Relationship of the Membrane ATPase from *Halobacterium saccharovorum* to Vacuolar ATPases[1]

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Polyclonal antiserum against subunit A (67 kDa) of the vacuolar ATPase from *Neurospora crassa* reacted with subunit I (87 kDa) from a membrane ATPase of the extremely halophilic archaebacterium *Halobacterium saccharovorum*. The halobacterial ATPase was inhibited by nitrate and *N*-ethylmaleimide; the extent of the latter inhibition was diminished in the presence of adenosine di- or triphosphates. 4-Chloro-7-nitrobenzofurazan inhibited the halobacterial ATPase also in a nucleotide-protectable manner; the bulk of inhibitor was associated with subunit II (60 kDa). The data suggested that this halobacterial ATPase may have conserved structural features from both the vacuolar and the F-type ATPases.
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F_1F_0 ATP synthases (F-type ATPases) are proton-pumping ATPases that utilize transmembrane proton gradients for the synthesis of ATP. They are found on the inner membranes of mitochondria and chloroplasts as well as the cytoplasmic membranes of eubacteria (1). Although membrane-bound ATPase activity (i.e., ATP hydrolysis) has been described in several archaebacteria (2–6), none of these enzymes has been shown to be an F-type ATPase. Vacuolar proton-pumping ATPases (V-type ATPases) are associated with the endomembranes of a variety of intracellular organelles found in eukaryotic cells. They resemble F-type ATPases in that they are large multimeric enzyme complexes consisting of a catalytic portion and a dicyclohexylcarbodiimide-sensitive proton

pore. However, V-type ATPases do not synthesize ATP [for reviews see (7, 8)].

DNA sequence data for the two major subunits of membrane ATPases from *Sulfolobus acidocaldarius* (9, 10) and *Methanosarcina barkeri* (11) as well as for a partial sequence from *Methanococcus thermolithotrophicus* (12) indicate extensive similarities between the ATPases from the sulfur-dependent thermoacidophiles and the methanogenes and the vacuolar ATPases (50% or more identical amino acid residues). Less extensive similarities (9–13) occur between archaebacterial ATPases and the F-type ATPases (ca. 25% identical amino acid residues). These observations imply that if these archaebacterial enzymes synthesize ATP, they are not F-type ATPases.

Two apparently distinct membrane-bound ATPases have been isolated from the extremely halophilic archaebacteria. Hochstein *et al.* described the purification and properties of the ATPase from *Halobacterium saccharovorum* (2, 14, 15); subsequently, a similar ATPase was isolated from *H. halobium* (6). While the molecular masses of the native enzymes and their major subunits appear to be identical, these ATPases differ with respect to pH optimum, ion activation, and detergent requirement (6, 16). Furthermore, there are differences with respect to their immunological reactivities. The ATPase from *H. halobium* reacts in immunoblots with the *Beta vulgaris* vacuolar ATPase (17). On the other hand, subunit II of the ATPase from *H. saccharovorum* reacts strongly with an antiserum against the β subunit of the ATPase from *S. acidocaldarius*, which in turn cross-reacts with the β subunit of F-type ATPase from mitochondria, eubacteria, and chloroplasts (18). This suggests a relationship of the ATPase from *H. saccharovorum* to the F-type ATPases. However, a comparison of the ATPase from *H. saccharovorum* with the F_1 ATPase from *Escherichia coli* leads to the conclusion that these latter two enzymes differ from each other on the basis of amino acid composition, trypsin sensitivity, peptide maps and isoelectric points of the major subunits (16).

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Consequently, it was of interest to compare the ATPase from *H. saccharovorum* to the V-type ATPases. We report here on the immunological cross-reaction and sensitivity to certain inhibitors of the ATPase from *H. saccharovorum* that suggest the enzyme possesses features indicating a relationship to V-type and F-type ATPases.

MATERIALS AND METHODS

Chemicals. The following materials were purchased from the companies indicated: Bio-Rad Protein assay reagent, γ-globulin, SDS[3] (Bio-Rad Laboratories); bovine serum albumine (fraction V), ATP, ADP, NEM, NBD-Cl (Sigma Chemical Co.).

