Properties of the Plasma Membrane ATPases of the Halophilic Archaebacteria

Haloferax mediterranei and Haloferax volcanii

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Both, Haloferax mediterranei and Haloferax volcanii membranes contain ATPases which are capable of hydrolyzing ATP in presence of Mg\(^{2+}\) or Mn\(^{2+}\). The ATPases require high concentrations of NaCl, a pH value of 9, and high temperatures up to 60 °C. Free manganese ions inhibited the enzyme activity of either ATPase. The ATPases of Hf. mediterranei and Hf. volcanii, respectively, show different sensitivities to inhibitors of ATP hydrolysis. ATP hydrolysis of isolated Hf. mediterranei ATPase was inhibited by NaN\(_3\), which was reported to be specific for F-ATPases, by nitrate and N-ethylmaleimide (NEM), which are specific inhibitors of V-ATPases. ATP hydrolysis of Haloferax mediterranei membranes was not inhibited by DCCD, but \(^{14}C\)DCCD was bound to a 14 kDa peptide of the isolated, partially purified enzyme. Furthermore, the ATPase was inactivated by preincubation with 7-chloro-4-nitrobenzofurazan (NBD-C\(_1\)). The ATPase activity of Hf. volcanii membranes was inhibited by NEM but not by nitrate and Na\(_2\)S\(_3\). SDS gel electrophoresis of the partially purified enzyme of Haloferax mediterranei showed putative ATPase subunits of 53.5, 49, 42, 22, 21, 14, 12, and 7.5 kDa. Immunoblots showed cross reactivity between a 53 kDa peptide and anti-\(\beta\)-chloroplast F\(_1\), as well as between 53, 50 and 47 kDa peptides and an ATPase antibody of Methanosa{rencia barkeri}. The results will be discussed in context with the placement of the archaebacterial ATPases (A-ATPases) between F- and V-ATPases.

Introduction

Up to now, the ATPases are classified into three families: The F-(F\(_1\)F\(_0\))ATPases which have been found in eubacteria as well as in mitochondria and chloroplasts of eukaryote organisms; the P-ATPases (also named E\(_1\)E\(_2\)-ATPases), the only covalently phosphorylated species which exist in plasma membranes of eukaryotic cells or in the sarcoplasmic and endoplasmic reticulum, transporting Ca\(^{2+}\) or other ions; and the V-(vacuolar) ATPases, found in vacuoles or tonoplast membranes of plants, and also in lysosomes, endosomes and Golgi vesicles. The H\(^{+}\)-ATPase of yeast vacuolar membranes also belongs to this family [1]. Among these three groups, the F-ATPases have been considered to be the only ATP-synthesizing ATPases in any living organism. Structure and function of the F-ATPases from eubacteria, higher plants, and animal tissues are very similar, showing that they indeed belong to the same family of proton translocating ATPases. F-ATPases catalyze the formation of ATP driven by a transmembrane proton potential. The catalytic sites are located on the F\(_1\) part of the enzymes, which can be easily solubilized from the membranes. The isolated F\(_1\) part of these ATPases has a subunit stoichiometry of \(\alpha_3\beta_3\gamma_0\) [2, 3]. The \(\alpha\)- and \(\beta\)-subunits have been well conserved during evolution, as could be seen by comparison of the amino acid sequence of the subunits of ATPases from different sources [4]. It has been suggested by several groups [5–7] that the archaebacterial ATPases are distinct from F-ATPases, inspite of the fact that they (so far as investigated) are able to synthesize ATP from ADP and phosphate [8]. Investigation of amino acid composition [6, 7, 9] immunological cross reactivity [10, 11] and inhibition of the enzyme activities by specific inhibitors [12–14] led to

Abbreviations: CF\(_1\), chloroplast coupling factor 1 (ATP-ase); DCCD, N,N\'-dicyclohexylcarbodiimide; DTT, dithiothreitol; NEM, N-ethylmaleimide; NBD-C\(_1\) (NBD), 7-chloro-4-nitrobenzofurazan; P\(_i\), inorganic phosphate; SDS, sodium dodecylsulfate; Tricine, N,N\'-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; Tris buffer, tris-hydroxymethyl aminomethane.

