Isolation, Characterization, and Substrate Specificity of the Plasma Membrane ATPase of the Halophilic Archaeon *Haloferax volcanii*

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Isolated membranes of the moderate halophilic bacterium *Haloferax volcanii* are able to hydrolyze ATP via an ATPase, which needs the presence of Mg^{2+} or Mn^{2+}, high concentrations of NaCl, a pH value of 9, and high temperatures with an optimum at 60 °C. We have not found any phosphatase activity in the preparations. We developed a purification method for the isolated enzyme with an enrichment factor of 90. SDS-gel electrophoresis of the partially purified enzyme of *Haloferax volcanii* showed putative ATPase subunits of 63, 51, 37, and 12 kDa. N-ethylmaleimide (NEM) a specific inhibitor for V-ATPases, which alkylates cysteines, inhibited the enzyme slightly. Binding of tritiated NEM to the isolated ATPase fractions resulted in labelling of the 63 and 51 kDa peptides. Using PCR with degenerate oligonucleotides, we could clone and sequence a gene cluster encoding the A_1 part of the halophilic ATPase. The described genes are organized in an operon in the order D, C, E, B, A, named alphabetically according to their decreasing size. The deduced products of 64.5, 52, 38.7, 22, and 11.6 kDa confirm the results of the partial purification of the ATPase.
Biochemical characterization of the *Haloferax volcanii* ATPase gave the following results: In presence of Mn^{2+} higher rates of ATP hydrolysis could be observed than in presence of Mg^{2+}, but free manganese ions inhibited the enzyme activity of the ATPase. Calculation of the true concentrations of the complex between ATP and the respective divalent metal ion led to determination of Michaelis-Menten constants for ATP in the hydrolysis direction of 1 mM in presence of $MgCl_2$ and 0.24 mM in presence of $MnCl_2$. Sodium chloride concentrations in the molar range induce changes in K_M by a factor of about 10. The enzyme is specific for ATP; other nucleotides including GTP and ADP are competitive inhibitors of ATP hydrolysis.

Introduction

Among different ATPases, the F-ATPases have been considered to be the only functional ATP synthases in eukaryotic cells or bacterial plasma membranes. 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. Their 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 $\alpha_3\beta_3\gamma\delta\varepsilon$ (Moase and Green, 1981). The subunits are named $\alpha\beta\gamma\delta\varepsilon$ according to their decreasing molecular weight. Subunit β is the catalytic subunit. In vacuolar ATPases, the subunits are called ABCDE, the catalytic subunit, A, has a higher molecular weight than the regulatory one (B), although B corresponds to F-ATPase α and A to F-ATPase β (Nelson, 1992). The α- (B-) and β-(A-) subunits have been well conserved during evolution, as can be seen by comparison of the amino acid sequences of the ATPase subunits derived from different sources (Walker *et al.*, 1985). The archaeal (formerly: archaebacterial) ATPases are found to be distinct from F-ATPases, inspite of the fact that they are able to synthesize ATP from ADP and phosphate (Mukohata and Yoshida, 1987a, own results).

Genetically derived amino acid sequences of the large subunits of archaeal ATPases (Denda *et al.*,

Abbreviations: AF_1, archaeal coupling factor 1 (ATPase); bp, base pair(s); NBD-Cl, 4-chloro-7-nitrobenzofurazan; NEM, N-ethylmaleimide; PEG, polyethylene glycol; P_i, inorganic phosphate; SDS, sodium dodecylsulfate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; Tris, tris-hydroxymethyl aminomethane.

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1988 a,b; Ihara and Mukohata, 1991), immunological cross-reactivity (Konishi *et al.*, 1990; Lübben *et al.*, 1988) and inhibition of the enzyme activities by specific inhibitors (Kristjansson and Hochstein, 1985; Schobert, 1991; Scheel and Schäfer, 1990; Dane *et al.*, 1992) revealed the finding that the A_1-part of archaeal ATPases resemble the eukaryotic V-ATPases rather than the eubacterial F-ATPases. The strong correspondence of archaeal ATPases to V-ATPases, in this respect, led to the nomenclature A and B, according to the V-ATPase nomenclature. On the other hand, V-ATPases are not able to synthesize ATP; their function is to generate a $\Delta\mu_H^+$ at the expense of ATP-hydrolysis. These findings require more detailed investigations on different archaeal ATPases and comparison of their structures and functions. This paper reports on the identification of subunits of an ATPase isolated from *Haloferax volcanii*, a representative of the aerobic, halophilic archaea.

Materials and Methods

1. Growth conditions

Haloferax volcanii WR 340 was a gift of Dr. J. Soppa (MPI Martinsried). The archaea were grown in a medium, containing a mixture of marine salts at a final concentration of 15% (wt/vol) (SW 15), and 0.5% (wt/vol) yeast extract. The pH value of the medium was adjusted to 6.8 using NaOH. The final concentrations of the salts in the saltwater solution were described in (Mullakhanbhai and Larsen, 1975). *Haloferax volcanii* was 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 3 days, corresponding to the optical density (520 nm) of about 1.

