The same is apparently true for subunit I, which has seven predicted transmembrane spans and which is, most likely, involved in ion transport. Overproduction of the homologue of subunit I of F_1F_O ATPases, subunit *a*, also proved to be difficult (Aris et al. 1985; Vik and Antonio 1994).

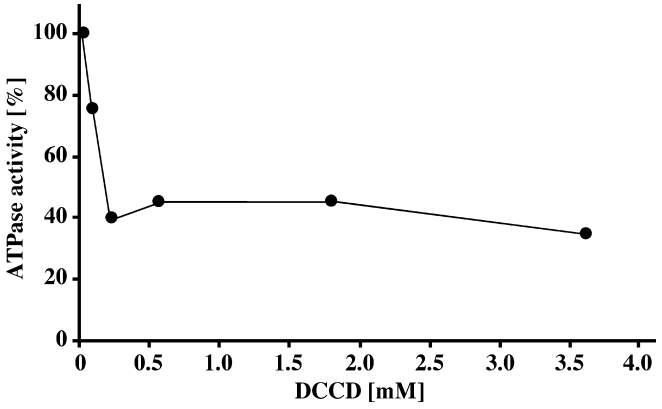

Fig. 7 Inhibition of the A_1A_O ATPase by DCCD. After preincubation with the inhibitor for 20 min at room temperature and 3 min at 80°C, the reaction was started by the addition of Na_2-ATP to a final concentration of 2.5 mM. DCCD was added as ethanolic solution, controls received the solvent only. The protein concentration was 0.3 mg/ml

Attempts to solubilize a complete A_1A_O ATPase from the mesophilic *M. mazei* Gö1 or other archaea have failed so far. Because it is widely assumed that multienzyme complexes from hyperthermophiles are much more stable than those from mesophiles, we investigated whether a complete A_1A_O ATPase could be solubilized from a hyperthermophile, *M. jannaschii*. As demonstrated here, subunit interactions in the A_1A_O ATPase from the hyperthermophile are apparently stronger and can withstand detergent treatment. However, this cannot be generalized, since attempts to solubilize complete A_1A_O ATPases from other thermophiles such as *Sulfolobus acidocaldarius* (Lübben et al. 1987; Lübben and Schäfer 1989) or *Methanothrix thermophila* (Inatomi et al. 1993) have failed.

It is apparent from the electron micrographs that the A_1A_O ATPase consists, like the F_1F_O and the V_1V_O ATPase, of a hydrophilic (A_1) and a hydrophobic domain (A_O) which are connected by two stalks. This two-domain structure has been observed before with membrane-bound enzymes or with the six-subunit-enzyme isolated from *M. mazei* Gö1 (Wilms et al. 1996). Here, with the nine-subunit-enzyme, similar overall dimensions of the enzyme complex were determined, but in addition to the previous six-subunit preparation additional masses can be seen, which are interpreted to represent the second stalk.

The ATPase operon of *M. mazei* Gö1 contains an open reading frame at its 3′ end, named *ahaG*. It is not clear whether this is indeed an authentic gene; a homologue is found in *Methanosarcina barkeri* (http://genome.ornl.gov/microbial/mbar/) and *Methanosarcina acetivorans* (Galagan et al. 2002), but not in the genome of *M. jannaschii*. Since a subunit G was not found in the purified enzyme, its presence seems unlikely, but cannot be excluded with certainty.

From the N-terminal sequences determined, it is now possible to delineate the gene–polypeptide correspondence (Table 2). All except subunit I have an ATG start codon, GTG is used in *atpI*. The N-terminal methionine

Table 2 Gene–polypeptide correspondence of the A_1A_O ATPase of *M. jannaschii*

Subunit										
A	DNA[a]	ATG	CCA	GTT	GGT	AAG	ATT	ATT		
	Protein[b]		P	V	V	G	K	I		
B	DNA	ATG	GCT	ACA	GCA	GCA	TCA	GCA	ATT	
	Protein		A	T	A	A	S	A	I	
C	DNA	ATG	GCG	ATG	GAT	ATQ	GAG	ACA	TTG	TTA
	Protein		A	M	D	I	E	T	L	L
D	DNA	ATG	CAG	AGA	GTG	AAT	CCA			
	Protein	M	Q	R	V	N	P			
E	DNA	ATG	GGA	GTT	GAT	AAG	ATA	AAG		
	Protein		G	V	D	K	I	K		
F	DNA	ATG	AAA	GTT	GGC	GTT	GTT			
	Protein	M	K	V	G	V	V			
H	DNA	ATG	AGC	GTT	AGT	GTT	ATG	GAA		
	Protein		S	V	S	V	M	E		
I	DNA	GTG	AGA	CCC	GTA	AGA	ATG	AAG	AAG	
	Protein	M	R	P	V	R	M	K	K	
K	DNA	ATG	GTA	GAT	CCT	TTA	ATC	TTA		
	Protein		V	D	P	L	I	L		