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the surprising finding that the archaebacterial ATPases resemble the eukaryotic V-ATPases more than they do the eubacterial F-ATPases. On the other hand, V-ATPases, so far as known, are not able to synthesize ATP. These findings raised the question whether archaebacteria (Archaea) possess a separate type of ATPase (A-ATPase), which has to be placed somewhere between V- and F-ATPases with respect to the evolution. A couple of reports about the properties of the ATPases of two different Halobacteria (Halobacterium halobium [7, 8] and Halobacterium saccharovorum, [12, 13, 15]) exist already. We have now investigated the properties of the ATPases of Haloferax mediterranei and of Haloferax volcanii, organisms, representatives of a genus of aerobic, halophilic archaebacteria (Archaea), distantly related to Halobacterium [16].

Materials and Methods

Growth conditions

Haloferax mediterranei ATCC 33560 and Haloferax volcanii NCMB 2012 were grown in a medium, containing a mixture of marine salts at a final concentration of 25% (wt./vol.) (SW 25) and 0.5% (wt./vol.) yeast extract [17]. The pH value of the medium was adjusted to 7 using NaOH. The final concentrations of the salts in the salt-water solution were described in ref. [18]. Hf. mediterranei and Hf. volcanii were cultured in 10 l flasks, aerated by a soft air flow, at 37 °C. In order to avoid foaming, five drops of silicone oil (AR 1000, Wacker Chemie, München) were added. The cells usually were harvested after 5 days, corresponding to the optical density (520 nm) of ca. 1.5, according to ref. [17].

Isolation of membranes

The cells were harvested by centrifugation at 10 °C in a Beckmann centrifuge (Model J2-21) at 5000 × g for 45 min. The pellets were pooled, resuspended in SW 25 (room temperature) and passed through a French press (SLM AMINCO FA-078). Rupture of cells occurred at 8000 psi and was usually controlled by a light microscope. After washing the membranes with the same volume of SW 25 and centrifugation (10 °C, 8000 × g, 30 min) in order to remove the remaining unrup-tured cells, the supernatants were collected and transferred to an ultracentrifuge (Beckmann L-8-70 M). Ultracentrifugation followed for 45 min at 140,000 × g and 4 °C. The resulting pellet, containing the membranes, was collected in 25% SW. The final protein yield was about 200–500 mg of protein, using 201 of culture. The membranes could be stored in the refrigerator at −70 °C, for a couple of weeks. The studies described here about the optimum conditions for the ATPase activity of Hf. volcanii were performed with these membrane fractions only, whereas Hf. mediterranei ATPase activity was measured either with membranes or with partially purified ATPase fractions, as indicated.

Isolation of Haloferax mediterranei ATPase

The applied methods for isolation of the Haloferax ATPase have been partially described by Schmidt and Gräber (1985) [19] for isolation of chloroplast F0F1 complex. Aliquots of the membranes were solubilized at room temperature for 30 min with a mixture of 19 m M octylglucoside plus 8 m M cholic acid, followed by centrifugation for 60 min at 25,000 × g (Beckmann centrifuge, Model J2-21, 10 °C). The supernatant contained the protein associated with the ATPase activity, which could be obtained by fractionated precipitation, using the following ammonium sulfate concentrations: 20, 35, 50, 70% (w/v). After both precipitation steps the samples were centrifuged for 30 min at 25,000 × g. ATPase activity tests showed that the 50% ammonium sulfate precipitates contained the highest activities. This fraction was usually further purified. The pellets were resuspended in 4 m NaCl and stored at room temperature, as storage in a refrigerator at −70 °C resulted in loss of activity. As the optimum of ATPase activity was found at a pH of 9, whereas the optimum of growth occurred at pH 7, we tested the storage conditions for membranes of Haloferax mediterranei at both pH values. Most of the activity was kept if the samples were stored at pH 9 and at room temperature.

