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Purification and Properties of a Plasma Membrane H^+-ATPase from the Extremely Acidophilic Alga *Dunaliella acidophila*

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This paper describes partial purification and characterization of a vanadate-sensitive H^+-ATPase from plasma membranes of *Dunaliella acidophila*, an extremely acidophilic unicellular alga (I. Sekler, H.U. Gläser, U. Pick [1991] J Membr Biol 121: 51–57). Purification is based on the insolubility in and stability of the enzyme in Triton X-100. The purified enzyme is highly enriched in a polypeptide of molecular mass 100 kD, which cross-reacts with antibodies against the plant plasma membrane H^+-ATPase. Upon reconstitution into proteoliposomes, the enzyme catalyzes an ATP-dependent electrogenic H^+ uptake. ATP hydrolysis is stimulated by lipids, is inhibited by vanadate, diethylstilbestrol, dicyclohexylcarbodiimide, erythrosine, and mercurials, and shows a sharp optimum at pH 6. Unusual properties of this enzyme, by comparison with plant plasma membrane H^+-ATPases, are a higher affinity for ATP (K_m = 40 μM) and a larger stimulation by K^+, which interacts with the enzyme from its cytoplasmic side. Comparative studies with cross-reacting antibodies, prepared against different domains of the plant H^+-ATPase, suggest that the central hydrophilic domain containing the catalytic site is more conserved than the C- and N-terminal ends. The high abundance and stability of the plasma membrane H^+-ATPase from *D. acidophila* make it an attractive model system for studies of the structure-function relations and regulation of this crucial enzyme.

Plasma membrane proton pumps are characteristic of plants, yeast, fungi, and related organisms. These vanadate-sensitive or "P-type" ATPases play a central role in the growth and development of cells, generating the electrical gradient across the plasma membrane that provides the driving force for nutrient and ion uptake, regulation of intracellular pH, and several other essential functions.

The H^+-ATPase has been characterized, purified, and cloned from plasma membrane of several higher plants (Vara and Serrano, 1982; Anthon and Spanswick, 1986; Harper et al., 1989; Pardo and Serrano, 1989; Serrano, 1989). However, the low yield and poor stability of the purified ATPase have hampered biochemical and kinetic characterization of this important enzyme.

An equally important role of the plasma membrane H^+-ATPase in lower plants has been suggested on the basis of several physiological studies (Spanswick, 1981; Shimmen and Tazawa, 1982; Takeuchi et al., 1985). However, there are few reports of preparations of plasma membrane from lower plants and virtually no studies on biochemical characterization of plasma membrane H^+-ATPases from unicellular algae (Gilmour et al., 1985; Weiss et al., 1989). Recently, we isolated a plasma membrane fraction from *Dunaliella acidophila*, an extremely acidophilic strain of the unicellular green alga *Dunaliella* (Sekler et al., 1991). This organism grows optimally at pH 1 and maintains an intracellular pH of 7 (Albertano et al., 1982; Gimmler et al., 1989b). The membrane preparation possesses a highly active vanadate-sensitive ATPase activity, suggesting that the plasma membrane H^+-ATPase is expressed at high density in this alga (Sekler et al., 1991). In the present work, we show that the plasma membrane ATPase of *D. acidophila* is insoluble and stable in high concentrations of Triton X-100. This observation was exploited to develop a purification procedure resulting in a highly purified and stable ATPase preparation, which catalyzes proton transport into proteoliposomes. The preparation has some unique characteristics as compared with the fungal and higher plant plasma membrane H^+-ATPases.

MATERIALS AND METHODS

Growing of Cells

Dunaliella acidophila (Masyuk strain No. SAG 19.85, from the algal collection of the Institute of Plant Physiology, University of Göttingen, Germany) was a generous gift from Dr. Schlösser in Göttingen. The cells were grown in 15- to 30-L bath culture tanks as previously described (Sekler et al., 1991) to a cell density of 2×10^7 cells/mL. The pH of the growth medium was adjusted to 0.5 with concentrated sulfuric acid.

Plasma Membranes

Crude plasma membrane preparations were obtained from a microsomal fraction of *D. acidophila* by a procedure utilizing a stepwise Suc gradient (Sekler et al., 1991).

ATPase Assay

Enzyme samples (0.5–2 μg of protein) were incubated in 200 μL of buffer containing 10 mM Tris-Mes, pH 6, 5 mM

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Abbreviations: ACMA, 9-aminochloro-2-methoxyacridine bis-(hexafluoracetonyl) acetone; IC_{50}, concentration required for 50% inhibition; Mega-9, nonanoyl-*N*-methyl-glucamide; PCMBS, *p*-chlorobezenesulfonate; SF-6848, 3,5-di(tert-buthyl)-4-hydroxybenzylidene malonitrile.

