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Structure and Function of the A_1A_0-ATPases from Methanogenic Archaea

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Recent molecular studies revealed nine to ten gene products involved in function/assembly of the methanoarchaeal ATPase and unravel a close relationship of the A_1A_0-ATPase and the V_1V_0-ATPase with respect to subunit composition and the structure of individual subunits. Most interestingly, there is an astonishing variability in the size of the proteolipids in methanoarchaeal A_1A_0-ATPases with six, four, or two transmembrane helices and a variable number of conserved protonizable groups per monomer. Despite the structural similarities the A_1A_0-ATPase differs fundamentally from the V_1V_0-ATPase by its ability to synthesize ATP, a feature shared with F_1F_0-ATPases. The discovery of duplicated and triplicated versions of the proteolipid in A_1A_0-ATP synthases questions older views of the structural requirements for ATP synthases *versus* ATP hydrolases and sheds new light on the evolution of these secondary energy converters.

KEY WORDS: Archaea; methanogens; bioenergetics; ATPases; ATP synthases; evolution; proteolipids.

INTRODUCTION

ATP synthases/ATP hydrolases are present in every organism and are essential for every living cell. The classical F_1F_0-ATPase found in most bacteria, mitochondria, and chloroplasts acts as an ATP synthase during respiration or photosynthesis. However, it is a reversible machine operating also as an ATP hydrolase; this function is of particular importance in strictly fermenting organisms, such as lactic acid bacteria, which produce ATP by substrate-level phosphorylation and use the ATPase to energize their cytoplasmic membrane. During the last decade, another class of ATPases was found in certain tissues and organelles of eukaryotes—the V_1V_0-ATPases (see other articles in this volume). Apparently, they have the same overall architecture, but are only distantly related to F_1F_0-ATPases. With respect to function the main difference is that V_1V_0-ATPases are unable to synthesize ATP under physiological conditions; their function is to generate a ΔpH across certain membranes.

Archaea represent the third domain in the universal tree of life beside the *Bacteria* and *Eukarya* (Woese *et al.*, 1990). From the recently published genome sequences it became evident that archaea have many features in common with bacteria as well as with eukarya (Bult *et al.*, 1996; Kawarabayasi *et al.*, 1998; Klenk *et al.*, 1997; Smith *et al.*, 1997). To date, we can distinguish three physiological groups of archaea: the hyperthermophiles, the halophiles, and the methanogens. The physiological differences of these groups have to be considered while attempting to depict a universal model of archaeal ATPases. The methanogenic archaea (methanogens) are strictly chemiosmotic, which means that an ATP synthase is essential (Deppenmeier *et al.*, 1996; Muller *et al.*, 1993). The halophiles can perform respiration/photosynthesis, but also fermentation; here we would expect an enzyme able to work in both directions, i. e., synthesis as well as hydrolysis (Bickel-Sandkotter *et al.*, 1996). Among the hyperthermophiles, a number of strictly fermenting organisms are known, and, therefore, there is no need

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for the enzyme to synthesize ATP. ATP hydrolysis would be sufficient to satisfy their cellular needs (Danson, 1993).

To date ATPases have been purified from a number of methanogenic archaea (Chen and Konisky, 1993; Inatomi, 1986; Inatomi *et al.*, 1989b, 1993; Scheel and Schafer, 1990; Wilms *et al.*, 1996). Biochemical, immunological, and molecular data revealed that the enzymes from archaea share structural features with the V_1V_0-ATPases but functional features with the F_1F_0-ATPases (Mukohata and Ihara, 1990; Schafer and Meyering-Vos, 1992); due to their unique structure and function they are named A_1A_0-ATPases. The ATPases from methanogens are hitherto the best investigated specimen of this unique class of ATPases. It is the purpose of this review to summarize current knowledge about the structure–function relation of A_1A_0-ATPases from methanogens. When necessary to draw a complete picture, we use information from other systems.

CELLULAR FUNCTION OF THE A_1A_0-ATPase FROM METHANOGENS

Methanogens are very unique because of several features (Deppenmeier *et al.*, 1996; Ferry, 1992; Thauer, 1998; Wolfe, 1993): they are found in extreme environments with respect to pH, salt concentration, or temperature; they are strictly anaerobic and gain energy by the conversion of only a small number of substrates, such as $H_2 + CO_2$, methanol, or acetate to methane; a number of their coenzymes are exclusively found in methanogens; they have a proton-translocating electron transport chain operating in the absence of oxygen; they have primary sodium ion pumps, which are coupled obligatory to the pathway of methane formation. Methanogens are the only microorganisms known to produce two primary ion gradients at the same time: an electrochemical sodium ion gradient ($\Delta\mu_{Na^+}$) and an electrochemical proton gradient ($\Delta\mu_{H^+}$). Therefore, they are posed with the problem to convert both ion gradients into ATP. How this is achieved is still a matter of debate but there is plenty of evidence that the A_1A_0-ATPase found in all methanogens tested so far does pump protons (Deppenmeier *et al.*, 1996).

A_1A_0-ATPases have been purified from various methanogens but, unfortunately, they are very unstable. In every case reported so far, only incomplete enzymes with two to six subunits were purified. Until now there are no reconstitution efforts and, therefore, direct experimental proof that the archaeal ATPase is indeed an ATP synthase is still lacking. However, there is overwhelming circumstantial evidence for the physiological function of the A_1A_0-ATPase as an ATP synthase. First, methane formation from all substrates known is not coupled to substrate-level phosphorylation but ion gradient-driven phosphorylation (Deppenmeier *et al.*, 1996; Thauer *et al.*, 1977). Studies by Blaut and Gottschalk gave the first clear evidence for the presence of a chemiosmotic mechanism of energy conservation in methanogens. Uncouplers led to a collapse of the ATP pool as well as $\Delta\mu_{H^+}$ but stimulated methane formation as catalyzed by *Methanosarcina barkeri*; ATP synthesis was inhibited by *N, N′*-dicyclohexylcarbodiimide (DCCD), and at the same time, the membrane potential increased while methane formation was inhibited. Inhibition of methane formation by DCCD could be relieved by addition of a protonophore (Blaut and Gottschalk, 1984). This is reminiscent of respiratory control observed in bacteria and mitochondria and clearly demonstrated the following sequence of events:

$$\text{methane formation} \rightarrow \text{generation of } \tilde{\Delta\mu}_{H^+}$$

$$\rightarrow \text{ATP synthesis}$$

Furthermore, inverted membrane vesicles of *Methanosarcina mazei* Go1 catalyzed bafilomycin-sensitive, $\Delta\mu_{H^+}$ driven ATP synthesis indicating the operation of an A_1A_0 rather than a F_1F_0-ATPase (Becher and Muller, 1994).