Further purification of ATPase

ATPase-containing fractions were suspended in a medium containing 30 m M Tris/Cl pH 6.5, 0.2 m M ATP, 0.5 m M EDTA, 0.2% (w/v) Triton
X-100, 2.5% (w/v) preformed asolectine vesicles, and 3.6 M NaCl, followed by layering on top of a discontinuous sucrose gradient. The preparation of asolectine vesicles was carried out according to ref. [19]. The suspension was layered on top of 12% sucrose containing the above described medium, the following concentrations of sucrose were 18, 24, 30 and 40% (w/v). Centrifugation was carried out for 16 h at 10 °C and 250,000 x g (Beckmann L8-70 M, swing-out rotor: SW 55 Ti). The vesicles containing the highest activity were collected from the 12% sucrose layer between coloured retinal proteins (on top) and a polysaccharide band at 18% sucrose. Table I summarizes this course of preparation.

### Electrophoresis and immunodetection

The protein distribution of crude membranes and ATPase fractions was studied in SDS polyacrylamide gels (15%) [20] after staining them by Coomassie Brilliant Blue (G 250) or after silver staining [21]. Molecular masses of polypeptides were estimated from the positions of standard proteins. Antisera against CF1 were raised in rabbits [22] with about 100 μg of the respective (see Results) CF1 subunits. The authors thank Dr. Radunz from Bielefeld for preparing a part of the used antibodies. For Western blot analysis, the peptides were transferred to nitrocellulose membranes and incubated with the indicated antisera. Detection followed by the enhanced chemiluminescence system (ECL) of Amersham [23]. The blotted protein standards were detected by ink stain using Pelikan Brilliant Black 4001, according to Hancock and Tsang [24].

### ATPase assay

ATPase activity was usually measured in a medium containing 50 mM Tris pH 9, 3 M NaCl, 5 mM MgCl2, 5 mM γ-32P-labelled ATP (Amersham-Buchler, specific activity 37 MBq/ml) and in the case of *Hf. mediterranei* either 4–10 μg of ATPase fractions (see point 3 or 4, as indicated) or 40–70 μg of crude membranes (see point 2). In the case of *Hf. volcanii* only crude membranes were used in the experiments, shown here. 32P contents were assayed in the isobutanol/toluol extracts of the phosphomolybdate complex [25]. In some experiments (those accomplished by M. Dane in Alicante) the Pj content was determined colorimetrically according to Taussky and Schorr [26]. Protein determinations were performed either according to Bensadoun and Weinstein [27] (samples containing SDS) or to Lowry [28].

### Results

**Optimum conditions for ATPase activity**

Each step of isolation and purification of ATPases of halophilic bacteria is especially difficult due to the high NaCl concentration (in molar range) which has to be kept. If NaCl was left out of one of the media, the ATPase activity of the enzyme dis-

<table>
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<th>Fractions</th>
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<th>Protein [mg/ml]</th>
<th>Yield of protein [mg]</th>
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appeared at once. KCl was not able to replace NaCl in case of *Haloferax mediterranei*, but in case of *Haloferax volcanii*, 70% of the initial activity could be measured in presence of KCl.

In Fig. 1 ATP hydrolysis of *Hf. mediterranei* and *Hf. volcanii* membranes is plotted against the concentration of NaCl in the incubation medium. The *Hf. mediterranei* ATPase shows an optimum activity in presence of about 3 M NaCl, whereas the *Hf. volcanii* ATPase shows optimal activity at 1.7 M NaCl (both measured at 40 °C). The optimum of the latter one is clearly related to the NaCl concentration in the Dead Sea, the natural environment of the bacterium [29]. The water of the Dead Sea contains 1.75–2.5 M NaCl [30]. *Halofe­rax volcanii* belongs to the more moderate halophilic archaebacteria, whereas *Halofe­rax mediterranei* can be collected from ponds with higher salt concentrations [16]. Furthermore, we found an optimum pH of 9 for ATP hydrolysis of both bacterial ATPases. In both cases, however, fairly good activity could be measured also at a pH value of 7 (Fig. 2). This is in accordance to data published by Schobert and Lanyi [43] about the ATPase of *Halobacterium saccharovorum*, whereas most of the investigated Archaea ATPases show pH optima of around 6.