$MgCl_2$, and 2 mM Tris-ATP. The released Pi was determined as described (Ames, 1966), except that 0.3% of SDS was added to the color-developing solution. One unit of enzyme activity is defined as hydrolysis of 1 μmol Pi per 1 min at 37°C. Unless indicated, ATPase activity represents the rate of total ATP hydrolyzed, which is over 90% vanadate sensitive. The K_m for ATP was determined by a regression analysis using a personal computer and the program Enzfitter (Elsevier Bio-Soft) in the presence of an ATP-regenerating system that included 5 units of pyruvate kinase and 5 mM phospho*enol*-pyruvate.

Purification of Plasma Membrane ATPase

Unless otherwise indicated, all operations were carried out at 0 to 4°C. Crude plasma membrane vesicles containing 2.5 to 6 mg of protein/mL (30 mL) were mixed with 90 mL of extraction buffer containing 20 mM Tris-Hepes, pH 7, 10% Suc; 2 mM EDTA, 1 mM DTT, and the protease inhibitors PMSF (1 mM) and leupeptin (5 μg/mL). Triton X-100 was added to a final concentration of 2% and the mixture was incubated for 20 min on ice. Ten milliliters of the Triton X-100-extracted membrane were overlaid on 10 mL of 28% Suc buffer (identical in its composition to the extraction buffer except that it contained 0.2% Triton X-100 instead of 2%) in 20-mL tubes and was sedimented for 2 h at $(3.3 \times 10^5)g$ in a Beckman Ti-60 rotor. The supernatant was discarded and the pellet was resuspended in 30 mL of 15% Suc buffer (identical to the extraction buffer but with 0.2% Mega-9 and 0.2% Triton X-100) and recentrifuged as above. The pellet was resuspended in 5 to 6 mL of Suc buffer (without detergents) and centrifuged for 5 min at 5000 rpm in Eppendorf microfuge 1-mL tubes to remove aggregated material, and the supernatant was diluted to 120 mL and sedimented for 1 h at $(3.3 \times 10^5)g$. The pellet was resuspended in 3 mL of buffer containing 20 mM Tris-Hepes, pH 7, 50 mM KCl, 2 mM EDTA, 1 mM DTT, 20% glycerol, and protease inhibitors. Insoluble material was removed by low-speed centrifugation, and the supernatant was kept frozen in liquid nitrogen.

SDS-PAGE

Protein samples (0.5–1 mg/mL) were treated with the protease inhibitors leupeptin (10 μg/mL), tosyl-L-lysine-chloromethyl-ketone (20 μg/mL), PMSF (1 mM), and *p*-chloromercuriphenylsulfonic acid (100 μM). After 5 min, 20 to 40 μL of the sample was solubilized in sample buffer containing 1% SDS and 1% β-mercaptoethanol at 20°C for 20 min and subjected to 7.5% SDS-PAGE as described previously (Laemmli, 1970).

Western Blot Analysis

After separation by SDS-PAGE, the polypeptides were electrophoretically transferred to nitrocellulose membranes and reacted with rabbit antiserum raised against the N-terminal (residues 6–51), the C-terminal (residues 851–949), and the central (residues 340–650) domains of *Arabidopsis thaliana* plasma membrane H^+-ATPase as described before (Parets-Soler et al., 1990).

Reconstitution of Proteoliposomes

The purified ATPase was diluted to 0.3 to 0.5 mg/mL in a medium containing 20% glycerol (v/v), 2 mM EDTA, 1 mM DTT, and 10 mM Tris-Hepes, pH 7. Soybean phospholipids were suspended at 50 mg/mL in water and sonicated to clarity under nitrogen in a bath sonicator. Purified protein (7.5–10 μg) was mixed with 2 mg of sonicated soybean lipids in 2 mL of reconstitution buffer containing 10 mM Tris-Mes, pH 6, 2 mM $MgSO_4$, and 20 mM of the different K^+ salts. The mixture was frozen in liquid nitrogen, thawed at room temperature, and sonicated once for 1 to 2 min. To determine the sidedness of the K^+ activation, the same amount of purified protein was mixed with 2 mg of sonicated soybean lipids in 100 μL of reconstitution buffer containing 10 mM Tris-Mes, pH 6, 2 mM $MgSO_4$, and 25 mM KNO_3 or choline nitrate. The mixture was passed through a 1-mL semi-dry Sephadex G-50 column equilibrated with the same buffer and reconstituted immediately as described above.