2. Isolation of membranes

The cells were harvested by centrifugation at 10 °C in a Beckman centrifuge (Model J2–21) at 5000 x *g* for 30 minutes. The pellets were pooled, resuspended in SW 15 (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 15 and centrifugation (10 °C, 8000 x *g*, 30 min) in order to remove the remaining unruptured cells, the supernatants were collected and transferred to an ultracentrifuge (Beckman L8–70M). Ultracentrifugation followed for 45 min at 140 000 x *g* and 4 °C. The resulting pellet, containing the membranes, was collected in 1.75 M NaCl, 50 mM Tricine-NaOH pH 7.5. The final protein yield was about 200–400 mg of protein, using 20 l of culture. The membranes could be stored during some weeks in the refrigerator at −70 °C.

3. ATPase isolation and purification

Membranes (see section 2) were diluted to a protein content of 20 mg per ml and passed through the French press (see above) at 20000 psi to remove the ATPase from the membrane (Hochstein *et al.*, 1987). After ultracentrifugation for 45 min at 140 000 x *g* (Beckman L8–70M), the ATPase activity could be found in the supernatant, and was obtained by fractionated precipitation using the following polyethylene glycol concentrations: 10, 15, 25 % (weight / volume). Solid polyethylene glycol (M_r 4000) was added in small portions on ice, under constant stirring, up to the indicated final concentrations. After 20 min of further stirring, the protein was collected by centrifugation for 20 minutes at 15 000 x *g* and 4 °C in a Beckman centrifuge (Model J2–21). We found the activity in the 25% PEG fraction. This pellet usually was resuspended in 1.75 M NaCl plus 50 mM Tricine pH 7.5, and stored at 4 °C, as storage in a refrigerator at −70 °C resulted in loss of activity.

The solution (200 µl) was applied to a superose 6 column HR 10/30 (Pharmacia) equilibrated with 1.75 M NaCl, 50 mM Tricine pH 7.5 and 100 mM $MgCl_2$. We used the same buffer to elute the ATPase.

The active peak fractions of the superose column were collected, pooled and subjected to ammonium sulfate mediated chromatography. This technique involves the absorption of the unfractionated proteins to the matrix at high ammonium sulfate concentration. Elution and separation of the proteins occurred by applying a decreasing concentration gradient of the same salt. The solution was applied to a Mono Q HR 5/5 (Pharmacia) column equilibrated with a buffer containing 2.4 M ammonium sulfate, 1 M NaCl, 50 mM $MgCl_2$ and

50 mM tris-hydroxymethyl aminomethane-HCl pH 7.5. Elution was achieved starting an increasing gradient of the following buffer: 0.8 M ammonium sulfate, 2.2 M NaCl, 50 mM $MgCl_2$ and 50 mM Tris pH 7.5.

5. Electrophoresis and immunodetection

We studied the protein distribution of crude membranes and ATPase fractions in SDS-polyacryl amide gels (15%) after silver staining. Molecular masses of polypeptides were estimated from the positions of standard proteins.

6. Cloning and sequencing

Genomic DNA from *Haloferax volcanii* WR 340 was prepared as described previously (Steinert *et al.*, 1995).

Northern and Southern Hybridization experiments were performed according to standard protocols. α-^{32}P-labelled gene probes were obtained by using the random primed labeling kit from Boehringer (Mannheim). Restriction fragments were subcloned in p Bluescript SK$^-$ and pUC19, respectively, using *E. coli* XL-1 Blue as hosts. Sequencing was carried out by employing the dideoxy chain termination method using the T7 sequencing kit (Pharmacia, Freiburg). Regions forming secondary structures during sequencing reactions or during electrophoresis were additionally sequenced with the TaQence kit (USB, Cleveland) using Taq-polymerase, both, with and without 7-deaza-dGTP, and with the T7 sequencing kit using 7-deaza-dGTP and 7-deaza-dATP.

7. ATPase assay

According to our earlier protocol (Dane *et al.*, 1992), ATPase activity was measured in a medium, containing 50 mM Tris pH 9, 1.75 M NaCl, 5 mM $MgCl_2$, 5 mM γ-^{32}P-labelled ATP (Amersham-Buchler, specific activity 37 MBq/ml) and 70 µg of membranes (see section 2) or 2 µg of purified enzyme. The temperature was 40 °C and the final volume 0.5 ml. $^{32}P_i$-contents were assayed in isobutanol/toluol extracts of the phosphomolybdate complex.

8. Chemical modification of cysteine residues

Binding of ^{3}H-labelled N-ethylmaleimide (Du Pont, Bad Homburg; specific activity 25 mCi/ ml) was achieved by incubation of 200 µg of the partially purified enzyme received by gel filtration (superose) with the tritiated NEM for 5 hours. Termination of the reaction and removal of unbound NEM was accomplished by twofold precipitation of the enzyme with trichloroacetic acid (final concentration 3 M). The amount of bound NEM per peptide band was studied in SDS polyacrylamide gels (12%), after staining with Coomassie blue. The putative ATPase subunit bands were cut out, solved in 30% alkaline H_2O_2 and measured by liquid scintillation counting.