The optimum temperature for ATPase activity of both organisms was about 60 °C, as shown in Table II. This is not surprising, as the investigated

![Fig. 1. NaCl concentration dependence of ATP hydrolysis of *Halofe­rax mediterranei* (■) and *Halofe­rax volcanii* (●). 70 μg of crude membranes of each bacterium were incubated at 40 °C in presence of 5 mM ATP and 5 mM MgCl₂ and the indicated NaCl concentrations. Each rate was calculated from kinetical analysis. Control activities: (■), 0.166 μmol P_i·mg protein⁻¹·min⁻¹; (●), 0.249 μmol P_i·mg protein⁻¹·min⁻¹.](image1)

![Fig. 2. ATP hydrolysis, dependent of the medium pH value. ATP hydrolysis of *Halofe­rax mediterranei* (○) and *Halofe­rax volcanii* (●) ATPases was performed with 70 μm of membranes in either case. Each rate was calculated from kinetical analysis. The following buffers were used: pH 6–6.5 MES, pH 6.5–7.5 HEPES, pH 7.5–8.5 Tricine, pH 8.5–10 glycylglycine. The rates of overlapping pH values were averaged. Maximum rates: (○), 0.675 μmol P_i·mg protein⁻¹·min⁻¹; (●), 0.279 μmol P_i·mg protein⁻¹·min⁻¹.](image2)

![Table II. ATP hydrolysis of *Halofe­rax* membranes, measured at different temperatures. The ATPase activity of 70 μg of crude archaebacteria membranes was tested in a medium containing 3 M NaCl, 5 mM ATP, 5 mM MgCl₂, and 50 mM Tris pH 9. The samples were incubated in a water-bath which was adjusted to the indicated temperatures. Each rate was calculated from 3 point kinetical analyses. The amount of unspecifically hydrolyzed ATP was measured at all temperatures and subsequently subtracted. Other conditions as in „Methods“.](table1)
organisms had to develop strategies to live in the very warm salt ponds, e.g. on the coasts of southern Spain. Since temperatures higher than 40 degrees in the control samples caused rather high unspecific ATP hydrolysis, we usually tested the ATPase activity of both organisms at 40 °C.

ATPase activity was dependent on Mg$^{2+}$, which could easily be replaced by Mn$^{2+}$, in both organisms. With Ca$^{2+}$, about 60% of the original activity could be found in *Hf. mediterranei* membranes (not shown). Fig. 3 shows the hydrolysis of ATP as a function of the Mg$^{2+}$ and Mn$^{2+}$ concentrations, respectively. Using MgCl$_2$, saturation of the reaction (*Haloferax mediterranei*) was reached at equimolar concentrations of the respective divalent ion and ATP. In contrast to this typical saturation slope, application of Mn$^{2+}$ resulted in an optimum of activity at less than equimolar concentrations, higher concentrations of Mn$^{2+}$ inhibited both enzymes. This resembles the dependency of divalent cations of isolated chloroplast ATPase: ATP hydrolysis of this enzyme also is inhibited by free Mn$^{2+}$ ions, but not by free Mg$^{2+}$ [31].

Fig. 4 shows kinetical analyses of the ATP hydrolysis by membranes of *Haloferax mediterranei* and by the most active sucrose gradient fraction. Investigating membranes, the activity usually slowed down after about 5–6 min. Thus, our results usually have been calculated from kinetical analysis of the first 3 min. Schobert and Lanyi [32] described a hysteretical behavior of *Halobacterium saccharovorum* ATPase, showing two different states of activity during the time curve of ATP hydrolysis. Using the partially purified enzyme (Fig. 4B), we found that the time-dependent hydrolysis of ATP is linear, for at least up to 20 min, which is not in line with a hysteretical behavior.