Measurements of ΔpH in Reconstituted Proteoliposomes

ATP-dependent acidification of the interior of the proteoliposomes was measured by recording the fluorescence quenching of the acridine dye ACMA. One micromolar ACMA was added to 2 mL of the reconstitution mixture and the reaction was started by addition of 0.5 mM ATP. Fluorescence changes were measured at 25°C in a Perkin-Elmer MPF-44A spectrofluorimeter at 412 nm (excitation) and 480 nm (emission).

Protein Determination

The concentration of protein was determined by a modification of the Lowry procedure (Markwell et al., 1978) with BSA as a standard.

Chemicals

Valinomycin, nigericin, diethylstilbestrol, Mega-9, Triton X-100, Na-ATP, and crude soybean phospholipids type II-S were obtained from Sigma. The acridine dye ACMA was donated by Dr. H.U. Gläser. The proton uncoupler SF-6847 was a gift from Dr. Evert Bakker in Osnabrück, Germany. All other reagents were of the highest commercially available grade.

RESULTS

Stability of the H^+-ATPase toward Detergents

The stability of the vanadate-sensitive H^+-ATPase from *D. acidophila* toward different detergents was tested to select an appropriate detergent for purification of this enzyme from plasma membrane preparations (Sekler et al., 1991). As demonstrated in Figure 1, treatment with 1% deoxycholate or octylglucoside results in a substantial inhibition of ATPase activity, whereas no such inhibition is obtained with Triton X-100. Triton X-100 and low concentrations of deoxycholate or octylglucoside actually stimulate vanadate-sensitive ATPase activity of the membrane preparation by 10 to 20%. The enzyme retains full activity even at 5% Triton X-100 (not

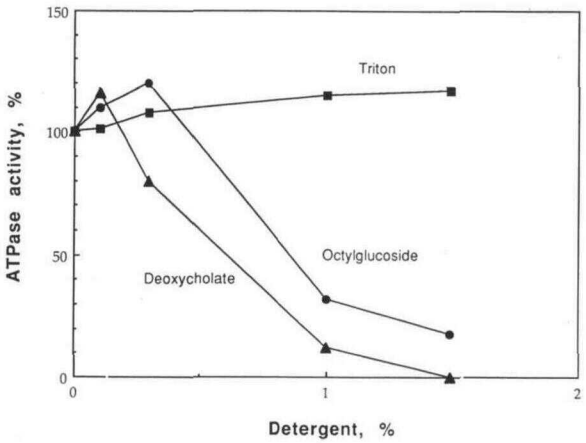

Figure 1. Effect of detergents on ATPase activity in plasma membrane preparations. Crude plasma membranes (0.5 mg of protein in 100 μL) were incubated for 10 min in the presence of the indicated detergents at room temperature. Ten micrograms of detergent-treated membranes were taken for ATPase activity as described in "Materials and Methods."

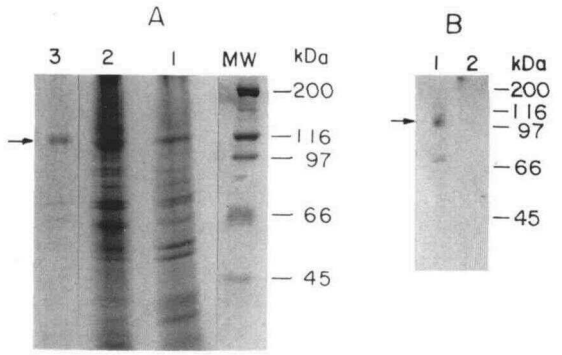

Figure 2. Coomassie blue staining and immunoblot analysis of the different purification stages. Electrophoresis staining and immunoblot were carried out as described under "Materials and Methods." A, Coomassie blue staining. Lane 1, Crude plasma membrane (20 μg); lane 2, Triton-extracted membrane (10 μg), and lane 3, Mega-9-extracted membranes (7 μg). B, Immunoblot. One microgram of the purified fraction was reacted with antisera (diluted 1:250) against the central domain of plasma membrane H+-ATPases of *A. thaliana* (lane 1) or with preimmune sera (lane 2) as described in "Materials and Methods." The arrows show the position of the ATPase.

shown). To determine whether the ATPase is solubilized by Triton X-100, membranes containing 2 mg of protein/mL were treated with 2% detergent as described in Figure 1 and centrifuged for 15 min at 50,000*g*, and the resulting pellet and supernatant were tested for ATPase activity. Seventy to 100% of the ATPase activity, but less than 10% of protein, was recovered in the pellet, and essentially no activity was observed in the supernatant. Thus, treatment with Triton X-100 does not solubilize the ATPase, but extracts most other proteins from the membrane preparation.