Second, very recently the genome sequences of two methanogens, *Methanococcus jannaschii* (Bult *et al.*, 1996) and *Methanobacterium thermoautotrophicum* ΔH (Smith *et al.*, 1997), were published. The genomic data revealed no genes encoding a F_1F_0-ATPase, but only the presence of genes encoding the A_1A_0-ATPase. Again, ion gradient-driven phosphorylation is the only way for these organisms to synthesize ATP and, therefore, this is the most conclusive evidence that the A_1A_0-ATPase functions as an ATP synthase *in vivo*. After cloning and expression of the genes encoding the A_1A_0-ATPase, it will be possible to test this hypothesis.

CATALYTIC PROPERTIES OF THE A_1A_0-ATPase FROM METHANOGENS

The catalytic properties of the A_1A_0-ATPase from methanogens have not been explored in great detail,

mainly due to the instability of the purified enzymes. All ATPases have a rather low pH optimum of around 5.0 to 5.2 (Chen and Konisky, 1993; Inatomi, 1986; Wilms *et al.*, 1996) with the exception of the enzyme from *Methanosarcina thermophila* (Inatomi *et al.*, 1993) and *Mc. jannaschii* (Ruppert *et al.*, 1999), which have an optimum at about pH 7.0. The enzymes hydrolyze ATP > GTP > ITP > TTP > UTP > CTP. They are stimulated by divalent cations, such as Mg^{2+} and Mn^{2+}, but, as far as described, not by Na^+. Sulfite, glycerol, and ethanol stimulate enzyme activity (Chen and Konisky, 1993; Inatomi, 1986, 1996; Inatomi *et al.*, 1989b, 1993; Scheel and Schafer, 1990; Wilms *et al.*, 1996).

Archaeal ATPases vary substantially with respect to inhibitor sensitivity. This might reflect the different primary structures but also the different assay conditions used (salt concentrations, pH). Methanoarchaeal A_1A_0-ATPases are not inhibited by *N*-ethylmaleimide whereas the enzymes from halobacteria are (Dane *et al.*, 1992; Stan-Lotter *et al.*, 1991; Steinert and Bickel-Sandkotter, 1996; Sulzner *et al.*, 1992). The structural basis for this diversity can easily be delineated from the primary structure. Subunit A of halobacterial A_1A_0-ATPases does contain a number of cysteine residues close to the catalytic center, whereas methanoarchaeal ATPases do not (Ihara and Mukohata, 1991; Inatomi *et al.*, 1989a; Steinert *et al.*, 1995; Wilms *et al.*, 1996). In general, A_1A_0-ATPases are insensitive to azide, oligomycin, and vanadate, but are inhibited by DCCD, diethylstilbestrol, and nitrate (Becher and Muller, 1994; Inatomi 1986; Inatomi *et al.*, 1993; Lubben *et al.*, 1987). In particular, nitrate inhibition was always seen to be specific for V_1V_0-A_1A_0-ATPases, but recently a nitrate-sensitive F_1F_0-ATPase was also described (Reidlinger and Muller, 1994), calling in question the classification of the ATPases based on inhibitor sensitivity. The methanoarchaeal ATPase is inhibited by bafilomycin, but in concentrations three orders of magnitude higher than those required for V_1V_0-ATPases: 50% inhibition of the enzyme from *Methanococcus voltae* is achieved at 10 μM (Chen and Konisky, 1993), whereas 50% inhibition of ATP synthesis as catalyzed by inverted vesicles of *Ms. mazei* is reached at 15 μM bafilomycin A_1 (Becher and Muller, 1994).

There is great uncertainty with respect to the coupling ion used by methanoarchaeal A_1A_0-ATPases. Since methanogens are strictly sodium ion-dependent and produce $\Delta\mu_{Na^+}$ and $\Delta\mu_{H^+}$ at the same time, there has always been the hypothesis that $\Delta\mu_{Na^+}$ might be used directly as driving force for ATP synthesis, and indications in support of this hypothesis have been obtained in several methanogens (Becher and Muller, 1994; Chen and Konisky, 1993; Smigan *et al.*, 1994). In fact, the ion specificity of the A_1A_0-ATPases has not yet been determined with a purified enzyme and we would not be surprised to find H^+-as well as Na^+-translocating A_1A_0-ATPases. From the F_1F_0-ATPases it is already known that only a few amino acid modifications will change the ion specificity of the ATPase (Kaim *et al.*, 1997; Rahlfs and Muller, 1997; Zhang and Fillingame, 1995). The situation is even more complicated by the finding of F_1F_0-ATPase activities (Becher and Muller, 1994) and genes (Sumi *et al.*, 1997) in methylotrophic methanogens, which, based on inhibitor studies, were assumed to pump Na^+. On the other hand, it seems possible to also convert the Na^+ gradient into a secondary proton gradient, which is then used to drive the synthesis of ATP via a H^+-A_1A_0-ATPases. Hydrogenotrophic methanogens such as *Mc. jannaschii* and *Mb. thermoautotrophicum* ΔH contain only A_1A_0-ATPases, but, in addition, several genes that could encode potential Na^+/H^+ antiporters (Bult *et al.*, 1996; Smith *et al.*, 1997). Clearly, much more data are needed to give an answer to this important question.

GENETIC ORGANIZATION OF KNOWN A_1A_0-ATPases

At present the A_1A_0-ATPase encoding genes have been cloned from three methanogens, two (*Mc. jannaschii, Mb. thermoautotrophicum* ΔH) by genome sequencing (Bult *et al.*, 1996; Smith *et al.*, 1997), and one [*Ms. mazei* Go1 (Ruppert *et al.*, 1998; Wilms *et al.*, 1996)] using conventional methods. However, we do expect to see more sequences in the near future because of the already started or intended genome sequencing projects. In addition, the genome sequences of the archaea *Archaeoglobus fulgidus* (Klenk *et al.*, 1997) and *Pyrococcus horikoshii* (Kawarabayasi *et al.*, 1998) were published and some of the genes encoding the A_1A_0-ATPase from the hyperthermophiles *Sulfolobus acidocaldarius* (Denda *et al.*, 1990) and *Desulfurococcus* sp. SY (Shibui *et al.*, 1997) as well as the halophile *Haloferax volcanii* (Steinert *et al.*, 1997) are known.