Results

Purification

In a previous paper (Dane *et al.*, 1992), we described the isolation and partial purification of *Haloferax mediterranei* ATPase by ammonium sulfate precipitation and successive sucrose gradient centrifugation. For *Haloferax volcanii*, this method was not useful. Most of the activity was lost *via* distribution in the step of ammonium sulfate precipitation. Purification of this halophilic enzyme means that about 2 mol/ l of sodium chloride has to be kept in all media. In fact most of the usual purification methods turned out to be not successful.

Nevertheless, we developed a purification method, which provides a high yield of active enzyme. The method described here, gave a 90 fold purification with a yield of 32%. The specific activity of the final product was 31.4 µmol P_i/mg protein per min. The results of the different isolation and purification steps are summarized in Table I.

Halobacterium saccharovorum ATPase has been purified 20 fold with a yield of 40% and a specific activity of 0.86 µmol P_i/mg of protein per min (Schobert and Lanyi, 1989). The same ATPase was reported to be purified by a factor of 70 by Hochstein *et al.* (1987), but with a yield of 13%. *Halobacterium halobium* ATPase has been purified by a factor of 23 (Nanba and Mukohata, 1987), the *Methanolobus tindarius* enzyme 80 fold by Scheel and Schäfer, (1990). The specific activity

Table I. Purification of *Haloferax volcanii* ATPase.
Activity test conditions: 1.75 M NaCl, 50 mM Tris pH 9, 10 mM $MgCl_2$, 10 mM ATP. The temperature was 40 °C. The final volume of 0.5 ml contained the following protein concentrations: membranes: 70 µg, isolated ATPase: 60 µg, PEG-precipitated fraction: 20 µg, column fraction: 2 µg. The purification factor was calculated by increasing activity. All other conditions are described in Methods.

Fractions	Total yield of protein [mg]	Specific activity (µmol P_i/mg protein per min)	Total activity (µmol P_i/min)	Yield (%)	Purification factor
Isolated membranes	290	0.35	101.5	100	1
Isolated ATPase	174	0.507	88.2	87	1.4
PEG-precipitate	4	9.0	36	35	26
Superose column fraction	1.04	31.4	32.7	32	90

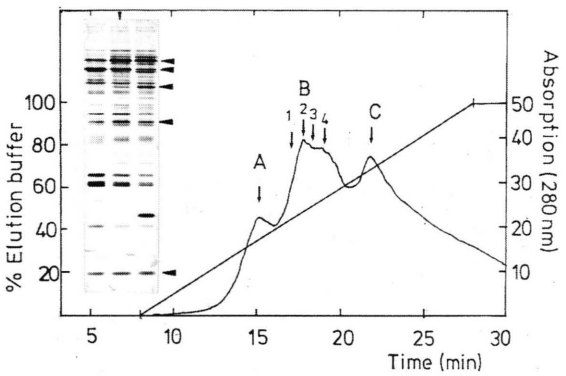

Fig. 1. Ammonium sulfate mediated chromatography (HPLC; high pressure liquid chromatography) of the pooled active fractions recieved by gel filtration. Chromatography was carried out at room temperature with a strong anion exchanger column (Mono Q, Pharmacia) operated at 1.5 MPa with a flow rate of 0.5 ml/min. 250 µg of protein were applied to the column, which was equilibrated with 2.4 M ammonium sulfate, 1 M NaCl, 50 mM $MgCl_2$, 50 mM Tris buffer pH 7.5. The ATPase was eluted with 0.8 M ammonium sulfate, 2.2 M NaCl, 50 mM $MgCl_2$, 50 mM Tris pH 7.5 with a linear gradient of 0–100% of this elution buffer within 20 minutes. Detection of the eluted proteins was achieved by measurement of the absorbance at 280 nm. Fractions of 250 µl were collected and 50 µl tested for ATPase activity. The active fractions (peak B) are indicated by numbers. Activities: 1; 3.8 µmol P_i/mg protein per min, 2; 4.8 µmol P_i/mg protein per min, 3; 3.5 µmol P_i/mg protein per min, 4; 2.8 µmol P_i/mg protein per min. Peak A and C contained no ATPase activity. The residual 200 µl of the active fractions (about 7 µg protein) were transferred to silver stained SDS PAGE (15 % acryl amide). Fractions 1 to 3 are shown as inset in the diagram (from left to right). The most active fraction (2) is marked by an arrow.

reported here is higher than those reported for the other halophilic bacteria.

Fig. 1 shows the accumulation of the subunits employing ammonium sulfate mediated HPLC (Mono Q column, Pharmacia). This method has been described to be very useful for the purification of halobacterial enzymes (Mevarech *et al.*, 1976; Leicht and Pundak, 1981). Fig. 2 shows the result of this separation together with the analysis of the fractions *via* SDS-PAGE. We indicated the fractions containing the remaining ATP hydrolysis activities by the numbers 1–4 in the elution profile as well as in the SDS gel. Unfortunately the total yield of activity was by a factor of 10 lower than that of the gel filtration products in presence of 200 mM $MgCl_2$ (Table I), which most probably is due to the relatively low Mg^{2+} content of the elution buffer (50 mM). Fraction 3 contained the specific activity of 4.8 µmol P_i/mg protein per minute. This problem is not easy to solve, as increasing any ion concentration leads to precipitation of the buffer due to the applied high ammonium sulfate concentrations. Thus, we did most of the examinations of the enzymatic activity with the more crude gel filtration product.