Purification of the Plasma Membrane H+-ATPase

As shown in Table I, extraction of plasma membranes with 2% Triton X-100 efficiently removes over 90% of the protein while preserving about two-thirds of the ATPase activity. Further purification was achieved by a second extraction with Mega-9 in combination with Triton X-100. It is interesting to note that Mega-9 was shown recently to be effective for purification of the plasma membrane H+-ATPase of *Saccharomyces cerevisiae* (Seto-Young and Perlin, 1991). Other detergents including octylglucoside, zwittergent 3-14, sodium cholate, and sodium deoxycholate were either ineffective or caused total and irreversible inhibition of ATPase activity. The addition of 2 mM EDTA, 1 mM DTT, and the protease inhibitors leupeptin and PMSF proved to be critical for stabilizing the ATPase activity.

In the absence of protease inhibitors, the preparation showed a very low vanadate-sensitive ATPase activity and the immunoblot analysis (see Fig. 2) revealed a major proteolytic fragment of about 70 kD. Sonicated soybean phospholipids activated the purified enzyme by up to 5-fold, with half-maximal activation at 6.5 μm/mL (not shown), and, hence, 200 μg of phospholipid/mL was added for all subsequent studies. The specific activity of the purified ATPase is 9 to 12 units/mg of protein at 37°C, and a typical recovery of ATPase activity is around 50%. The high specific activity and recovery can probably be explained by the fact that the purification procedure does not involve solubilization of the ATPase, a step that causes the greatest loss in activity of plant and fungal ATPases (Serrano, 1988b).

Polypeptide Composition and Immunoblot Analysis

Analysis of the polypeptide composition at the different purification steps by SDS-PAGE (Fig. 2A, lanes 1–3) indicates

Table I. *Purification of the plasma membrane ATPase*

The data correspond to a typical preparation starting with 30 mL of crude plasma membranes. ATPase and protein determination were performed as described in "Materials and Methods."

Purification Stage	Protein	Specific Activity	Purification	Yield
	mg	*units/mg protein*	*fold*	*%*
Crude plasma membrane	90	0.5	1	100
Triton X-100 extracted membranes	7.4	4.0	8	66
Mega-9 wash	2.1	10	20	47

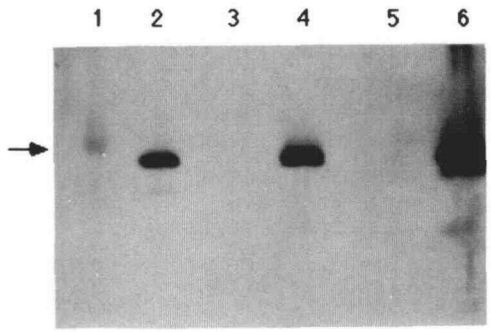

Figure 3. Immunoblot of cotton and *Dunaliella* plasma membrane preparations. Electrophoresis transfer and immunoblot were as described in "Materials and Methods." Lanes 1, 3, and 5 are Triton X-100-extracted plasma membrane preparations of *Dunaliella* (4 μg); lanes 2, 4, and 6 are plasma membrane preparations of cotton (20 μg). Lanes 1 and 2, 3 and 4, 5 and 6 were reacted with antiserum raised against the central, N-terminal, and the C-terminal domain, respectively, of the plasma membrane H^+-ATPase of *A. thaliana*. The arrow shows the position of the *D. acidophila* ATPase.

that a polypeptide of 100 kD, which is a minor component in the intact membrane, becomes highly enriched in the purified preparation. Apart from one minor band with a molecular mass of 70 kD, the 100-kD polypeptide is the major band at the last step of the purification (Figs. 2A and 3). To confirm that the purified 100-kD protein is the plasma membrane H^+-ATPase, we tested its cross-reactivity with polyclonal antibodies raised against the central domain of *Arabidopsis* plasma membrane H^+-ATPase. As shown in Figure 2B, the antibodies specifically cross-react with the 100-kD band. Thus, this polypeptide corresponds to the plasma membrane H^+-ATPase. The minor 70-kD band also cross-reacts and is, therefore, presumably a proteolytic fragment of the ATPase.