As can be seen from Fig. 1, the overall genetic organization of the A_1A_0-ATPase genes in the three methanoarchaea as well as in the hyperthermophilic

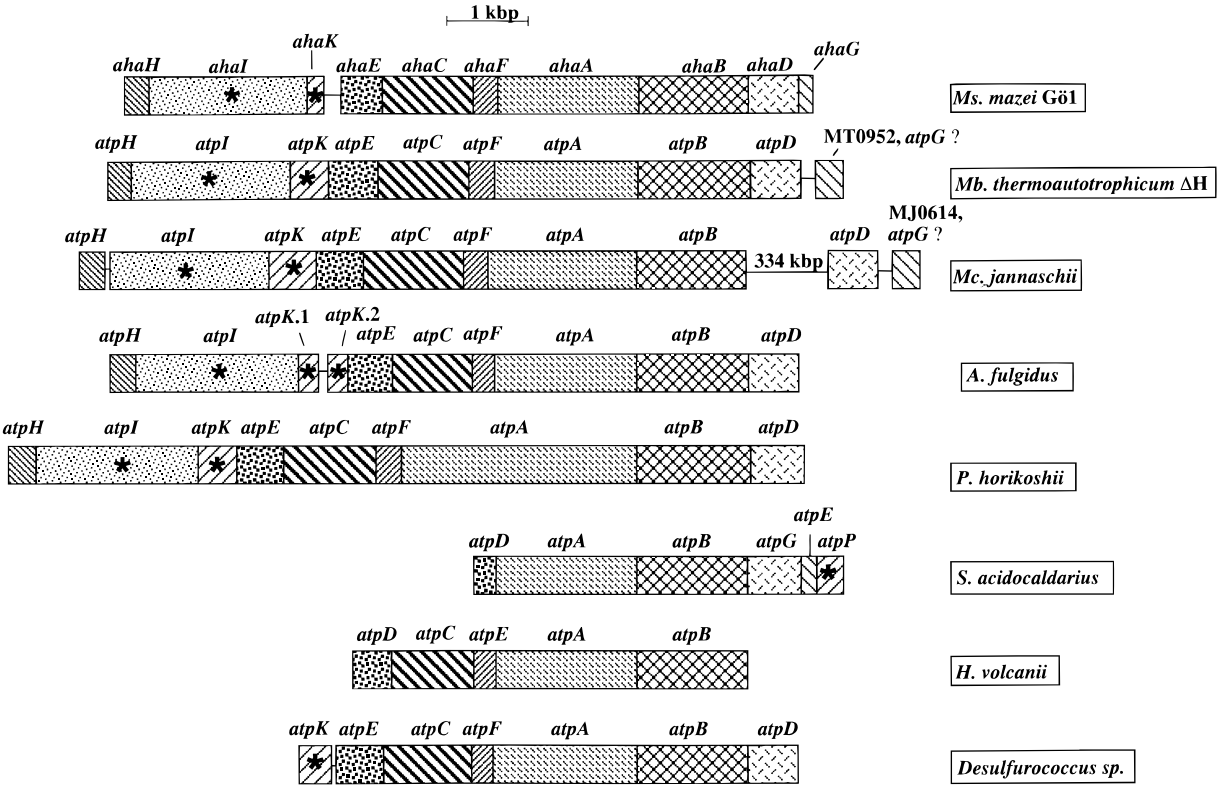

Fig. 1. Organization of genes in the archaeal A_1A_0-ATPase operons. Genes encoding hydrophobic subunits are marked by asterisks. Homologous genes are depicted by the same pattern. AhaG homologs could not be identified unambiguously in *Mb. thermoautotrophicum* ΔH and *Mc. jannaschii*. Note the gap of 334 kbp in *Mc. jannaschii*, which was not drawn to scale.

sulfate-reducing archaeon *A. fulgidus* is almost identical. The first gene of every cluster encodes a hydrophilic polypeptide, and it is followed by two genes encoding hydrophobic subunits. The rest of the genes encode hydrophilic subunits. The last open reading frame in the *aha* operon of *Ms. mazei Go1*, *ahaG*, is an authentic gene since it is expressed in *Escherichia coli* (Lemker and Muller, 1999). There are homologous genes at the same position in the V_1V_0-ATPase operon from *E. hirae* (*ntpH*) (Takase *et al.*, 1994) and the A_1A_0-ATPase operon from *S. acidocaldarius (atpE*) (Denda *et al.*, 1990), but there are no unequivocal homologs in the other archaea. Therefore, it is still an open question whether AhaG is related to ATPase function/assembly. In *Mc. jannaschii* and *Mb. thermoautotrophicum* ΔH the open reading frames upstream of *atpD*, MJ0614 and MT0952 respectively, which code for hydrophilic peptides, are tentatively designated *atpG* based on sequence comparisons. Of the amino acids 22% and 20%, respectively, are identical to AhaG from *Ms. mazei*. Go1.

There is a small intergenic region of 211 bp between *ahaK* and *ahaE* of *Ms. mazei*, Go but, nevertheless, the entire gene cluster *ahaH* through *ahaG* is transcribed as one 9.0-kb message (Ruppert *et al.*, 1998). Upstream of *ahaH* is an AT-rich region, which contains two potential archaeal promotor sequences. Interestingly, there is an additional message of 0.64 kb hybridizing with a probe derived from *ahaK*, the gene encoding the proteolipid (Ruppert *et al.*, 1998). This is of particular importance since the 8-kDa proteolipid, in analogy to *E. coli* F_1F_0-ATPase (Jones and Fillingame, 1998), is likely to be present in twelve copies per enzyme molecule and, therefore, the organisms have to develop mechanisms to guarantee enhanced synthesis of the proteolipid relative to other subunits. In *E. coli*, the ATPase encoding genes form a polycistronic operon and enhanced synthesis of the

proteolipid is achieved by translational regulation (McCarthy *et al.*, 1985). On the other hand, there is also a polycistronic message including the proteolipid-encoding gene in *Ms. mazei* Go1, but there is no translational enhancer motif present. However, the additional separate transcription of the proteolipid-encoding gene only is likely to lead to the relatively high copy number of the proteolipid.

The ATPase genes of *Mc. jannaschii* are not organized in one but two clusters. The same is probably true for *S. acidocaldarius* and *H. volcanii* where the remaining genes are distantly organized on the chromosome. At present, we can assume a number of nine to ten genes related to ATPase function/assembly in archaea.

PROPERTIES AND FUNCTION OF INDIVIDUAL SUBUNITS

The deduced properties of the methanoarchaeal A_1A_0-ATPase gene products are given below. Only subunits I and K are hydrophobic proteins, the others are hydrophilic. Homologous proteins in V_1V_0- and F_1F_0-ATPases are given in Table I.

Subunit H (M_r = 11,800–12,200) is hydrophilic and highly charged. Data base searches did not reveal a homolog in eukaryal V_1V_0-ATPases. The fact that *ahaH* and its homologs in other prokaryotes are the first genes in their operons could suggest that they are homologous to *uncI* of bacterial F_1F_0-ATPases (Walker *et al.*, 1984), but only 12% of the amino acids of UncI of *E. coli* and AhaH of *Ms. mazei* Go1 were found to be identical.