[a] The DNA sequences given start at base pair 208, 947 (A), 207, 137 (B), 210, 514 (C), 546, 344 (D), 211, 232 (E), 209, 304 (F), 214, 471 (H), 214, 142 (I), and 211, 957 (K) of the *M. jannaschii* genome

[b] The experimentally determined amino-acid sequence is given

was removed from subunits A, B, C, E, H, and K. The annotated start codons of *atpA*, *atpE*, and *atpI* differ from the start codons deduced from the N-terminal sequences. *atpA* starts 21 base pairs downstream of the annotated start codon, the start codon of *atpE* is annotated as GTG but translation apparently starts six nucleotides downstream, and *atpI* starts with a GTG two triplets downstream from the annotated GTG.

In summary, the purification procedure described here enables the isolation of a structurally complete A_1A_O ATPase comprising nine subunits and will allow a thorough analysis of the structure and function of this unique class of enzymes from archaea.

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References

Abrahams JP, Leslie AGW, Lutter R, Walker JE (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. Nature 370:621–628

Aris JP, Klionsky DJ, Simoni RD (1985) The F_O subunits of the *Escherichia coli* F_1F_O-ATP synthase are sufficient to form a functional proton pore. J Biol Chem 260:11207–11215

Becher B, Müller V (1994) $\Delta\tilde{\mu}_{Na}$drives the synthesis of ATP via an $\Delta\tilde{\mu}_{Na^+}$translocating F_1F_O-ATP synthase in membrane vesicles of the archaeon *Methanosarcina mazei* Gö1. J Bacteriol 176:2543–2550

Boekema EJ, Ubbink-Kok T, Lolkema JS, Brisson A, Konings WN (1997) Visualization of a peripheral stalk in V-type ATPase: evidence for the stator structure essential for rotational catalysis. Proc Natl Acad Sci USA 94:14291–14293

Böttcher B, Gräber P (2000) The structure of the H^+-ATP synthase from chloroplasts and its subcomplexes as revealed by electron microscopy. Biochim Biophys Acta 1458:404–416

Chen W, Konisky J (1993) Characterization of a membrane-associated ATPase from *Methanococcus voltae*, a methanogenic member of the Archaea. J Bacteriol 175:5677–5682

Chien LF, Wu JJ, Tzeng CM, Pan RL (1993) ATPase of *Rhodospirillum rubrum* requires three functional copies of *β*-subunit as determined by radiation inactivation analysis. Biochem Mol Biol Int 31:13–18

Coskun Ü, Grüber G, Koch MH, Godovac-Zimmermann J, Lemker T, Müller V (2002) Crosstalk in the A_1-ATPase from *Methanosarcina mazei* Gö1 due to nucleotide-binding. J Biol Chem 277:17327–17333

Deppenmeier U, Müller V, Gottschalk G (1996) Pathways of energy conservation in methanogenic archaea. Arch Microbiol 165:149–163

Dimroth P (1997) Primary sodium ion translocating enzymes. Biochim Biophys Acta 1318:11–51

Fillingame RH (2000) Getting to the bottom of the F_1-ATPase. Nat Struct Biol 7:1002–1004

Fillingame RH, Jiang W, Dmitriev OY (2000) Coupling H^+ transport to rotary catalysis in F-type ATP synthases: structure and organization of the transmembrane rotary motor. J Exp Biol 203:9–17

Forgac M (1999) Structure and properties of the vacuolar H^+-ATPases. J Biol Chem 274:12951–12954

Forgac M (2000) Structure, mechanism and regulation of the clathrin-coated vesicle and yeast vacuolar H^+-ATPases. J Exp Biol 203:71–80

Futai M, Omote H, Maeda M (1995) *Escherichia coli* H^+-ATPase (ATP synthase): catalytic site and roles of subunit interactions in energy coupling. Biochem Soc Trans 23:785–789

Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P, FitzHugh W, Calvo S, Engels R, Smirnov S, Atnoor D, Brown A, Allen N, Naylor J, Stange-Thomann N, DeArellano K, Johnson R, Linton L, McEwan P, McKernan K, Talamas J, Tirrell A, Ye W, Zimmer A, Barber RD, Cann I, Graham DE, Grahame DA, Guss AM, Hedderich R, Ingram-Smith C, Kuettner HC, Krzycki JA, Leigh JA, Li W, Liu J, Mukhopadhyay B, Reeve JN, Smith K, Springer TA, Umayam LA, White O, White RH, Conway de Macario E, Ferry JG, Jarrell KF, Jing H, Macario AJ, Paulsen I, Pritchett M, Sowers KR, Swanson RV, Zinder SH, Lander E, Metcalf WW, Birren B (2002) The genome of *Methanosarcina acetivorans* reveals extensive metabolic and physiological diversity. Genome Res 12:532–542

Gallagher S, Winston SE, Fuller SA, Hurrell JGR (1993). Analysis of proteins In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York

Gogarten JP, Taiz L (1992) Evolution of proton pumping ATPases: rooting the tree of life. Photosynth Res 33:137–146

Grüber G, Svergun DI, Coskun Ü, Lemker T, Koch MH, Schägger H, Müller V (2001a) Structural insights into the A_1 ATPase from the archaeon, *Methanosarcina mazei* Gö1. Biochemistry 40:1890–1896

Grüber G, Wieczorek H, Harvey WR, Müller V (2001b) Structure–function relationships of A-, F- and V-ATPases. J Exp Biol 204:2597–2605

Heinonen JE, Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal Biochem 113:313–317

Hochstein LI, Kristjansson H, Altekar W (1987) The purification and subunit structure of a membrane-bound ATPase from the Archaebacterium *Halobacterium saccharovorum*. Biochem Biophys Res Commun 147:295–300

Ihara K, Watanabe S, Sugimura K, Mukohata Y (1997) Identification of proteolipid from an extremely halophilic archaeon *Halobacterium salinarum* as an N′,N′-dicyclohexyl-carbodiimide binding subunit of ATP synthase. Arch Biochem Biophys 341:267–272

Inatomi KI (1986) Characterization and purification of the membrane-bound ATPase of the archae bacterium *Methanosarcina barkeri*. J Bacteriol 167:837–841

Inatomi KI, Kamagata Y, Nakamura K (1993) Membrane ATPase from the aceticlastic methanogen *Methanothrix thermophila*. J Bacteriol 175:80–84

Jones WJ, Leigh JA, Mayer F, Woese CR, Wolfe RS (1983) *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch Microbiol 136:254–261

Kane PM (1995) Disassembly and reassembly of the yeast vacuolar H^+-ATPase *in vivo*. J Biol Chem 270:17025–17032

Laubinger W, Dimroth P (1988) Characterization of the ATP synthase of *Propionigenium modestum* as a primary sodium pump. Biochemistry 27:7531–7537

Lemker T, Ruppert C, Stöger H, Wimmers S, Müller V (2001) Overproduction of a functional A_1 ATPase from the archaeon *Methanosarcina mazei* Gö1 in *Escherichia coli*. Eur J Biochem 268:3744–3750

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265–275

Lübben M, Schäfer G (1989) Chemiosmotic energy conservation of the thermoacidophile *Sulfolobus acidocaldarius*: oxidative phosphorylation and the presence of an F_O-related N′,N′-dicyclohexylcarbodiimide-binding proteolipid. J Bacteriol 171:6106–6116

Lübben M, Lünsdorf H, Schäfer G (1987) A plasma membrane ATPase of the thermophilic archaebacterium *Sulfolobus acido-*

caldarius: purification and immunological relationships to F_1-ATPases. Eur J Biochem 167:211–219

Menz RI, Walker JE, Leslie AG (2001) Structure of bovine mitochondrial F_1-ATPase with nucleotide bound to all three catalytic sites: implications for the mechanism of rotary catalysis. Cell 106:331–341

Michel H, Oesterhelt D (1980) Electrochemical proton gradient across the cell membrane of *Halobacterium halobium*: comparison of the light-induced increase with the increase of intracellular adenosine triphosphate under steady-state illumination. Biochemistry 19:4615–4619

Mountfort DO (1978) Evidence for ATP synthesis driven by a proton gradient in *Methanosarcina barkeri*. Biochem Biophys Res Commun 85:1346–1350