Preparation and chemical modification of the ATPase from H. saccharovorum. The membrane fraction from *H. saccharovorum* (ATCC 29252) was prepared as described earlier (2, 15, 16). Prior to modification, the enzyme (0.66-1.2 mg protein/ml) was equilibrated in 50 mM triethanolamine-HCl, pH 7.15, 4 M NaCl, 2 mM EDTA, by centrifuging it through Sephadex G 50 columns according to the procedure described by Penefsky (19). NEM was dissolved in pentane and added to a final concentration of 1 mM. NBD-Cl was dissolved in methanol and added to the enzyme to a final concentration of 1.25 mM. Incubation was at ambient temperature. Excess NBD-Cl was removed by centrifuging the reaction mixture through Sephadex G 50 columns, which were equilibrated in 50 mM Tris-HCl, pH 8.0, 4 M NaCl, 10 mM $MgCl_2$. Nucleotide protection experiments were carried out by incubating the enzyme at ambient temperature with either ATP or ADP for 15 min prior to the addition of the inhibitor.

Immunological assay (Western blot). The preparation of antiserum against subunit A from the vacuolar ATPase from *Neurospora crassa* was carried out as described previously for the corn ATPase (20). The immunoblot procedure of Rott and Nelson (21) was used and the immunoconjugates were visualized with alkaline phosphatase (22).

Other methods. ATPase activity was measured as published previously using MnATP as the substrate (2) except that Triton X-165 instead of Triton X-100 was used as suggested by Schobert and Lanyi (23). Gel electrophoresis was performed according to Laemmli (24). Gels were stained with Coomassie blue according to Fairbanks *et al.* (25). Fluorescence was observed under ultraviolet light prior to staining of the gels, and was recorded using Polaroid film 67. Protein was determined by the method of Bradford (26) or Lowry *et al.* (27) with γ-globulin or bovine serum albumin, respectively, as standards.

RESULTS AND DISCUSSION

Figure 1 shows that the polyclonal antiserum raised against the 67-kDa subunit (subunit A) from the vacuolar ATPase of *N. crassa* (Fig. 1, lane 1) reacted with the 87-kDa subunit (subunit I) from the purified membrane ATPase of *H. saccharovorum* (Fig. 1, lane 3) and with a polypeptide of similar size in the membrane fraction of this organism (Fig. 1, lane 2). Thus, in addition to the cross-reaction with the β subunits from the ATPase from *S. acidocaldarius* and F-type ATPases (18), the ATPase from *H. saccharovorum* showed an immunological relationship to the V-type ATPases. We conclude that halobacterial ATPases can cross-react immunologically with both F-type and V-type ATPases.

[3] Abbreviations used: NBD-Cl, 4-chloro-7-nitrobenzofurazan (or 7-chloro-4-nitrobenzo-2-oxa-1,3,diazole); NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.
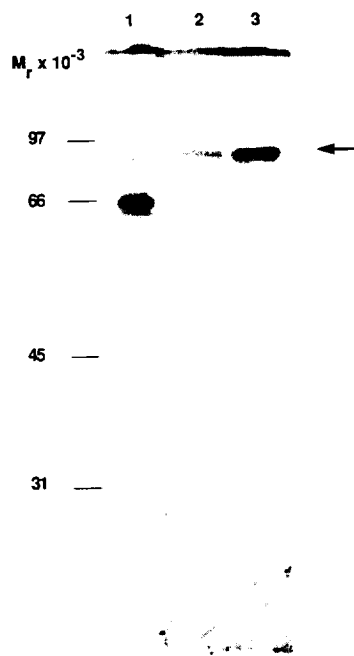

FIG. 1. Immunoreactivity of halobacterial ATPase and membranes with an antiserum to subunit A from the vacuolar ATPase of *Neurospora crassa*. Immunoblotting was performed with polyclonal rabbit antiserum and alkaline phosphatase linked to protein A. Lane 1, vacuolar ATPase, 6 μg; lane 2, membranes (P2 fraction) of *H. saccharovorum*, 80 μg; lane 3, purified ATPase of *H. saccharovorum*, 2.5 μg. Molecular mass standards are indicated on the left; the position of halobacterial subunit I (87 kDa) is marked by the arrow.