The molecular mass of subunit I ranges from 72 to 76 kDa. It is very similar to the 100-kDa subunit [subunit *a* or VpH1p/Stv1p (Manolson *et al.*, 1992, 1994)] of V_1V_0-ATPases. Hydrophobicity plots propose a hydrophilic N-terminal and a hydrophobic C-terminal domain which are in *Ms. mazei* Go1 39 and 33 kDa, respectively. Garnier analysis of the hydrophilic domain predicts a highly α helical structure, as it is the case with subunit *b* of F_1F_0-ATPases. Interestingly, the identity of the hydrophilic domain of subunit *b* of F_1F_0-ATPases to the hydrophilic domain of subunit I is 22.3 to 31.7%. The hydrophobic C-terminus of subunit I is predicted to have seven transmembrane helices; similarity to subunit *a* of F_1F_0-ATPases is below 20%. The difference in molecular mass of around 30% between subunit I and the 100-kDa subunit of V_1V_0-ATPases is due to a drastic shortage of the hydrophilic loops connecting the transmembrane helices.

Subunit K is synonymous with proteolipid. Proteolipids have been purified and characterized from some archaea and, in almost all cases, they were shown to be of $M_r \approx 8000$ with two transmembrane helices (Ihara *et al.*, 1997; Inatomi, 1986; Lubben and Schafer, 1989, Steinert and Bickel-Sandkotter, 1996; Wilms *et al.*, 1996). This size corresponds to the size of the proteolipid from F_1F_0-ATPases and was hitherto

Table I. Listing of Similar ATPase Gene Products in the Three Types of ATPases[a]

Ms. mazei Go1 A_1A_0		*S. cerevisiae* V_1V_0			*E. coli* F_1F_0	
A	A_1 subunits	Vma1p	(A)	V_1 subunits	β	F_1 subunits
B		Vma2p	(B)		α	
C		Vma6p	(d)	V_0 subunit	—	
D		Vma8p	(D)	V_1 subunits	γ	
E		Vma4p	(E)		δ	
F		Vma7p	(F)		ε	
—		Vma13p	(H)		—	
—		Vma5p	(C)		—	
H	structure?	Vma10p	(G)		soluble b?	
G	assembly?	—	—		—	
I	A_0 subunits	Vph1p/Stv1p	(a)	V_0 subunits	a + b ?	F_0 subunits
K		Vma3p	(c)		c	
—		Vma11p	(c′)		—	
—		Vma16p	(c″)		—	

[a] There are no homologs of Vma12p, Vma14p, Vma15p, Vma21p, Vma22p, and Vma23p nor of AtpI of F_1F_0-ATPases found in A_1A_0-ATPase.

assumed to be the reason for the F_1-like properties of the A_1A_0-ATPases, i. e., their function as ATP synthases. However, from the recently published genome sequences proteolipids with four (*Mb. thermoautotrophicum* ΔH) and six (*Mc. jannaschii*) membrane-spanning helices are predicted. In case of *Mb. thermoautotrophicum* ΔH, the active carboxylate is conserved in helix two and four, but in *Mc. jannaschii* it is only conserved in helix four and six, but not in two (Fig. 2); in helix two, the active carboxylate is substituted by a glutamine residue. Since these data emerged from genome sequencing projects, it was essential to verify the size of the proteolipid, especially in *Mc. jannaschii*. The proteolipid of *Mc. jannaschii* was purified and subjected to MALDI analysis, which revealed a molecular mass of 21.183 kDa (Ruppert *et al.*, 1999). This provides evidence that the proteolipid from *Mc. jannaschii* does indeed consist of six transmembrane helices. Apparently, the proteolipids from *Mb. thermoautotrophicum* ΔH and *Mc. jannaschii* arose by gene duplication and triplication, respectively, with subsequent fusion of the genes. The ATP synthases of *Mb. thermoautotrophicum* ΔH and *Mc. jannaschii* are the first ones reported to contain proteolipids with four and six transmembrane helices. Most interestingly, the proteolipid from *Mc. jannaschii* has only two active carboxylates per six membrane-spanning helices, which is of particular importance for the mechanism by which ion transport is coupled to ATP synthesis.

The proteolipids from methanoarchaea are very similar to each other (≈ 50%) and to those from *A. fulgidus* (45–52% identity) (Klenk *et al.*, 1997), *S. acidocaldarius* (30–34% identity) (Denda *et al.*, 1989), and *Halobacterium salinarum* (31–52% identity) (Ihara *et al.*, 1997), and to proteolipids of V_1V_0-ATPases from bacteria or eukarya, the degree of identities range from 26.7 to 33%. A leader sequence as observed in *H. salinarum* (Ihara *et al.*, 1997) or *S. acidocaldarius* (Lubben and Schafer, 1989) is apparently not present in the methanoarchaeal proteolipids.

An interesting feature apart from the different sizes is the conserved PET motif found in all methanoarchaeal proteolipids known so far; this motif includes the protonizable group involved in H^+ transport. A threonine at the same position was also found in the proteolipids of the Na^+-translocating F_1F_0-ATPases from *Acetobacterium woodii* (Rahlfs and Muller, 1997) and *Propionigenium modestum* (Kaim *et al.*, 1997). Apparently, there are no conserved residues in the hydrophilic loops, which is in contrast to the proteolipids of F_1F_0-ATPases.