Mukohata Y, Ihara K (1990). Situation of archaebacterial ATPase among ion-translocating ATPase In: Kim CH, Ozawa T (eds) Bioenergetics. Plenum, New York, pp 205–216

Müller V, Ruppert C, Lemker T (1999) Structure and function of the A_1A_O ATPases from methanogenic archaea. J Bioenerg Biomembrane 31:15–28

Müller V, Aufurth S, Rahlfs S (2001) The Na^+ cycle in *Acetobacterium woodii*: identification and characterization of a Na^+ translocating F_1F_O-ATPase with a mixed oligomer of 8 and 16 kDa proteolipids. Biochim Biophys Acta 1505:108–120

Neumann S, Matthey U, Kaim G, Dimroth P (1998) Purification and properties of the F_1F_O ATPase of *Ilyobacter tartaricus*, a sodium ion pump. J Bacteriol 180:3312–3316

Reidlinger J, Müller V (1994) Purification of ATP synthase from *Acetobacterium woodii* and identification as a Na^+-translocating F_1F_O-type enzyme. Eur J Biochem 223:275–283

Ruppert C, Wimmers S, Lemker T, Müller V (1998) The A_1A_O ATPase from *Methanosarcina mazei*: cloning of the 5′ end of the *aha* operon encoding the membrane domain and expression of the proteolipid in a membrane-bound form in *Escherichia coli*. J Bacteriol 180:3448–3452

Ruppert C, Kavermann H, Wimmers S, Schmid R, Kellermann J, Lottspeich F, Huber H, Stetter KO, Müller V (1999) The proteolipid of the A_1A_O ATP synthase from *Methanococcus jannaschii* has six predicted transmembrane helices but only two proton-translocating carboxyl groups. J Biol Chem 274:25281–25284

Ruppert C, Schmid R, Hedderich R, Müller V (2001) Selective extraction of subunit D of the Na^+-translocating methyltransferase and subunit *c* of the A_1A_O ATPase from the cytoplasmic membrane of methanogenic archaea by chloroform/methanol and characterization of subunit *c* of *Methanothermobacter thermoautotrophicus* as a 16-kDa proteolipid. FEMS Microbiol Lett 195:47–51

Schäfer G, Meyering-Vos M (1992) F-Type or V-Type? The chimeric nature of the archaebacterial ATP synthase. Biochim Biophys Acta 1101:232–235

Schäfer G, Engelhard M, Müller V (1999) Bioenergetics of the Archaea. Microbiol Mol Biol Rev 63:570–620

Scheel E, Schäfer G (1990) Chemiosmotic energy conservation and the membrane ATPase of *Methanolobus tindarius*. Eur J Biochem 187:727–735

Senior AE, Nadanaciva S, Weber J (2000) Rate acceleration of ATP hydrolysis by F_1F_O ATP synthase. J Exp Biol 203:35–40

Steinert K, Bickel-Sandkötter S (1996) Isolation, characterization, and substrate specificity of the plasma membrane ATPase of the halophilic archaeon *Haloferax volcanii*. Z Naturforsch 51:29–39

Steinert K, Wagner V, Kroth-Pancic PG, Bickel-Sandkötter S (1997) Characterization and subunit structure of the ATP synthase of the halophilic archaeon *Haloferax volcanii* and organization of the ATP synthase genes. J Biol Chem 272:6261–6269

Stock D, Gibbons C, Arechaga I, Leslie AG, Walker JE (2000) The rotary mechanism of ATP synthase. Curr Opin Struct Biol 10:672–679

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Valentine RC, Chignell DA (1968) Electron microscopy of muscle phosphorylase molecules: significance of a rhombic shape. Nature 218:950–953

Vik SB, Antonio BJ (1994) A mechanism of proton translocation by F_1F_O ATP synthases suggested by double mutants of the *a* subunit. J Biol Chem 269:30364–30369

Wilkens S, Capaldi RA (1998) Electron microscopic evidence of two stalks linking the F_1 and F_O parts of the *Escherichia coli* ATP synthase. Biochim Biophys Acta 1365:93–97

Wilms R, Freiberg C, Wegerle E, Meier I, Mayer F, Müller V (1996) Subunit structure and organization of the genes of the A_1A_O ATPase from the archaeon *Methanosarcina mazei* Gö1. J Biol Chem 271:18843–18852