Nitrate is a useful compound for distinguishing between V-type and F-type ATPases, since the latter are unaffected by nitrate whereas the former are inhibited by millimolar concentrations of this anion (28). Inhibition by nitrate has also been found for the archaebacterial ATPases from *S. acidocaldarius* and *H. halobium* (4, 17). The ATPase from *H. saccharovorum* was inhibited by nitrate at an $I_{50\%}$ of 5 mM, a concentration similar to that reported for other archaebacterial ATPases.

V-type ATPases are inhibited by micromolar concentrations of NEM. Subunit A of the vacuolar ATPase from *N. crassa* is labeled by NEM in a nucleotide protectable manner (20). The ATPase from *H. saccharovorum* was also inhibited by NEM. The inhibition by NEM was characterized by its time dependence, the relatively high concentration of NEM, and the protective action of nucleotides. As shown in Fig. 2, hydrolytic activity was reduced to about 30% of that of the untreated enzyme following 3 h of incubation with 1 mM NEM. When the enzyme was preincubated with 5 mM ATP prior to the addition of NEM, the inhibition developed at a slower rate and the remaining activity was significantly higher (about 70% of that of the control enzyme). Similar results were obtained, when ADP replaced ATP (not shown). The results suggested that the sulfhydryl reagent NEM
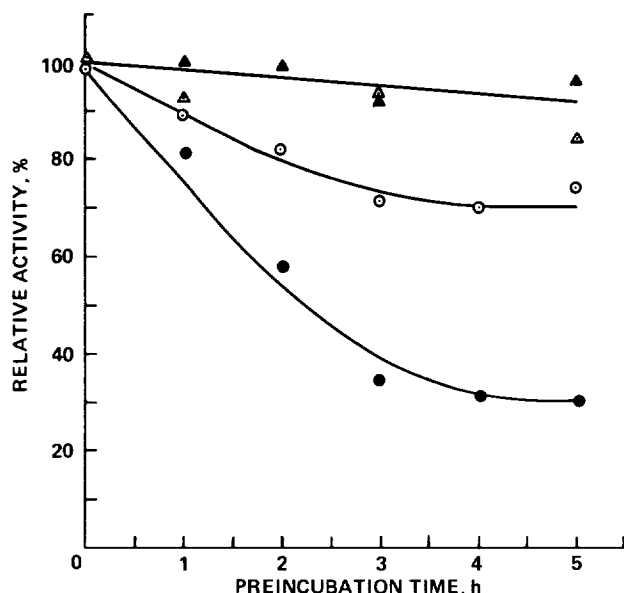
FIG. 2. Inhibition of the activity of the halobacterial ATPase by NEM. Purified ATPase from *H. saccharovorum* (0.66 mg protein/ml) was incubated without (●) or with 5 mM ATP (○) for 15 min. Subsequently, incubation with 1 mM NEM was carried out for the times indicated. Control experiments with 9% pentane without (▲) and with 5 mM ATP (△) were performed simultaneously. Remaining ATPase activity was assayed following dilution of 1:100 into assay buffer and is shown as percentage of the pentane-containing control.

binds at or near the active site of the halobacterial enzyme and were consistent with the presence of cysteinyl residues in subunit I and subunit II (16).

The nucleotide analogue NBD-Cl inhibits the hydrolytic activity of F-type ATPases, when one molecule of NBD-Cl is bound to one β subunit (29). NBD-Cl inhibits also the vacuolar ATPases of fungi and plants; the inhibitor is incorporated predominantly into subunit A (20, 30–32). The ATPase from *H. halobium* is also inhibited by NBD-Cl, with partial protection by ATP (6). The ATPase from *H. saccharovorum* is inhibited by NBD-Cl in a time-dependent manner by a reaction that is stopped, but not reversed, by dithiothreitol (2). To demonstrate where NBD-Cl was bound, the halobacterial enzyme was preincubated in the presence of 1.25 mM NBD-Cl at pH 7.15 for 3 h, which reduced the activity to about 12% of that of the untreated enzyme. When NBD-Cl incubation was carried out in the presence of 12 mM ADP or 12 mM ATP, the remaining activity was 65 and 49%, respectively, of that of the untreated enzyme. Figure 3 shows SDS gels of these three halobacterial ATPase samples. The two major subunits I and II were both labeled by NBD-Cl; the bulk of label was in subunit II, as judged by its fluorescence (B, lane 3). The presence of nucleotides prevented almost completely the incorporation of NBD-Cl into the enzyme, leaving traces of fluorescence associated with the subunits (B, lanes 1 and 2).