Resemblance between Plant and *Dunaliella* Plasma Membrane H^+-ATPases

Recently, the genes encoding the plant and fungal H^+-ATPases have been cloned and found to be homologous with respect to their primary amino acid sequence. The central domain, containing the proposed ATP binding and the autophosphorylation site, is the most highly conserved region, whereas the C- and N-terminal domains are much less conserved (Briskin, 1990). A comparison between higher plants (cotton) and *Dunaliella* plasma membrane H^+-ATPase was carried out by immunoblot analysis using polyclonal antibodies raised against the central (residues 340–650), the C-terminal (residues 851–941), and the N-terminal (residues 6–51) domains of the *Arabidopsis* H^+-ATPase. The purified *Dunaliella* ATPase and a purified plasma membrane preparation from cotton contain equivalent amounts of 100-kD polypeptide bands according to Coomassie blue staining (not shown). As shown in Figure 3, all the antibodies interact strongly with a 95-kD band of cotton, indicating that these domains are conserved among higher plants. However, only the antibodies against the central domain recognize a 100-kD band of the *Dunaliella* ATPase preparation, indicating that only the central domain containing the ATP binding site is partially conserved between *Dunaliella* and higher plants.

Reconstitution of Proton Transport

Conclusive identification of an H^+-ATPase requires demonstration of ATP-dependent H^+ uptake. For this purpose, the purified ATPase was reconstituted into proteoliposomes by the freeze-thaw-sonication procedure and internal acidification was measured with the fluorescent acridine dye ACMA. As shown in Figure 4, the addition of ATP results in proton transport that is stimulated by the K^+ ionophore valinomycin. The effect of valinomycin is less prominent when a permeant anion nitrate is present in the medium (Fig. 4, a and b), whereas in the presence of nonpermeable anion such as SO_4^{2-}, no ΔpH is observed in the absence of valinomycin. The latter finding indicates that proton transport is electrogenic, and formation of the membrane potential inhibits build-up of the ΔpH. The observed quenching of fluorescence could be reversed by the protonophore SF-6847, by the H^+/K^+ exchange ionophore nigericin, by the weak base NH_3, or by the ATPase inhibitor vanadate (Fig. 4, a-d). The latter finding proves that the observed fluorescence changes reflect proton movements driven by the plasma membrane ATPase.

Kinetic Properties of the ATPase

ATP hydrolysis is strongly inhibited by the classical inhibitors of plasma membrane H^+-ATPases: vanadate, dicyclo-

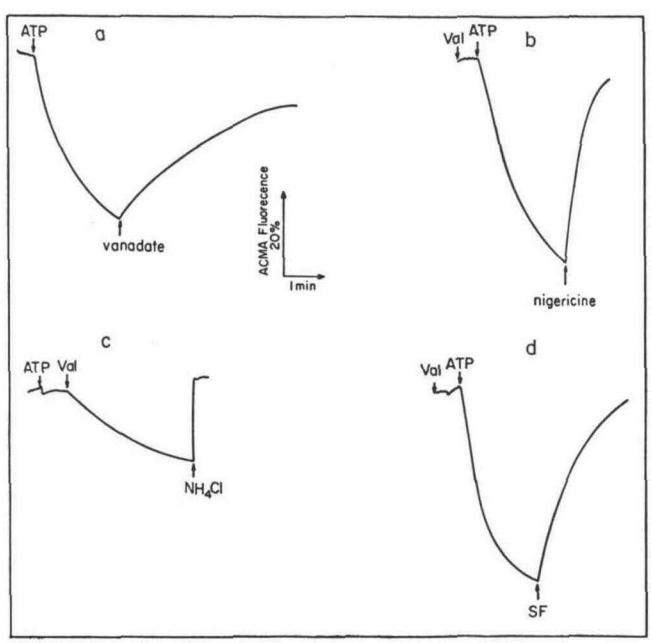

Figure 4. ATP-dependent ΔpH formation across proteoliposomes. Proteoliposomes were formed as described in "Materials and Methods." ACMA fluorescence quenching was measured in 2 mL containing proteoliposomes (7 μg), 10 mM Tris-Mes, pH 6, 5 mM $MgSO_4$, 50 mM KNO_3 (a and b) or K_2SO_4 (c and d), and 1 μM ACMA. ATP (0.5 mM), valinomycin (1 μM), vanadate (200 μM), SF-6847 (1 μM), nigericine (1 μM), and NH_4Cl (10 mM) were added as indicated.