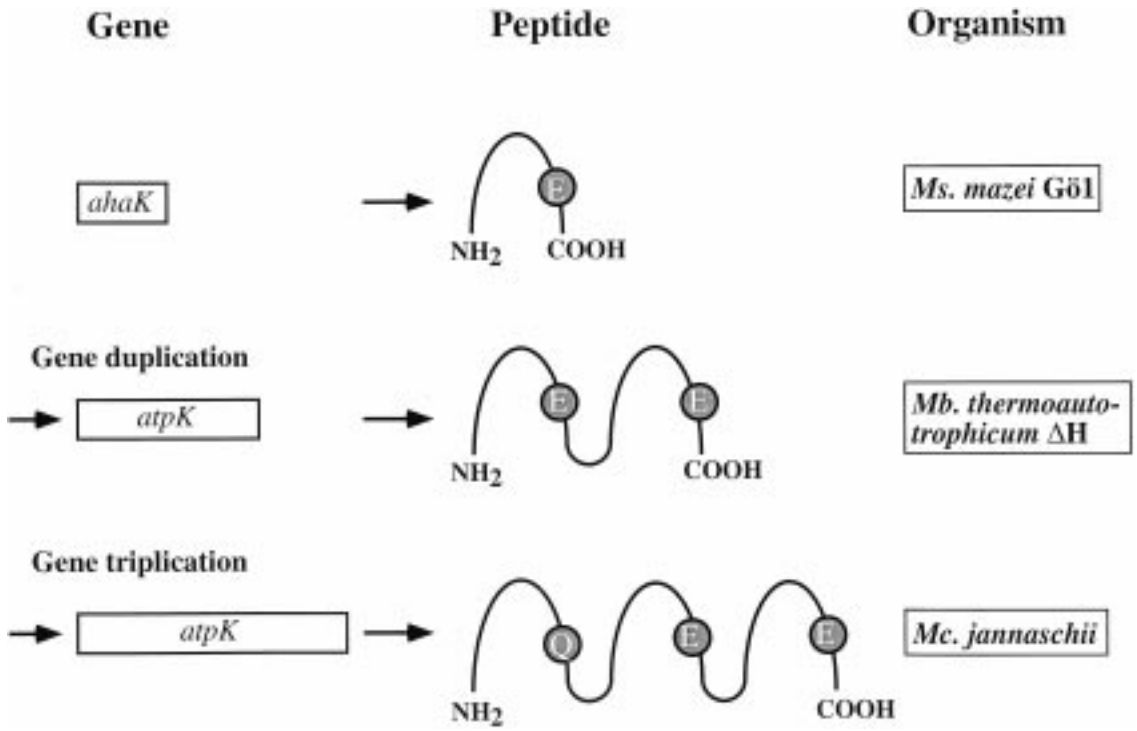

Fig. 2. The DNA sequences predict proteolipids with molecular masses of 8, 16, and 21 kDa in methanoarchaea.

Subunit E (M_r, = 20,400-22,900) is similar to subunit E of V_1V_0-ATPases [Vma4p in yeast (Foury, 1990)]. Secondary structure analysis predicts that AhaE and its homologs are largely α-helical. A multiple alignment shows that the peptides are well conserved between prokaryal, eukaryal, and archaeal subunits. According to its size, subunit E could well be the homolog of subunit δ of F_1F_0-ATPases (Walker *et al.*, 1984).

Subunit C (M_r = 41,300–42,400) is similar to subunit *d* of V_1V_0-ATPases [Vma6p in yeast (Bauerle *et al.*, 1993)]. Apparently, there is no homologous polypeptide in F_1F_0-ATPases. Subunit *d* of V_1V_0-ATPases is a hydrophilic polypeptide but copurifies with the V_0 domain and, therefore, it is regarded as a V_0 polypeptide (Stevens and Forgac, 1997; Zhang *et al.*, 1992). Currently, there is no information on the localization of subunit C in A_1A_0-ATPases. However, a 40-kDa polypeptide copurifies with the A_1 domain, but it is not known whether this is subunit C (Wilms *et al.*, 1996).

Subunit F is a hydrophilic polypeptide of 10.8 to 11.8 kDa, which could suggest that it is the homolog of subunit F [or Vma7p in yeast (Graham *et al.*, 1994; Nelson *et al.*, 1994)] of V_1V_0-ATPases and subunit ε of F_1F_0-ATPases (Walker *et al.*, 1984). Secondary structure analysis predicts a small α-helical region at its N-terminus and a β-sheet region at its C-terminus.

Subunit A (M_r = 63,800–66,400) was purified and the encoding gene was sequenced from a number of archaea (Bult *et al.*, 1996; Denda *et al.*, 1988; Ihara *et al.*, 1992, Inatomi *et al.*, 1989a; Klenk *et al.*, 1997; Shibui *et al.*, 1997; Smith *et al.*, 1997; Steinert *et al.*, 1995; Wilms *et al.*, 1996). It is similar to subunit A [Vma1p of yeast (Hirata *et al.*, 1990)] of V_1V_0-ATPases (about 50% identity). On the other hand, 26.9–27.6% of the residues are identical to subunit β of the F_1F_0-ATPase of *E. coli* (Walker *et al.*, 1984), indicating a common precursor of subunit A of A_1A_0-ATPases and subunit β of F_1F_0-ATPases. Subunit A contains the Walker motifs A and B (Walker *et al.*, 1982), which are part of the nucleotide binding domain (Abrahams *et al.*, 1994) and, therefore, it can be concluded that it is the catalytic subunit. This is in agreement with the finding that antisubunit A-specific antibodies were four times more effective in inhibiting ATPase activity of the purified enzyme from *Ms. mazei* Go1 than antisubunit B-specific antibodies (Wilms *et al.*, 1996). From a multiple alignment it is evident that all of the residues involved in nucleotide binding in the mitochondrial F_1F_0-ATPase (Abrahams *et al.*, 1994) are conserved in subunit A of A_1A_0-ATPase and, therefore, this general scheme seems also valid for nucleotide binding in subunit A of A_1A_0-ATPases.

Subunit B (M_r = 50,600–51,900) is closely related to subunit B [or Vma2p of yeast (Nelson *et al.*, 1989)] from V_1V_0-ATPases and similar to subunit α from F_1F_0-ATPase (Walker *et al.*, 1984). The Walker site A present in subunit α is not conserved in subunit B at the equivalent position. However, there is a conserved motif G-X-X-X-G-K-T 195 amino acids downstream of the expected site. Studies with *S. acidocaldarius* revealed the presence of six nucleotide binding sites in the ATPase and, therefore, this subunit is also suggested to bind ATP (Schafer and Meyering-Vos, 1992). However, because of sequence analysis and experimental data, subunit B from A_1A_0- as well as from V_1V_0-ATPases seems to be non-catalytic.

Subunit D (M_r = 23,900–24,900) is similar to subunit D [(Vma8p of yeast (Graham *et al.*, 1995; Nelson *et al.*, 1995)] of V_1V_0-ATPase. Of the residues of subunit D from *Ms. mazei* Go1, 21.7% are identical to subunit γ of the *E. coli* F_1F_0-ATPase (Walker *et al.*, 1984). Secondary structure analysis predicts a largely α-helical structure, which is a striking homology to subunit γ of F_1F_0-ATPases. Thus, we suggest that AhaD and the corresponding subunits in V_1V_0- and A_1A_0-ATPases are the homologs of subunit γ of F_1F_0-ATPases. However, there are no conserved residues in these polypeptides.

AhaG (M_r = 6135 in *Ms. mazei* Go1) is the only polypeptide for which no homologs can be found by data base searches. *ahaG* is expressed in *E. coli* as part of the *aha* operon, but it is unknown whether the gene product is related to ATPase function/assembly (Lemker and Muller, 1999).