Subunit distribution

The most active fractions shown in Fig. 1 (inset) show several bands in the silver stained gel (indicated by arrows.) It should be noted, that this staining method means an increased sensitivity by a factor of at least 100 compared to the in this field

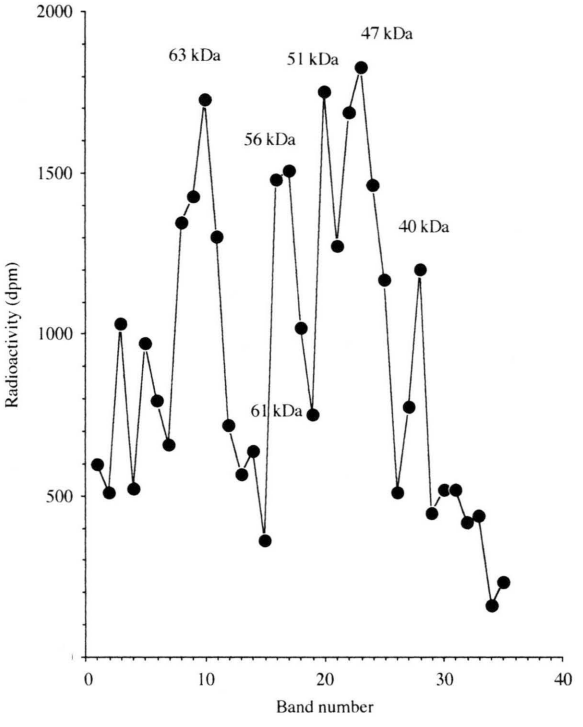

Fig. 2. Radioactivity profile of [^{3}H]NEM-labelled peptides of the partially purified *Haloferax volcanii* ATPase. The peptide bands were cut out of the gel, dissolved in H_2O_2 as described in Materials and Methods and the radioactivity was measured by liquid-scintillation counting. The numbers indicate the molecular masses of the bands, calculated from the gel. The 63 and 51 kDa bands are putative ATPase subunits, whereas the 61 kDa band belongs to the pyruvate decarboxylase of *Haloferax* (identified by blotting and transferring to a gas-phase sequencer).

commonly used Coomassie blue staining method. Nevertheless, we were able to discriminate a few bands out of all fractions, which were accumulated exclusively in the active fractions. These accumulated peptides, which are shown in Fig. 1, have apparent molecular masses of 63, 56, 51, 37 and 12 kDa.

Examining immunological cross-reactivity of the column fractions with different antisera gave evidence that the 51 kDa- and the 63 kDa- peptides belong to the ATPase (Steinert *et al.* 1995a). Furthermore, the large subunits of the ATPase could be labelled by tritiated NEM (N-ethylmaleimide). NEM is a potent inhibitor of V-ATPases, alkylating SH-groups of cysteines within the enzymes. It inhibited the halophilic ATPases too, as shown in our previous paper (Dane *et al.*, 1992). Inhibition of ATP hydrolysis in case of the *Haloferax* ATPase was less effective compared to V-ATPases (Zimniak *et al.*, 1988). Incubation of the enzyme for five hours with tritiated NEM, however, led to high amounts of radioactivity in the 63 kDa and 51 kDa-peptides, but also in the 56 kDa band and some other peptides. Fig. 2 shows the radioactivity profile of the labelled peptides. The result confirms the previously done immuno-labelling despite the fact that more peptides have been labelled, which do not belong to the ATPase as judged from the following results.

Structure of the atp gene cluster

In a recent paper (Steinert *et al.*, 1995), we describe the cloning and sequencing of the large ATPase subunit (A, B) encoding genes. The deduced amino acid sequences have calculated molecular weights of 64.5 and 52 kDa. We were now able to clone two genomic DNA fragments which are overlapping with the previously described fragment containing the genes atpA and atpB. These fragments, received with the enzymes XhoI and PstI, were completely sequenced in both directions. We obtained three additional reading frames upstream of atpA. With respect to their size and by similarity to known ATPase subunits, they were designated atpD, atpC, and atpE. Upstream of atpD, we identified a 5' untranslated region of 550 bp, whereas the non-coding regions between the reading frames are extremely short. AtpA is separated from atpB by only four nucleotides; there is a spacer of six bp between atpA and atpE, whereas the start and stop codons of the reading frames in front of atpE are even overlapping by 4 bp, each. The reading frames atpD, C, and E were translated into amino acid sequences of 194, 348, and 106 residues. The calculated molecular weights are 22.03 (D), 38.76 (C) and 11.58 kDa (E). These molecular weights fit well with those recieved from SDS gel electrophoresis, except of the 22 kDa subunit. This subunit could not be identified without doubt by enrichement in the active fractions and therefore is not indicated in Fig. 1.