Table II. *Inhibitors of the purified ATPase*

Purified enzyme samples (2 μg) were preincubated with the indicated inhibitors for 10 min at 0°C before the ATPase assay.

Inhibitor	IC_{50}[a]
	μM
Vanadate	1
Dicyclohexylcarbodiimide	30
Diethylstilbestrol	50
Erythrosine	0.9
$HgCl_2$	8
PCMBS	25

[a] IC_{50} is the inhibitor concentration required for 50% inhibition. The control activity was 9.4 units/mg protein.

hexylcarbodiimide, diethylstilbestrol, and erythrosin with $K_{0.5}$ values of inhibition of 1, 30, 50, 0.9 μM, respectively (Table II). ATPase activity is also extremely sensitive to mercurial compounds, with 50% inhibition by 8 and 25 μM for $HgCl_2$ and PCMBS, respectively. This indicates the existence of essential sulfhydryl groups and may explain the need for DTT during the purification process.

The pH dependence of the ATPase, shown in Figure 5, reveals a sharp optimum at pH 6. The enzyme is highly specific for ATP. Other nucleotides such as GTP, UTP, ITP, CTP, and ADP sustain less than 10% of the activity observed with ATP. In the absence of Mg^{2+}, the activity was less than 3% of that in its presence, indicating a requirement for divalent cations. Maximal activity was obtained with either 5 mM $MgCl_2$ or $MgSO_4$. With Co^{2+} and Mn^{2+}, the activity was 90% and 50% of that with Mg^{2+}, respectively, whereas substitution by Zn^{2+} or Ca^{2+} completely abolished ATPase activity. With 5 mM Mg^{2+} and variable ATP from 1 to 1000 μM, the kinetics obey Michaelis-Menten behavior. A K_m of about 40 μM could be extrapolated from the Endzffiter linear regression analysis (Fig. 6). This value is about 1 order of magnitude lower than that observed previously for plasma membrane H^+-ATPases (Goffeau and Slayman, 1981; Serrano, 1988a).

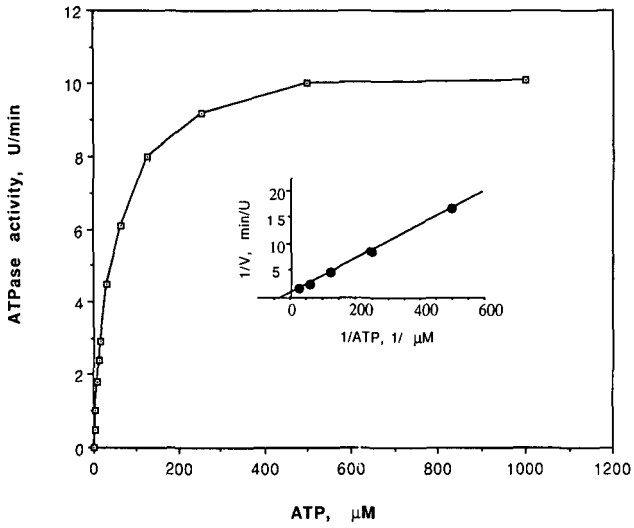

Figure 6. Dependence of ATPase activity on ATP concentrations. ATPase activity of the purified enzyme was measured as described in "Materials and Methods" in the presence of the indicated ATP concentrations. The fitted K_m and V_{max} from the regression analysis are 39.5 ± 4.5 μM and 9.1 ± 0.3 units/min, respectively.

Stimulation of ATPase and H^+-Pumping Activities by K^+

A characteristic feature of plant and fungal plasma membrane H^+-ATPase is stimulation of up to 2-fold by K^+. The *D. acidophila* ATP is stimulated 3- to 4-fold by K^+ (Fig. 7; Sekler et al., 1991). The stimulation is relatively specific for K^+ with an IC_{50} of 3 mM for K^+, about 12 mM for Rb^+, Cs^+, or Na^+, and no stimulation at all by Li^+ or $Tris^+$. The large stimulation by K^+ presumably indicates a specific interaction