ENZYME STRUCTURE

The molecular data revealed that the A_1A_0-ATPase is composed of at least nine non-identical subunits. A_1A_0 like F_1F_0-and V_1V_0-ATPases are composed of a head and a base connected by a stalk. The "traditional" stalk is supposed to be part of the rotor, whereas a second stalk is the stator of the rotatory enzyme (Boekema *et al.*, 1997; Rodgers and Capaldi, 1998; Wilkens and Capaldi, 1998). The dimensions of the domains and the entire complex are comparable to F_1F_0 and V_1V_0-ATPases. The subunit composition of each domain is still speculative. A model of the A_1A_0-ATPase of *Ms. mazei* Go1 is given in Fig. 3. Note that the number of proteolipids, as shown, does not apply

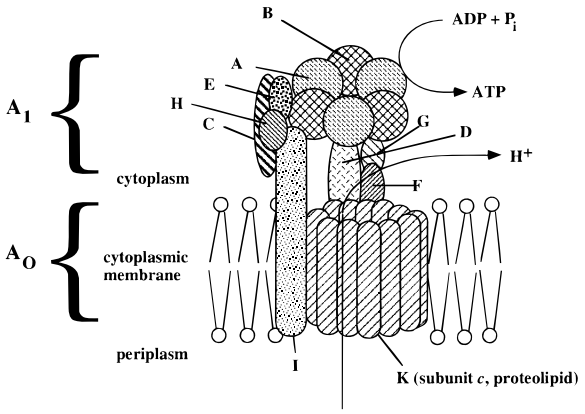

Fig. 3. Hypothetical structure of the A_1A_0-ATPase of *Ms. mazei* Go1. Note that this model does not apply to all methanoarcheal ATPases. The enzyme of *Ms. mazei* Go1 presumably contains twelve copies of a 8-kDa proteolipid, whereas the enzyme from *Mb. thermoautotrophicum* ΔH has six copies of a 16-kDa proteolipid and the *Mc. jannaschii* ATPase possesses four copies of the 21-kDa proteolipid. The localization of AhaC, AhaD, AhaE, AhaF, AhaG, and AhaH is speculative.

to the enzymes from *Mb. thermoautotrophicum* and *Mc. jannaschii* (see below).

A_1 is composed of subunits A and B which, based on electron microscopy, are suggested to be present in three copies, each arranged alternating around a central mass (Wilms *et al.*, 1996). In F_1F_0-ATPases this central mass is built by subunit γ, which plays an important role in transmitting energy from the membrane domain to the catalytic domain (Abrahams *et al.*, 1994; Noji *et al.*, 1997; Sabbert *et al.*, 1996; Zhou *et al.*, 1997). Based on sequence comparisons, subunit D of A_1A_0- and V_1V_0-ATPases is suggested to be the homolog of subunit γ of F_1F_0-ATPases. Nothing is known about the function of subunit C despite the fact that its homolog of V_1V_0-ATPases copurifies with the membrane domain, indicating that subunit C could be part of the stalk and be associated with the membrane domain. Subunits E and F could be the homologs of subunits δ and ε of F_1F_0-ATPases, and, therefore, be part of the stator and rotor, respectively.

The situation is much clearer in the A_0 domain. For a long time, the only membrane-bound subunit known was the proteolipid, but recent sequence analysis revealed the presence of a second membrane-bound subunit, subunit I, which is composed of a hydrophobic and a hydrophilic domain (Ruppert *et al.*, 1998). No gene was found for the 36-kDa subunit previously assigned to the A_0 domain (Wilms *et al.*, 1996). Preliminary immunological data suggest that subunit I is proteolytically degraded giving rise to a membrane-bound 36-kDa polypeptide (Kavermann and Müller, 1999). The stoichiometry of the polypetides in either of the domains, A_0 as well as A_1 is still unknown.

STRUCTURE AND FUNCTION OF THE A_0 DOMAIN

The 8-kDa proteolipid folds in the membrane like a hair pin with two transmembrane helices and contains one protonizable group (in helix two) catalyzing H^+ transport. For *E. coli*, it was demonstrated experimentally that twelve copies of the 8-kDa proteolipid are present per F_1F_0-ATPase molecule (Jones and Fillingame, 1998), arranged in a ringlike structure in a "front-to-back" type of packing in the membrane (Fillingame *et al.*, 1998). In analogy to F_1F_0-ATPases it is likely that the proteolipid of archaea is also arranged in a ringlike structure and a constant number of twenty four helices per enzyme is assumed. This would require twelve copies of the two helix–proteolipid from *Ms. mazei* Go1, six copies of the four helix–proteolipid from *Mb. thermoautotrophicum* ΔH, and four copies of the six helix–proteolipid from *Mc. jannaschii*. The possible arrangement of the *c*-ring oligomer in the membrane is shown in Fig. 4. The determination of the exact number of copies is extremely important for *Mc. jannaschii* since its six helix–proteolipid contains only two protonizable groups (Fig. 4).

It was always hypothesized that the apparent inability of the V_1V_0-ATPases to synthesize ATP is due to their 16-kDa proteolipid. However, from the recent finding of duplicated and triplicated versions of proteolipids in A_1A_0-ATP synthases, it is evident that not the size of the proteolipid *per se* determines whether a given enzyme can catalyze not only ATP hydrolysis, but also ATP synthesis. The important point is the number of protonizable groups per enzyme. If one assumes a constant number of twenty four helices per enzyme, than the ATP synthase from *Mc. jannaschii* has eight protonizable groups per enzyme, whereas all other A_1A_0-ATPases as well as the F_1F_0-ATPases have twelve; the V_1V_0-ATPase has only six. Two protonizable groups per catalytic center as in V_1V_0-ATPases is apparently not sufficient for ATP synthesis but allows the generation of a relatively large proton gradient (ΔpH). Apparently, 2.6 carboxyl groups per catalytic center center, as in *Mc. jannaschii*, are already sufficient for ATP synthesis, whereas all known A_1A_0 and F_1F_0-ATP synthases operate at four protonizable groups per catalytic center. In this context