A Northern hybridization experiment resulted in a single transcript of about 5 kbp which may cover all the genes of the atp gene cluster and indi-

cates that we have found an operon encoding the whole A_1 part of the ATPase. Fig. 3 shows the operon compared to the *Sulfolobus acidocaldarius* operon and to the V-ATPase operon of *Enterococcus hirae* (formerly *Streptococcus faecalis*).

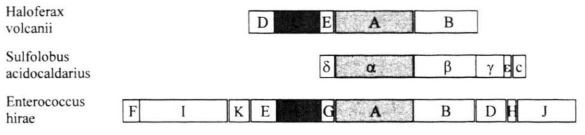

Fig. 3. Arrangement of the genes in the atp-operon of (from top to bottom) *Haloferax volcanii* (archaeal H^+-ATPase), *Sulfolobus acidocaldarius* (archaeal H^+-ATPase), *Enterococcus hirae* (eubacterial Na^+-ATPase, V-type). Related subunits are printed in the same gray. The letters indicate the subunits encoded by the respective genes. Citations are given in the text.

The nucleotide sequence has been submitted to the EMBL Data Library and is accessible under number X79516. The deduced amino acid sequences as well as the alignments to related ATPases will be shown in a different paper.

Since the *Haloferax volcanii* ATPase operon is the first example of an archaeal halophilic ATPase gene cluster, we compared the arrangement of genes to the respective operon in the archaeon *Sulfolobus acidocaldarius* (Denda *et al.*, 1989), and the V-ATPase-operon (Na^+-ATPase) of *Enterococcus hirae* (Takase *et al.*, 1994). In the acidothermophile archaeon *Sulfolobus acidocaldarius*, five genes encoding hydrophilic polypeptides (atp D, A, B, G, E) precede a gene (atp P) coding for a very hydrophobic subunit (c or "proteolipid"). The small subunits are described to have neither significant similarity to F-ATPase- nor to eukaryotic V-ATPase subunits, whereas α,β, and c have homologous counterparts in other ATPases.

The *Haloferax* operon is not similar to that one of *Sulfolobus acidocaldarius*, whereas complete correspondence could be found comparing the eubacterial *Enterococcus hirae* Na^+-ATPase gene cluster. In *Enterococcus*, the proteolipid-encoding gene is atpK, positioned in front of atpE, which encodes the homologous peptide to *Haloferax volcanii* D. We suggest that the A_1 and A_0 subunits of the *Haloferax* ATPase may be encoded by different operons that were transcribed independently, as it is in the photosynthetic bacterium *Rhodospirillum rubrum* (Falk *et al.*, 1985).

Ion dependencies

Haloferax volcanii belongs to the more moderate halophilic archaebacteria. Its natural environment, the Dead Sea, contains 1.75–2.5 M NaCl. Measuring the ATP-hydrolysis activity, NaCl can be replaced by KCl without loss of activity. Replacement of the Cl^--ion by NO^{3-} resulted in loss of 50% of the activity, whereas the enzyme lost its activity in presence of Br^- instead of Cl^-. Furthermore, the enzyme requires divalent cations for ATP hydrolysis, with $Ca^{2+} < Mg^{2+} < Mn^{2+}$, using optimal ratios of ATP:Me^{2+}.

The true substrate for the chloroplast F-ATPase is the complex between a divalent cation and ATP *via* P_β and P_γ of ATP (Bickel-Sandkötter, 1985). Most probably, this is the same for A-ATPases and especially for *Haloferax volcanii.* Inhibition of ATP-hydrolysis by free Mn^{2+}-ions has been found, working with isolated chloroplast ATPase (own result) and with *Haloferax mediterranei-* ATPase (Dane *et al.*, 1992). Furthermore, the ATPases of *Halobacterium saccharovorum* (Schobert 1992) and *Sulfolobus acidocaldarius* (Lübben and Schäfer, 1987) are sensitive to free divalent cations.

Fig. 4 shows hydrolysis of ATP as a function of Mg^{2+} and Mn^{2+} concentrations, respectively. Using

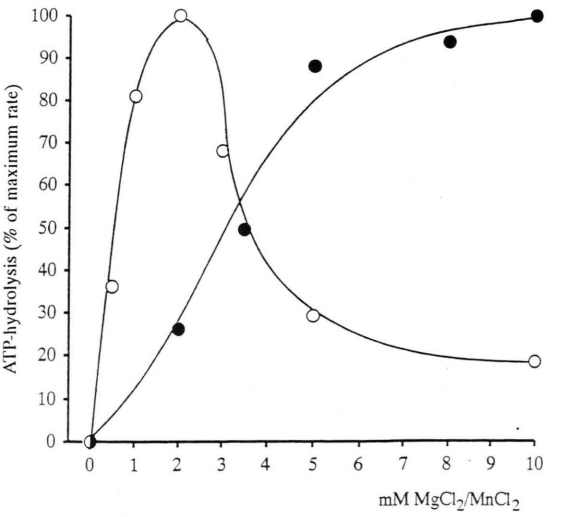

Fig. 4. ATPase activity of *Haloferax volcanii* membranes (70 µg) as a function of the concentrations of $MgCl_2$ or $MnCl_2$. Conditions: (○) $MnCl_2$, 5 mM ATP, 100% = 0.129 µmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$; (●) $MgCl_2$, 5 mM ATP, 100 % = 0.105 µmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$; Each rate is determined by 3 point kinetics.