We found $K_M$ values for ATP of 5 mM for *Hf. mediterranei* and of 12 mM for *Hf. volcanii* in presence of equimolar concentrations of Mg$^{2+}$.

To test whether the activity of the ATPases can be increased by reduction, membranes have been incubated in presence of 50 mM DTT, a usual application method for activation of the chloroplast F-ATPase. Compared to CF$_1$, which via reduction shows an increase in activity by a factor of 34, the archaeabacterial ATPases show only a small stimulation effect of a factor of about 2 (*Hf. mediterranei*) or 1.4 (*Hf. volcanii*) measured after 2 h of preincubation in presence of DTT. A factor of 2 could also be found by addition of 80 mM (final concentration) sodium sulfite (results not shown).

As preparations from *Halobacterium saccharovorum* showed a 20-fold increase in activity if Tri-
ton X-100 was added [12], we tested the effect of different concentrations of this detergent on either Haloferax membranes. We found no stimulation in case of Hf. mediterranei membranes and a minor one of a factor of 1.3 in case of Hf. volcanii using 0.01% of Triton (here not shown).

Inhibition studies

Several inhibitors of different ATPase types were tested for their inhibitory effects on both ATPases. ATP hydrolysis was not inhibited by DCCD, which is not corresponding to the result found with Halobacterium saccharovorum ATPase. We tested Haloferax mediterranei and Haloferax volcanii membranes, which were preincubated with 500 μM DCCD up to 2 h at pH 7 or at pH 9, and found no inhibition of ATPase activity. The DCCD-binding proteolipids in F-type ATPases belong to the F₀ part and have proton conducting functions. Testing membranes, the proton conducting part of the enzyme, if it is buried in the membrane, may not be accessible for DCCD. In preliminary experiments concerning isolated and partially purified ATPase of Hf. mediterranei, we found a membrane peptide of about 14 kDa (compare Fig. 7), difficult to detect by Coomassie staining, which was labelled by [¹⁴C]DCCD, and which most probably belongs to the subunits of the membrane part of the ATPase. This peptide may be identical with the proteolipide which forms the proton channel.

Na₃VO₄, which is a potent inhibitor of P-type ATPases and competes with phosphate, does neither inhibit Hf. mediterranei — nor Hf. volcanii — ATPase activity. We tested the effect of this P-ATPase inhibitor using concentrations up to 1 mM.

The partially purified enzyme of Hf. mediterranei is inhibited by NBD-Cl, a potent inhibitor of F-ATPases, which binds covalently to tyrosines and/or lysines within the ATPase [33]. As shown in Fig. 5, the concentration of NBD-Cl for the half maximal inhibition was 300 μM.

Sodium azide had no inhibition effect, if tested in concentration ranges effective for chloroplast ATPase (up to 500 μM), but if applied at higher concentrations (1–10 mM) inhibition of ATPase activity could be found. The enzyme was completely inactivated at 10 mM azide. Half maximal inhibition was achieved at 3 mM NaN₃ (Fig. 5). In contrary to this finding, Hf. volcanii membranes showed no sensitivity against azide. Nitrate, which inhibits vacuolar ATPases very effectively in different manners, depending on the concentration added (see Discussion), is effective in the inhibition of Haloferax mediterranei ATPase, too: The half maximal inhibition was found at 2.5 mM NaN₃ (Fig. 5). The Haloferax volcanii ATPase surprisingly is not inhibited by nitrate, added up to 20 mM. Mullakhanbhai and Larsen [29] reported that they could replace NaCl in the culture medium partially by NaN₃, which had a positive effect on the growth of the bacteria. This observation may deliver the explanation for the specific behavior of the ATPase.