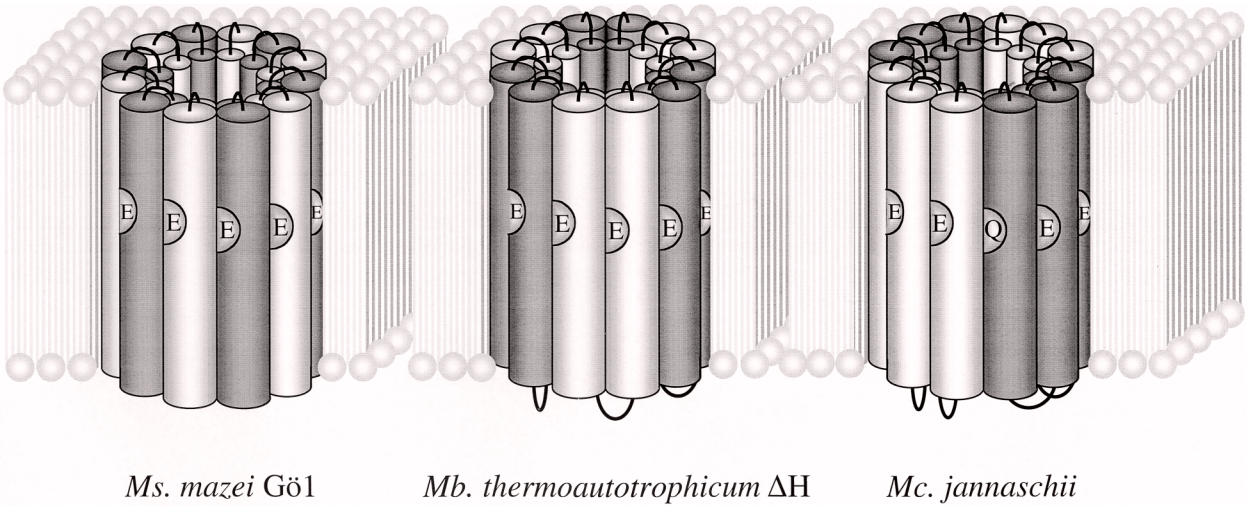

Fig. 4. Hypothetical structure of the *c*-ring oligomer in methanoarchaeal ATPases. The methanoarchaeal proteolipid monomers differ in size. Therefore, different numbers of proteolipids with a constant number of twenty four transmembrane helices are present in the enzymes.

it would be interesting to analyze the dependence of ATP synthesis on $\Delta\mu_{H^+}$ in *Mc. jannaschii* and to determine the threshold values for ATP synthesis.

Since no other membrane-bound subunit, apart from the proteolipid, was known for any archaeon, it was assumed that the proteolipid alone is responsible for proton translocation. We have tested this hypothesis by expressing the proteolipid-encoding gene from *Ms. mazei* Go1 in a ATPase-negative *E. coli* strain. The proteolipid of *Ms. mazei* Go1 is incorporated into membranes of *E. coli* despite the different chemical nature of lipids from bacteria and archaea. However, membrane preparations from this transformant did not show altered proton permeabilities; upon oxidation of NADH, a ΔpH was produced irrespective of the absence or presence of the archaeal proteolipid (Ruppert and Muller, 1998). These experiments indicate that another polypeptide apart from the proteolipid, i. e., most likely subunit I, is involved in proton translocation.

Subunit I is a two-domain subunit (Fig. 5) and it is very tempting to speculate that the two domains have distinct functions, which are separated in F_1F_0-ATPases on two polypeptides, subunit *a* and *b*. The hydrophobic domain of Vph1p as well as subunit *a* of F_1F_0-ATPases are known to be involved in H^+ translocation (Deckers-Hebestreit and Altendorf, 1996; Fillingame *et al.*, 1998; Leng *et al.*, 1996, 1998). A multiple alignment of archaeal subunit I and vacuolar 100-kDa subunits reveals a high degree of conservation. Thirteen residues of the hydrophobic domain are apparently invariant in the archaeal and eukaryal pep-

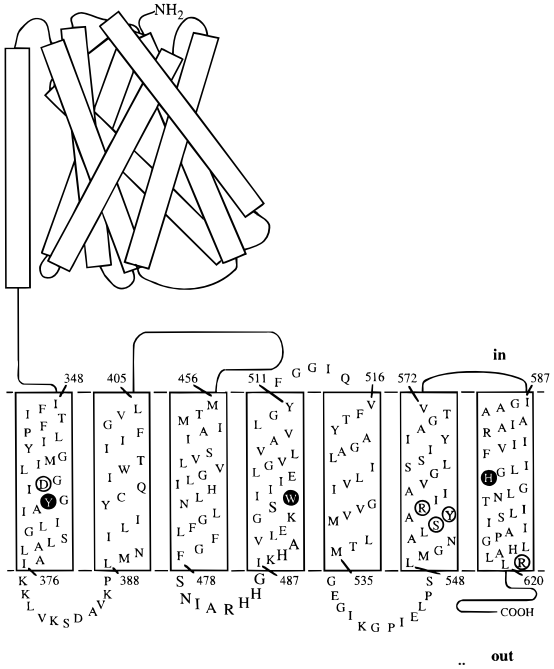

Fig. 5. Topological model for AhaI of *Ms. mazei* Go1. Of the 13 amino acid residues invariant in the hydrophobic domain of archaeal and eukaryal 100-kDa peptides five charged or polar residues located in putative transmembrane segments are marked by open circles; three residues that are only conserved in archaeal peptides are marked by black circles. The model is based on sequence comparisons, hydrophobicity plots, and Garnier analysis.

tides; five of these (Asp-362, Ser-554, Tyr-555, Arg-557, and Arg-619) are charged or polar and located in putative transmembrane segments. Of these, the Asp-362 homolog in yeast (Asp-425) was shown to be important for assembly of the V_0 domain. In addition, mutation of the residue resulted in reduced activity of the ATPases assembled (Leng *et al.*, 1996). A mutation of Arg-557 (Arg-735 in *S. cerevisiae*) led to a loss of stability of the ATPase (Leng *et al.*, 1996). However, none of the residues, which were shown by mutational studies to be important for proton translocation in Vph1p, are conserved in the prokaryotic homologs. Tyr-365, Trp-497, and His-602 are only conserved in archaeal ATPases, but whether they are involved in ion transport remains to be established.

It was demonstrated recently that subunit γ of F_1F_0-ATPases rotates relative to the $\alpha_3\beta_3$ assembly (Noji *et al.*, 1997; Sabbert *et al.*, 1996; Zhou *et al.*, 1997); such a rotation requires a stator and it is suggested that subunit *b* and subunit δ fix the F_1 domain to the membrane (Rodgers and Capaldi, 1998). The relatively large and α-helical hydrophilic domain of subunit I could serve this function in A_1A_0-ATPases. In the hydrophilic domain of subunit I of A_1A_0-ATPases and subunit *a* from V_1V_0-ATPases a region of approximately twenty five amino acids amino-terminal to the first putative membrane spanning helix are very similar. Only four of the residues of the hydrophilic domain seem to be conserved between vacuolar and archaeal subunits (Gly-148, Pro-315, Tyr-339, and Glu-341), whereas eight additional residues seem invariant in the archaeal N-terminus of subunit I. These are Leu-26, His-27, Leu-93, Glu-251, Glu-310, Lys-338, Asp-343, and Pro-344. Subunit *b* of F_1F_0-ATPases (M_r = 14,500) is present in two copies per enzyme, but the 39-kDa hydrophilic domain of subunit I of A_1A_0-ATPases could intrinsingly combine the two copies of the hydrophilic domain of subunit *b*. Inspired by the similarity of AhaH to the hydrophilic domain of subunit *b*, it seems also possible that AhaH serves the function of the soluble domain of subunit *b*. In this regard, it is interesting to note that Vma10p of the V_1V_0-ATPase is considered to be part of the membrane domain, but since it does not contain transmembrane segments it is suggested to interact as a "soluble" *b* homolog with the membrane domain (Supekova *et al.*, 1995). Vma10p is, indeed, 29% similar to AhaH.