The ATPase from *H. saccharovorum* resembled V-type ATPases in its sensitivity to NEM and nitrate. In addition, subunit I showed immunological cross-reactivity with antiserum to subunit A of the *N. crassa* vacuolar ATPase. However, the inhibition by NBD-Cl and the immunoreaction of subunit II indicated distinct differences. NBD-Cl labels the larger subunit (A) of vacuolar ATPases and this subunit has been proposed to contain the catalytic site (20, 31). In the case of the ATPase from *H. saccharovorum*, NBD-Cl bound predominantly to the smaller of the two major subunits (subunit II) of the enzyme. Interestingly, another ATPase inhibitor, DCCD, also binds to subunit II of the *H. saccharovorum* ATPase (14), with the concomitant loss of hydrolytic activity. The conditions required to affect inhibition are similar to those that result in binding of DCCD to the β subunit of F-type ATPases (33). These results suggested that in the ATPase from *H. saccharovorum* subunit II rather than subunit I may behave as the functional equivalent of the β subunits of F-type ATPases. The immunological cross-reaction between subunit II and several β subunits of archaebacterial and F-type ATPases (18, 34) are consistent with this possibility.

Amino acid sequence data suggest that proton-pumping ATPases may have originated from a common ancestral enzyme (10, 13, 35), a vacuolar type enzyme which possessed the ability to synthesize ATP, and that the archaebacterial ATPases evolved from this enzyme (35). The extensive amount of amino acid identities between the two large subunits of the *Sulfolobus* ATPase and V-
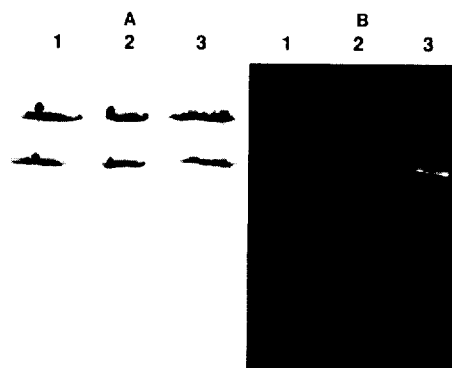

FIG. 3. Incorporation of NBD-Cl into the subunits of the halobacterial ATPase. Purified ATPase from *H. saccharovorum* was incubated with 12 mM ATP (lane 1), 12 mM ADP (lane 2), or without nucleotides (lane 3) prior to treatment with NBD-Cl. Following removal of unbound reagent, the enzyme subunits were separated on SDS–PAGE gels. Acrylamide concentration was 11%. A, gel stained with Coomassie blue; B, gel photographed under ultraviolet light prior to staining. Subunits I (upper band) and II (lower band) of the ATPase are shown. Residual fluorescence in the subunits in B, lanes 1 and 2, was visible in the original gel. Protein content per lane was 31, 24, and 34 μg, respectively. Remaining ATPase activity of NBD-Cl-treated samples was determined prior to electrophoresis (see methods) and was 65, 49, and 12%, respectively, of that of an untreated control, corresponding to enzyme samples shown in lanes 1, 2, and 3, respectively.

type ATPases is consistent with this proposal. However, Denda *et al.* (36) found that the proteolipid subunit of the *Sulfolobus* ATPase is more related to the proteolipid from the F-type ATPases rather than V-type ATPases, which suggests a chimeric nature of this archaebacterial ATPase. The results we report in this communication as well as earlier observations (14) imply that the ATPase from *H. saccharovorum* possesses structural features characteristic of F-type and V-type ATPases at the level of their major subunits.

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