NEM was less effective, as can be seen in Fig. 6. Inhibition was not complete and the C₁₅₀ value is about 6 mM for Hf. mediterranei ATPases. Hf. volcanii ATPases (membranes) are inhibited only up to 15%. Membrane fractions are often less inhibited than isolated ATPases; we assume that the reason for this discrepancy may be found in the high
Fig. 6. Influence of NEM on *Haloferax mediterranei* ATPases (partially purified, sucrose gradient fraction, 4 µg) (●) and *Haloferax volcanii* ATPase (membranes, 70 µg) (○). Conditions as in Fig. 5. Control activities: 12.22 (●) and 0.605 µmol P\textsubscript{i} mg protein\textsuperscript{-1}·min\textsuperscript{-1} (○).

amount of negative charges on the surface of membranes of halophilic organisms.

**Subunit composition and immunological cross reactivity of *Haloferax mediterranei* ATPase**

Fig. 7 shows the silver-stained gel of the membranes (lane 1) and the most active sucrose gra-

![Silver-stained gel](image)

**Fig. 7.** Part I: Silver-stained SDS-PAGE of the 50% ammonium sulfate precipitation (lane 1), the 12% sucrose fraction from the density gradient (lane 2). Part II: Western blots of *Haloferax mediterranei* membranes with anti-γ CF\textsubscript{1} (lane 3) and anti-*Methanosarcina barkeri* antibodies (lane 4). Lane 5 shows a Western blot of CF\textsubscript{1} against anti-CF\textsubscript{1} as a marker for the molecular masses.

The resulting subunit patterns are shown in Fig. 7, part II. *Anti-γ-CF\textsubscript{1}* (not shown), which specifically labelled γ- and β-subunits of CF\textsubscript{1} but not α, reacted mainly with bands at 58/56, 52 and 47 kDa of *Haloferax mediterranei* membranes; a weak reaction could also be seen in the region of 53 kDa. *Anti-Met*, however, which reacts with β and γ of CF\textsubscript{1} (not shown), mainly labelled a 53, a 49 and again the 47 kDa peptide; additionally to a 22 kDa peptide of the *Hf. mediterranei* membranes. *Anti-β* antibodies (specific against β-subunit of CF\textsubscript{1}), reacted with mainly two subunits of *Hf. mediterranei* ATPase with an apparent molecular mass of about 54 and 49 kDa (M. Dane, personal communication). To sum up: Our results permit the conclusion that at least the 53.5, 49, and 22 kDa peptides of Fig. 7, lane 2 belong to the *Haloferax mediterranei* ATPase.

**Discussion**

Archaebacterial ATPases have been described to lack sensitivity to azide inhibition [37, 38], and to lack minor subunits [7, 10]. Both criteria (sensitivity to azide and minor subunits) are common characteristics for F-ATPases. In contrast to these reports, *Haloferax mediterranei* membranes contain an ATPase which is inhibited by azide. Recently, it could be shown that Mg\textsuperscript{2+}-dependent ATP hydrolysis of isolated, differently activated chloroplast F-ATPase is sensitive to azide, irrespective of the applied method of enzyme activation, whereas the Ca\textsuperscript{2+}-ATPase activity was less inhibited [39]. This, in our opinion, may be inter-
interpreted to be an effect of binding of the negatively charged azide ion via the positive charge of Mg\(^{2+}\) to the Mg-binding site(s) of the enzyme. The molecular mechanism of azide inhibition, however, is not yet understood. Takeda et al. [40] found that F\(_1\) of an *E. coli* mutant in which serine 174 (in β) was replaced by phenylalanine, was less sensitive to azide. The authors suggest from their results, that the azide-binding site may be in the near of serine 174. The sensitivity of *Hf. mediterranei* ATPase against azide may be an indication for the position of halophilic archaeabacterial ATPases between F-ATPases of eubacteria, chloroplasts, and mitochondria, on one side, and vacuolar ATPases of higher plants, and yeast on the other. In accordance with the reports concerning *Sulfolobus acidocaldarius* and *Halobacterium halobium* [37, 38], and in contrast to *Haloferax mediterranei*, neither the membrane fraction nor the partially purified enzyme of *Haloferax volcanii* is inhibited by azide. This may be due to the diversity of the archaea, and moreover to the diversity of the halophilic bacteria.