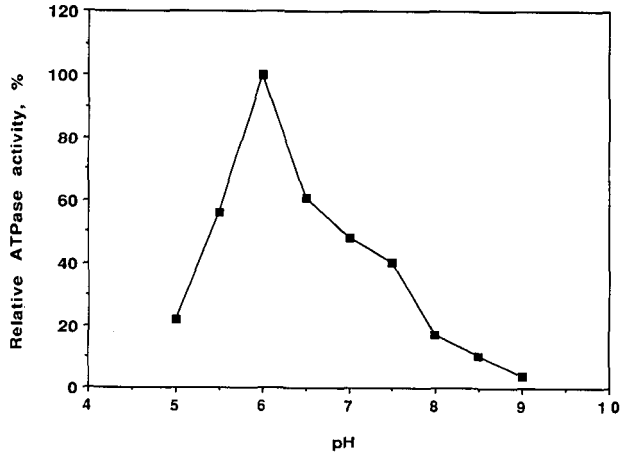

Figure 5. Effect of pH on ATPase activity. ATPase activity of the purified enzyme (2 μg) was determined at the indicated pH values as described in "Materials and Methods." Control (100%) activity is the rate of ATP hydrolysis at pH 6 (10.3 units/mg of protein).

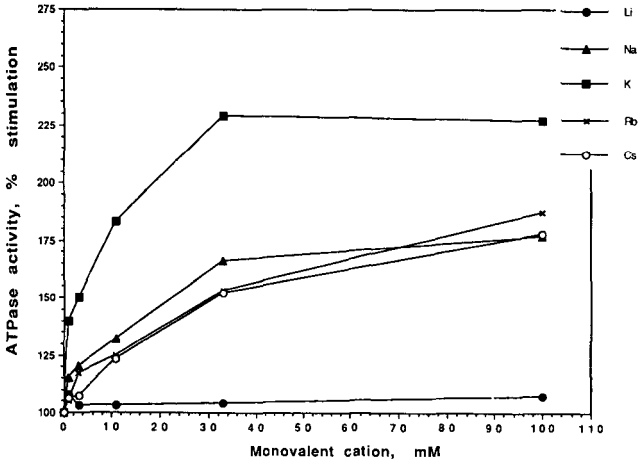

Figure 7. Stimulation of ATPase activity by monovalent cations. The activity of the purified ATPase was measured as described in "Materials and Methods" in the presence of the indicated concentrations of cations added as chloride salts at pH 6.

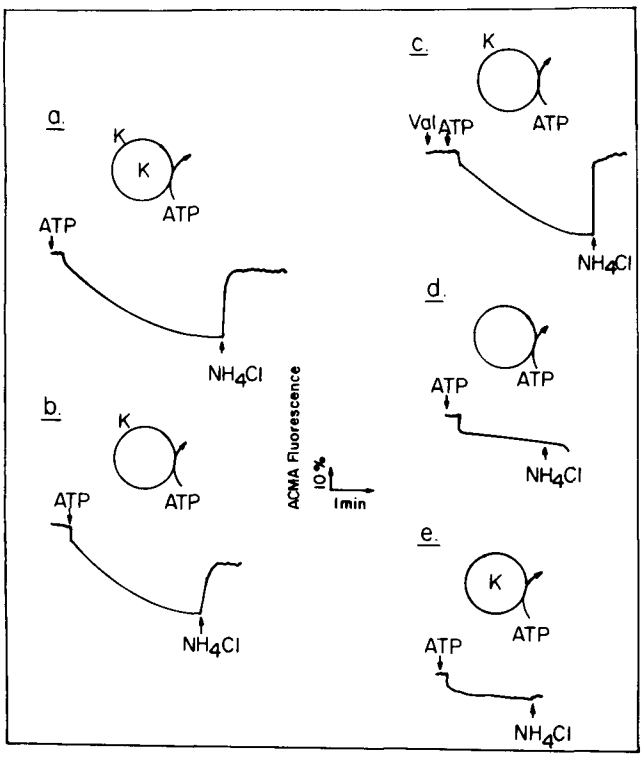

Figure 8. Sidedness of K^+ activation of the ATP-dependent ΔpH formation across proteoliposomes. Proteoliposomes were formed as described in "Materials and Methods" in a medium containing either 25 mM choline nitrate (b–d) or 25 mM KNO_3 (a and e). ACMA fluorescence quenching was measured as described in Figure 5. The assay medium contained either 25 mM KNO_3 (a–c) or 25 mM choline nitrate (d and e) and 1 μM ACMA. ATP (0.5 mM), valinomycin (1 μM), and NH_4Cl (10 mM) were added as indicated.