$MgCl_2$, the reaction was saturated at equimolar concentrations of the divalent ion and ATP. Application of Mn^{2+} resulted in an optimum at less than equimolar concentrations; exceeding concentrations of Mn^{2+} inhibited the enzyme. Application of $MnCl_2$ resulted in an optimum-curve with hyperbolic increase. In contrast to this, application of $MgCl_2$ on *Haloferax volcanii* membranes resulted in a sigmoidal curve.

High salt conditions (molar range) and high temperatures influence the complex constants for Me^{2+}/ATP very effectively. Brigitte Schobert (1992), calculated from her experiments (*Halobacterium saccharovorum*) dissociation constants for the Me/ATP complexes for high salt conditions, which may be used to calculate the real substrate concentrations:

$ATP^{4-} + Mg^{2+} \leftrightarrow MgATP^{2-}$ $K_D = 9 \times 10^{-3}$ M
$ATP^{4-} + Mn^{2+} \leftrightarrow MnATP^{2-}$ $K_D = 2.5 \times 10^{-3}$ M

Using these constants that approximate to our high salt conditions, we calculated relative amounts of actual MeATP complex and free divalent ions for the conditions of our experiments by the following equations:

$$[MA] \xleftrightarrow{K_D} [M]+[A]$$

MA = Metal-ATP-complex
M = Metal
A = ATP

$$K_D = \frac{[M]\cdot[A]}{[MA]} = \frac{A_f\cdot(M_t-A_t+A_f)}{A_t-A_f} \quad (1)$$

$$MA = M_{total}-M_{free} = (M_t-M_f) \quad (2)$$

$$MA = A_{total}-A_{free} = (A_t-A_f) \quad (3)$$

The solution of Equ. (1) results in the following quadratic equation:

$$A_f^2+A_f(M_t+K_D-A_t)-A_t\cdot K_D = 0$$

with the solution

$$A_f = \frac{M_t+K_D-A_t}{2} \pm \sqrt{\left(\frac{M_t+K_D-A_t}{2}\right)^2+(A_t\cdot K_D)}$$

The true complex concentrations calculated in this way as well as the concentrations of free metal ions are drawn in Fig. 5 in dependency of the respective added divalent cation concentration. This result delivers the reason for the optimum slope of the Mn^{2+} dependency shown in Fig. 4. Up to a concentration of 5 mM $MnCl_2$, the complex concentration (substrate) exceeds the concentration of free divalent cations. Addition of higher Mn-concentrations leads to rising free ion levels, which inhibit the ATPase very strongly. In contrast to this, the free Mg^{2+} level rises almost linearly and exceeds the complex concentration from the beginning.

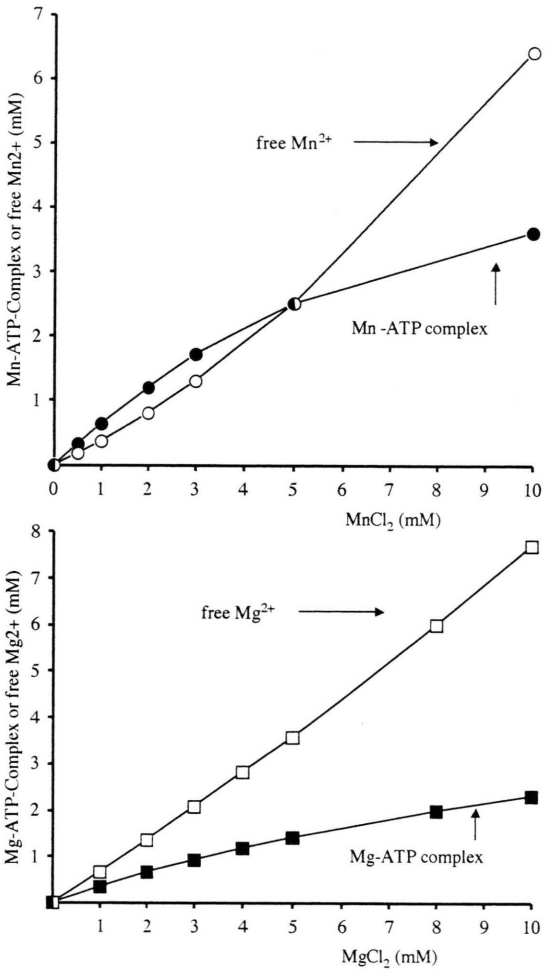

Fig. 5. MeATP complex concentrations and amount of free metal ions as calculated from the conditions of Fig. 4 in dependency of the added $MnCl_2$ respective $MgCl_2$ concentrations.