We tested sensitivities against a couple of different ATPase inhibitors. Among these, N-ethylmaleimide (NEM) seems to be interesting, as it covalently binds to sulfhydryl residues of cysteines in the proteins [41]. F-ATPases, so far as known, have less cysteines in their big subunits. CF\(_1\), for instance, contains only one cysteine per β and one per α-subunit, and both are not associated to nucleotide-binding sites or catalytic centers. ATP hydrolysis of both, isolated and membrane-bound CF\(_1\) is not inhibited by NEM (own results). In contrast to this, V-ATPases, so far as known, were inhibited by NEM. Zimniak et al. [41] pointed out, that the 69 kDa subunit of carrot vacuolar ATPase contains three cysteines within the catalytic region, one of them at the Mg\(^{2+}\)-binding site (Cys 279). This, surely, is the reason for the sensitivity of the enzyme to NEM. In view of this, *Haloferax* ATPases occupy an intermediate position: The ATPase activity is inhibited, but not complete and in a weaker manner. A very similar finding has been described for *Halobacterium halobium* ATP hydrolysis as well as synthesis by Mukohata and Yoshida [8]. The amino acid sequence of *Halobacterium halobium* α-subunit shows, that there are two cysteines, one of which is quite near to a proposed nucleotide-binding site [7]. This one is conserved in *Methanosarcina barkeri* – [42] as well as in *Sulfolobus acidocaldarius* – α-subunits [9] and we can expect, that it also will be found in *Haloferax mediterranei* and in *Haloferax volcanii* α-subunits. *Halobacterium halobium* α-subunits are 47% homologous to A-subunits of H\(^{+}\)-ATPases of higher plants [7] and about 50–65% among other archaeabacteria. In contrast to this, the homology to F-ATPases is only 20–30%.

*Haloferax mediterranei* ATPase as well as *Haloferax volcanii* ATPase, both contain small subunits, at least one 22 kDa peptide, but most probably more. The subunit distribution of *Haloferax volcanii* ATPase will be described elsewhere. Schobert and Lanyi [43] reported that *Halobacterium saccharovorum* contains 5 putative subunits, the smaller ones of 31, 22, and 14 kDa. Lübben et al. [11] found a 20 kDa and a 12 kDa subunit additional to the big subunits of *Sulfolobus acidocaldarius* ATPase.

Our results show that the big subunits of *Haloferax mediterranei* ATPase have apparent molecular masses which are comparable to F-ATPases (53 and 49 kDa). In contrast to this result, most other described archaeabacteria ATPases have been described to be closer related to V-ATPases with respect to the molecular masses of their big subunits (compare Table III).

The *Haloferax mediterranei* membrane ATPase seems to resemble F-ATPases of eubacteria and higher organisms more than V-ATPases. *Methanosarcina* antibodies reacted with two big subunits of *Hf. mediterranei* ATPase. This is consistent with the close relationship of archaeabacterial ATPases among another. On the other hand, we could observe cross reactivity between antibodies against subunits of CF\(_1\) and *Hf. mediterranei* membranes. This is consistent with the idea that *Haloferax* ATPase resembles the F-type ATPases. On the other hand, the enzyme is inhibited by nitrate and NEM, which are called specific inhibitors for V-ATPases.