with the enzyme, which enhances a rate-limiting step in ATP hydrolysis. The sidedness of the site of interaction with K^+ was determined in reconstituted proteoliposomes. K^+ (Fig. 8, a and e) or the inert cation choline (Fig. 8, b–d) were trapped within proteoliposomes during the reconstitution, and the proteoliposomes were diluted into assay media containing either K^+ nitrate (Fig. 8, a–c) or choline nitrate (Fig. 8, d and e). The lipophilic nitrate anion was selected because it should be relatively permeable and should eliminate electrophoretic limitations of charge compensation during H^+ uptake. To avoid possible equilibration of K^+, ATP was added within seconds following dilution of the vesicles into the assay medium. Previous studies using the same reconstitution procedure have shown that the leakage of trapped Rb^+, a K^+ analog, from the reconstituted vesicles is fairly slow and cannot lead to considerable loss of trapped K^+ within this time scale (Gläser et al., 1990). As demonstrated in Figure 8, K^+ trapped inside the proteoliposomes does not stimulate H^+ uptake (compare traces d and e), whereas external K^+ fully stimulates H^+ uptake, either in the presence (trace a) or absence (trace b) of internal K^+. Thus, the site of interaction of K^+ is at the outer side of the proteoliposomes, which corresponds to the cytoplasmic surface of the pumps, oriented inside-out by comparison with their normal cellular orientation.

DISCUSSION

To our knowledge, the ATPase preparation described in this work is the first that has been purified and functionally reconstituted from a lower plant or an extreme acidophile. The yield of the purified enzyme compares very favorably with typical yields of higher plant ATPase preparations. The reason for the high yield is probably the unique resistance to nonionic detergents that allows an essentially complete extraction of contaminating proteins without solubilization of the ATPase. The solubilization is often the stage in which destabilization and loss of activity occur.

The H^+-ATPase 100-kD polypeptide appears to be a major plasma membrane protein in *D. acidophila*, as indicated from the staining intensity of the 100-kD band in crude plasma membrane preparations (Fig. 2A, lane 3) and from estimation of ATPase recovery, which suggests that it comprises about 5% of the total membrane proteins by assuming that the enzyme is neither activated nor inactivated during purification (Table II). This estimate is considerably higher than is usually assumed for plants (Serrano, 1988a), which suggests that the plasma membrane H^+-ATPase is overexpressed in *D. acidophila*. The *D. acidophila* H^+-ATPase resembles the plant and fungal ATPases in its size, in the sensitivity to mercurials, vanadate, dicyclohexylcarbodiimide, and diethylstilbestrol, and in the high selectivity for ATP.

Previously, we have demonstrated that the *D. acidophila* enzyme is stimulated by fusicoccin, similar to the plant plasma membrane H^+-ATPase (Sekler et al., 1991). These results and the presence of immunological cross-reactivity indicate a significant homology between the *D. acidophila* and the plant plasma membrane H^+-ATPases. However, there are also several distinct differences between the *D. acidophila* and higher plant enzymes, including a higher stability toward nonionic detergents and proteases (not shown) and the lack of cross-reactivity with antibodies against the C- and N-terminal domains of the plant enzyme. The structural differences may be required for adaptation to the extreme acidic pH outside the cell. Kinetic differences include the low K_m value for ATP, about 1 order of magnitude lower than that in plant and fungi plasma membrane H^+-ATPases (Goffeau and Slayman, 1981; Serrano, 1988a), the greater stimulation by K^+, and a slightly more acidic pH optimum. These properties may also have an important physiological significance in enabling *D. acidophila* to withstand acid stress, which may be encountered upon exposure to weak acid or high CO_2. Previously, we have observed that acid stress induced by acetic acid leads to a rapid drop of about 95% of the cellular ATP level (Gimmler et al., 1989a). A high affinity for ATP may enable the H^+ pump to function at a maximal rate even under these conditions.

The mechanism of K^+ stimulation, observed previously in both plant and fungal plasma membrane H^+-ATPases, is not clear. It has been attributed either to stimulation of a rate-limiting step in ATP hydrolysis, such as the hydrolysis of the phosphorylated intermediate E_2-P (Gläser and Höffer, 1987), or to K^+ transport by a primary H^+/K^+ pump (Villalobo, 1984;

Briskin, 1986). Previously, we have observed that ATP stimulates $^{86}Rb^+$ efflux from proteoliposomes reconstituted with *D. acidophila* plasma membranes and have demonstrated that this efflux is catalyzed by an H^+/K^+ symport mechanism in response to internal acidification by the H^+ pump (Gläser et al., 1990). These results have clearly demonstrated that K^+ is not a substrate of the ATPase. The present study, carried out with a purified enzyme preparation, is consistent with these observations and demonstrates that K^+ is an activating ligand of the H^+-ATPase and that it interacts with the enzyme from the cytoplasmic side of the plasma membrane.

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