Substrate kinetics

We determined different Michaelis-Menten constants, using either MnATP or MgATP as a substrate. In order to avoid inhibition of the reaction by free metal ions, we used a $MgCl_2$: ATP ratio of 1:1 and a Mn: ATP ratio of 2:5, according to the results shown in Fig. 4. Fig. 6 shows the determination of K_M-values for ATP in presence of Mn^{2+} and in presence of equimolar concentrations of Mg^{2+}. The K_M-values differ by a factor of about 4.

By the use of the above calculated complex concentrations, the kinetic constants for ATP hydrolysis approximate to more "normal" values (in comparable order to F-ATPases). The K_M for the MnATP complex reduces to 0.24 mM, and that for the MgATP complex becomes about 1 mM.

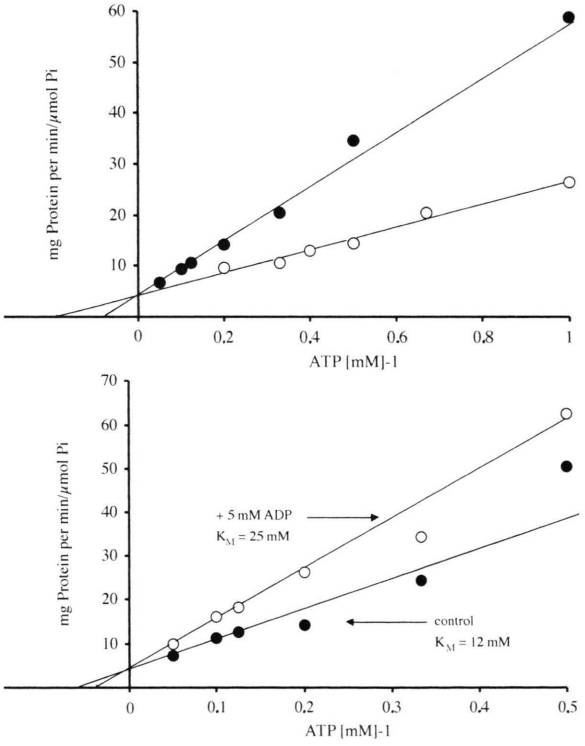

Fig. 6. Upper part: ATPase activity of *Haloferax volcanii* membranes (70 µg) as a function of ATP/Mn^{2+} concentration (○) (ratio: 5/2); and as a function of ATP/Mg^{2+} concentration (●) (equimolar) shown as Lineweaver-Burk plots. The conditions are given in Methods. The rates are determined by 3 point kinetics (1, 2, 3 min). Apparent $K_M(Mg^{2+})$; 12.5 mM; (Mn^{2+}); 3 mM. Lower part: Inhibition of ATP hydrolysis by ADP, shown as Lineweaver-Burk plot. Conditions: 30 mM $MgCl_2$, 5 mM ADP, other conditions as described in Methods. The K_I calculated from this experiment is 4.4 mM.

Measuring kinetics up to half an hour, we routinely observed nonlinearity in ATP hydrolysis above 5–6 minutes of incubation, which is due to the increasing amount of free ADP. We calculated every rate, which is given in this paper from the linear increase in P_i-production of the first three minutes. ADP is a potent competitive inhibitor of ATP hydrolysis. We calculated a K_i of 4.4 mM in presence of excess Mg^{2+} (Fig. 6, lower part).

The enzyme is specific for ATP; other nucleoside triphosphates were competitive inhibitors of the ATP hydrolysis reaction in descending extent: GTP > ADP > ITP > UTP. GTP was more effective as ADP, with a K_i of 2.5 mM in presence of Mg^{2+} (not shown). The calculated K_I values for the latter nucleotides are: ITP; 12.3 mM, UTP; 69.4 mM.

Discussion

Isolated cell membranes from *Haloferax volcanii* exhibit an ATPase activity, which is sensitive to free Mn^{2+} ions and ADP, but is neither inhibited by nitrate nor bafilomycin (V-type-ATPases) nor by vanadate (P-type-ATPases, Steinert *et al.*, 1995). The isolated ATPase shares several properties with F_1-type ATPases. Among these are the following criteria: a high pH optimum (Dane *et al.*, 1992), inhibition by free Mn^{2+}-ions, competitive inhibition by ADP, GTP and other nucleotides, and cross reactivity between antibodies against subunits of CF_1 and *Haloferax volcanii* membranes (Steinert *et al.*, 1995).

In spite of this, usual inhibitors of F-ATPases, like phlorizin, oligomycin, NBD-Cl, and azide, did not affect the ATPase activity (Steinert *et al.*, 1995). NEM, which inhibits the *Haloferax* enzyme only slightly when incubated up to 10 minutes (Dane *et al.*, 1992), binds to the 63 and 51 kDa peptides, which are subunits of the ATPase, as shown in this paper.