From the overall similarity one can conclude that the A_1A_0-ATPase like the F_1F_0-ATPase is a rotatory enzyme. Rotation of subunit γ relative to $\alpha_3\beta_3$ was shown unequivocally for F_1, and it is now widely assumed that ion flow through the membrane is coupled to rotational movement of the *c*-ring oligomer, which then drives rotation of the γ subunit (Abrahams *et al.*, 1994; Noji *et al.*, 1997; Sabbert *et al.*, 1996; Zhou *et al.*, 1997). Since the membrane domain of the A_1A_0-ATPase is composed of two subunits only, the proteolipid and subunit I, it is evident that, if rotation occurs, the *c*-ring oligomer rotates relative to subunit I. The A_0 domain is, with respect to number of nonidentical subunits, the simplest membrane domain of any ATPase and could become a model system for analyzing the mechanism of ion transport and its coupling with rotational movement of subunits.

EVOLUTIONARY ASPECTS: A_1A_0 *VERSUS* V_1V_0

The same structure of two domains connected by a stalk is found in ATPases from all three branches of the evolutionary tree and it is assumed that all ATPases arose from a common ancestor (Gogarten and Taiz, 1992; Nelson and Taiz, 1989). The major polypeptides (A and B) originated from duplication of one ancestral gene. In the line leading to the A_1A_0-/V_1V_0-ATPases a number of deletions and insertions occurred, which, on the one hand, led to the loss of catalytic activity from one subunit of the A_3B_3 core particle, which still needs to bind nucleotides in order to achieve its proper folding, and, on the other hand, to an enlargement of the catalytic subunit relative to the noncatalytic that is *vice versa* in F_1F_0-ATPases. There was always the hypothesis that the diversion of the A_1A_0-and V_1V_0-ATPases took place by a duplication and subsequent fusion of the genes encoding the proteolipid; this was assumed to be the reason for the apparent inability of V_1V_0-ATPases to synthesize ATP. However, multiplied proteolipid-encoding genes are now found in ATP synthases of archaea. Interestingly, the multiplied genes were fused in *Mb. thermoautotrophicum* ΔH and *Mc. jannaschii*, but not in *A. fulgidus*, which apparently represents an intermediate in evolution (cf. Fig. 1). The identity of proteolipid one and two of *A. fulgidus* is 100%, 73% of hair pin one and two of the proteolipid of *Mb. thermoautotrophicum* ΔH are identical, and 58, 57, and 46% of the amino acids of hair pins one and two, two and three, and one and three, respectively, of *Mc. jannaschii* are identical. This is clear evidence for gene multiplication. Furthermore, the high similarity values indicate a selective pressure to maintain structure and H^+-translocation of each hair pin in the

multiplied proteolipids and, from looking at the physiology of the organisms, it is clear that the selection pressure is to keep the ability of the enzyme to synthesize ATP, the most prominent cellular function of the ATPases from methanogens. Very recently, duplications and triplications of the two helix–proteolipid from *E. coli* were generated by molecular methods. Interestingly, transformants were able to grow on succinate, demonstrating that these F_1F_0-ATPases with multiplied proteolipids retained their function to synthesize ATP (Jones and Fillingame, 1998). Taken together these experiments gave clear evidence that not simply the size of the proteolipids *per se* determines whether a given enzyme is able to synthesize ATP, but rather the conservation of structure and the capability of each hair pin to participate in ion translocation. In the duplicated four helix–proteolipids of V_1V_0-ATPases hair pin one is apparently degenerated, accompanied by a loss of the protonizable group and the capability to pump protons, which led to the apparent inability to synthesize ATP.

Whether the discovery of multiplied proteolipids in archaea can be taken as argument against the hypothesis that the divergence of A_1A_0/V_1V_0 took place by the duplication of the proteolipid-encoding gene is difficult to decide. However, it should be kept in mind that while most archaeal proteolipids have a molecular mass of $\approx$ 8 kDa, only a few underwent multiplication events. Recently, it was hypothesized that the duplication of the eukaryal and archaeal proteolipid genes were independent events (Hilario and Gogarten, 1998). The discussion of the evolution of ATPases is complicated by the possibility of a horizontal gene transfer, which could explain the occurrence of multiplied proteolipids found in some A_1A_0-ATPases. The existence of V_1V_0-ATPases in bacterial species, as well as the coexistence of A_1A_0- and F_1F_0-ATPase genes in *Ms. barkeri*, was already assumed to result from a horizontal gene transfer (Olendzenski *et al.*, 1998). Very recently, multiplication of a proteolipid-encoding gene with and without subsequent fusion of the multiplied genes was also found in the operon encoding the Na^+-F_1F_0-ATPase of the Gram-positive bacterium *Acetobacterium woodii* (Rahlfs and Muller, 1999). What could be the reason for the multiplications of the proteolipid-encoding gene, which is apparently found in all domains of life? The proteolipid is present in a four to twelve times higher copy number than the other subunits. As long as the proteolipid-encoding gene is part of a polycistronic message (as apparently observed in all prokaryotes), only few mechanisms for enhanced synthesis of the proteolipid are conceivable. First, enhancement of translation, as observed in *E. coli* (McCarthy *et al.*, 1985). Second, an additional transcription of the proteolipid-encoding gene only, as observed in *Ms. mazei* Go1. Third, multiplication of the proteolipid-encoding gene and insertion the copies into the same polycistronic message.

CONCLUDING REMARKS

The study of enzymes from nonstandard organisms already did and will also broaden in future our understanding of certain aspects of the structure and function of these interesting enzymes. Because of the fascinating variability of the physiology of archaea, we see a variation in structure and function of archaeal ATPases. Proteolipids that arose by gene duplication and triplication events have already been discovered, but perhaps we will see higher degrees of multiplication in the future. We also expect to find different ion specificities of A_1A_0-ATPases, which have already been observed in F_1F_0-ATPases. The overall structure of the ATPases as well as their catalytic properties, ion transport, and coupling mechanism have been well conserved during evolution and, therefore, results obtained from the study of one enzyme can often be generalized. The study of the enzymes from archaea is especially interesting in view of their V_1V_0-like structure. Because of the genetic organization of the genes in operons, a delineation of the exact number of subunits will be possible. The genes from *Ms. mazei* Go1 are functionally expressed in *E. coli*; this will lead to the development of new experimental designs to approach fundamental aspects of the structure and function of A_1A_0-ATPases.

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