Nitrate is reported to react competitive with respect to Mg-ATP in experiments with the tonoplast H\(^{+}\)-ATPase from *Beta vulgaris* roots [48]. This possibly can be interpreted in a way that the anion may become bound to a positive counter charge in the region of the nucleotide-binding site. At least one positive charge is required for the binding of a substrate nucleotide, that is for the
Table III. Molecular masses and numbers of large subunits of diverse ATPase types.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type</th>
<th>Molecular mass [kDa]</th>
<th>Number of subunits</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Halofax mediterranei</em></td>
<td>A-ATPase</td>
<td>$x$: 53, $\beta$: 49</td>
<td>at least 3</td>
<td>this paper</td>
</tr>
<tr>
<td><em>Halobacterium halobium</em></td>
<td>A-ATPase</td>
<td>$x$: 86, $\beta$: 64</td>
<td>2</td>
<td>[7]</td>
</tr>
<tr>
<td><em>Halobacterium saccharovororum</em></td>
<td>A-ATPase</td>
<td>$x$: 110, 87*, $\beta$: 71, 60*</td>
<td>5, 4*</td>
<td>[15*, 43]</td>
</tr>
<tr>
<td><em>Sulfolobus acidocaldarius</em></td>
<td>A-ATPase</td>
<td>$x$: 65, $\beta$: 51</td>
<td>4</td>
<td>[6, 9]</td>
</tr>
<tr>
<td><em>Methanobacterium tindarius</em></td>
<td>A-ATPase</td>
<td>$x$: 67, $\beta$: 52</td>
<td>4</td>
<td>[14]</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>A-ATPase</td>
<td>$x$: 62, $\beta$: 49</td>
<td>2</td>
<td>[42]</td>
</tr>
<tr>
<td>Lysoosomal $H^+$-ATPase</td>
<td>V-ATPase</td>
<td>A: 72, B: 57</td>
<td></td>
<td>[45]</td>
</tr>
<tr>
<td>$H^+$-ATPase of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromaffin granules</td>
<td>V-ATPase</td>
<td>A: 72, B: 57</td>
<td></td>
<td>[44]</td>
</tr>
<tr>
<td>Carrot vacuolar ATPase</td>
<td>V-ATPase</td>
<td>A: 69</td>
<td></td>
<td>[41]</td>
</tr>
<tr>
<td>Chloroplast ATPase, F$_1$</td>
<td>F-ATPase</td>
<td>$x$: 54.8, $\beta$: 53.7</td>
<td>5</td>
<td>[34, 35]</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, F$_1$</td>
<td>F-ATPase</td>
<td>$x$: 55.3, $\beta$: 50.2</td>
<td>5</td>
<td>[46, 47]</td>
</tr>
</tbody>
</table>

counter charge of $P_k$ of the phosphate chain, as could be shown by studies using chiral phosphoro-
thioate analogues of ADP and ATP [49]. At higher concentrations, nitrate works as a chaotropic an-
ion [50], disturbing electrostatic and hydrophobic interactions which are essential for the main-
tenance of the function of any enzyme.

The functional role of F-ATPases is the synthe-
sis of ATP. The ATPases of archaebacteria (Ar-
chaea) are expected to synthesize ATP, though this
directly could be demonstrated only for *Halobac-
terium halobium* [8, 37]. Evidences for ATP syn-
thesis in whole cells of *Methanosarcina barkeri*
have been reported by Mountfort already in 1978
[51] and by Blaut and Gottschalk in 1985 [52]. This
function together with some F-ATPase like prop-
erties, in the case of *Halofax mediterranei* the
size and putative distribution of subunits, and the
inhibition by azide, lead to the suggestion that
archaebacteria (Archaea) possess F-type ATPases.
F-ATPases exist in prokaryotes as well as in mito-
chondria and chloroplasts of eukaryotes. V-ATP-
ases, so far as known, are not able to synthesize
ATP.

Available sequences from different archaebac-
teria, for instance the $\alpha$- and $\beta$-sequences of *Me-
thisorarcina barkeri* ATPase [42], of *Halobacte-
rium halobium* [7] and of *Sulfolobus acidocaldarius*
[6], on the other hand, substanciated a close relation-
ship of A-ATPases to vacuolar $H^+$-ATPases.
This is supported by an 80 amino acid long inser-
tion which is present in the catalytic subunits of
the cited A- and V-ATPases, but neither in the ca-
talytic nor non-catalytic subunits of F-ATPases
[5]. These similarities recently led to the specula-
tion, that vacuolar ATPases, which can only be
found in eukaryotic cells, have been derived from
an archaebacterium-like organism [53]. This re-
 mains pure speculation, until more information is
available about A-ATPases, especially about the
morphological differences which lead to different
functions (ATP hydrolysis/ATP synthesis) of both
ATPase types.

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