Meanwhile, we have received the true molecular masses for all A_1 subunits of the *Haloferax* enzyme from their sequences. As we could show by these recently published (Steinert *et al.*, 1995) amino acid sequences of the large subunits A and B, subunit A contains 4 cysteines, one of them in close neighbourhood to the Walker consensus sequence A. The *Daucus carota* V-ATPase contains three of five cysteines in this region. One of the

cysteines is lying in the Walker consensus motif A. Recently, Taiz *et al.,* (1994) could verify by site-directed mutagenesis of the respective cysteines (cys-ser) in the *Saccharomyces cerevisia* V-ATPase, that NEM binds mainly to this central cysteine. This explains the more effective inhibition of V-ATPases by NEM. In contrast to this, the chloroplast F-ATPase β-subunit contains no cysteine (Fig. 7; *S.ole.* β). The alignment in Fig. 7 makes it possible to compare the respective parts of the sequences of different ATPases.

Despite the differences found when studying different halophilic ATPases, their catalytic subunits (A or α) show a very high similarity in their primary structures. Available sequences from different archaea, for instance the large subunits of *Methanosarcina barkeri - ATPase (Inatomi et al.*, 1989), of *Halobacterium halobium* (Ihara and Mukohata 1991) and of *Haloferax volcanii* (Steinert *et al.,* 1995) substantiated the close relationship of A-ATPases to vacuolar H^+-ATPases. This is supported by an 80-amino acid insertion, which has been found in the catalytic subunits of the cited A- ATPases as well as V-ATPases. This insertion is the reason for the larger size of the respective catalytic subunits. Our results show, that the *Haloferax volcanii* ATPase is no exceptional case concerning this point.

The *Haloferax volcanii* -ATPase consists of subunits A and B and three additional small subunits of 39, 22 and 12 kDa. The gel (Fig. 1) shows a 37 and a 12 kDa-peptide, which clearly was accumulated by purification. In region of 22 kDa, however, we could not recognize an accumulated peptide band. Most probably, this subunit is lost in one of the purification steps. Schobert and Lanyi (1989) reported that *Halobacterium saccharovorum* contains 5 putative subunits, the smaller ones of 31, 22, and 14 kDa. Denda *et al.,* (1990) published data about an *Sulfolobus acidocaldarius* ATPase operon, which encodes five different subunits of 66, 51, 25, 13 and 7 kDa.

Halophilic archaeal ATPases differ in their properties beginning with the NaCl concentration needed for optimal activity and ending with different specific inhibitors (Steinert *et al.,* 1995). But they all should be able to synthesize ATP on the expense of an ion gradient, which is a feature of F-ATPases. ATP-synthesis could directly be demonstrated for *Halobacterium halobium* (Mukohata and Yoshida, 1987a, b) and for *Haloferax volcanii* (Steinert, 1995). The catalytic subunit is larger than the regulatory one, like in V-ATPases, but the molecular mass of the proteolipid in the A_0-part is comparable to that one of F-ATPases. The *Haloferax volcanii*-ATPase contains a 9.7 kDa proteolipid (Steinert, 1995), Sulfolobus acidocaldarius a 7 kDa one (Denda *et al.,* 1990), whereas V-ATPases possess proteolipids with molecular masses of about 16 kDa. Mandel *et al.,* (1988) proposed that both peptides have got their origin from a common ancestral gene that underwent gene duplication to bring about the V-ATPase proteolipid.

Summarizing the results, we conclude that the membrane standing A_1 part of the archaeal ATPases is closely related to that of V-ATPases, as shown comparing the different operon structures.

*H.vol.*A	TAAIPGPFGSGKTVTQHQLAKW---ADADIVVYVG**C**GERGNE	271
*H.hal.*A	TAAIPGPFGSGKTVTQQSLAKF---ADADIVVYIG**C**GERGNE	268
*M.bar.*A	TAAIPGPFGSGKTVTQQSLAKW---SDTEIVVYIG**C**GERGNE	261
*D.car.*A	TCAIPGAFG**C**GKTVISQALSKY---SNSDTVVYVG**C**GERGNE	283
*S.ole.*β	KIGLFGGAGVGKTVLIMELINNIAKAHGGVSVFGGVGERTRE	208

Fig. 7. Detail of the amino acid sequence of *Haloferax volcanii* ATPase (subunit A) (*H.vol.*A; Steinert *et al.,* 1995a) concerning a nucleotide binding stretch, aligned to the respective sequences of *Halobacterium halobium* (*H.hal.*A; Ihara and Mukohata, 1991), *Methanosarcina barkeri* (*M.bar.*A; Inatomi *et al.,* 1989), *Daucus carota* V-ATPase (*D.car.*A; Zimniak *et al.,* 1988), and spinach chloroplast ATPase β (*S.ole.*β; Hudson *et al.,* 1987). Numbering corresponds to the original papers. The reported cysteines (possible NEM-binding) are shown in bold letters.

The membrane integral A_0-part, however, may be closer related to that of the F-ATPase class, as shown by the size of the proteolipid. This leads to the idea that the function of the enzyme as an ATP synthase may be reduced to a structural factor in the A_0-part of the ATPase. Further work on this part is in progress.

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