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Regulation and Reversibility of Vacuolar H^+-ATPase*

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Arabidopsis thaliana **vacuolar H^+-translocating pyrophosphatase (V-PPase) was expressed functionally in yeast vacuoles with endogenous vacuolar H^+-ATPase (V-ATPase), and the regulation and reversibility of V-ATPase were studied using these vacuoles. Analysis of electrochemical proton gradient ($\Delta\mu H$) formation with ATP and pyrophosphate indicated that the proton transport by V-ATPase or V-PPase is not regulated strictly by the proton chemical gradient (ΔpH). On the other hand, vacuolar membranes may have a regulatory mechanism for maintaining a constant membrane potential ($\Delta\Psi$). Chimeric vacuolar membranes showed ATP synthesis coupled with $\Delta\mu H$ established by V-PPase. The ATP synthesis was sensitive to bafilomycin A_1 and exhibited two apparent K_m values for ADP. These results indicate that V-ATPase is a reversible enzyme. The ATP synthesis was not observed in the presence of nigericin, which dissipates ΔpH but not $\Delta\Psi$, suggesting that ΔpH is essential for ATP synthesis.**

Eukaryotic cells develop highly differentiated endomembrane organelles including vacuoles, lysosomes, endosomes, the Golgi apparatus, and synaptic vesicles (1–3). Their lumens are maintained at a specific acidic pH value by vacuolar H^+-ATPase (V-ATPase)[1] in combination with other ion transporters and channels (4, 5). V-ATPase transports protons coupled with the hydrolysis of ATP and establishes electrochemical gradients of protons. The V-ATPase exhibits catalytic cooperativity similar to F-type ATPase in ATP hydrolysis (6–8). However, they have different physiological roles; V-ATPase is involved in organelle acidification, whereas F-type ATPase is an ATP synthase (9).

Fungal and yeast vacuoles possess the V-ATPase as the sole proton pump, whereas those of higher plants contain V-ATPase and a proton translocating pyrophosphatase (V-PPase). V-PPase, a dimer of an 80-kDa polypeptide, transports protons into the vacuolar lumen by hydrolyzing cytosolic pyrophosphate (10). Kim *et al*. (11) showed recently that *Arabidopsis thaliana* V-PPase can be expressed in yeast as a functional vacuolar proton pump. This heterologous expression provided a system for studying the structural requirements for pyrophosphate-dependent proton transport (12, 13). We realized that this expression system also constitutes a unique approach for studying the regulation of V-ATPase if the two proton pumps, V-ATPase and V-PPase, are expressed functionally within the same vacuolar compartment. In this study we demonstrated that V-ATPase can synthesize ATP coupled with $\Delta\mu H$ generated by V-PPase and that proton translocation by the two pumps is not strictly regulated by ΔpH.

EXPERIMENTAL PROCEDURES

Expression of A. thaliana V-PPase in Yeast—*Arabidopsis* vacuolar pyrophosphatase (*AVP3*) cDNA (14) was isolated from an *Arabidopsis* cDNA library by polymerase chain reaction-assisted screening using a specific primer set designed from the *AVP3* structure. The coding and 3′-untranslated regions were amplified by polymerase chain reaction and subcloned into a yeast expression vector, pKT10-*N*-myc-1 (*URA3*, 2-μM *ori*) (15). The resultant plasmid, pKT10-*N*-myc-AVP3 (*AVP3* cDNA with the Myc epitope sequence), was introduced into strain YW12 (16). The *in vitro* amplified segments were verified by sequencing.

Preparation of Vacuolar Membranes—Yeast cells were grown up to 2×10^7 cells/ml in SCD medium (minimal medium containing 2% glucose and 0.5% casamino acids) and then for an additional 2 h after dilution with an equal volume of fresh YPD medium (2% glucose, 1% yeast extract, and 2% polypeptone). Vacuolar membranes were prepared (17), suspended in 5 mM MES-Tris buffer, pH 7.2, containing 400 mM sucrose, and then stored at −80 °C until use.

Immunological Analysis—For immunoblotting analysis, samples were denatured at 50 °C for 20 min in 10 mM Tris-HCl buffer, pH 8.0, containing 12.5% glycerol, 5% SDS, and 2% β-mercaptoethanol and then subjected to polyacrylamide gel electrophoresis in the presence of SDS. Affinity-purified rabbit anti-V-PPase (a gift from Dr. M. Maeshima) (18) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmnoResearch Laboratories) antibodies were used for immunoblotting. Indirect immunofluorescence microscopy was carried out (19) using early log phase cells grown in SCD medium. Cells were incubated with affinity-purified rabbit anti-V-PPase and the mouse monoclonal anti-yeast vacuolar H^+-ATPase 60-kDa subunit (Molecular Probes). Cy3-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmnoResearch Laboratories) were used as secondary antibodies. Confocal images were acquired with a Zeiss LSM510 microscope system. Subcellular fractionation of yeast was performed, and organelle marker proteins were detected as described previously (20).

Assays for Electrochemical Proton Gradient Formation and ATP Synthesis—The formation of ΔpH and $\Delta\Psi$ were assayed by measuring the fluorescence quenching of quinacrine (excitation, 425 nm; emission, 495 nm) and oxonol V (excitation, 580 nm; emission, 620 nm), respectively, using a Hitachi F-3000 fluorescence spectrophotometer. Vacuolar membranes were suspended in 1 ml of buffer A (20 mM MES-Tris buffer, pH 7.2, 150 mM sucrose, 5 mM $MgSO_4$, 0.1 mM EGTA-Tris) containing 75 mM of various salts such as KCl, choline chloride, LiCl, NaCl, RbCl, or potassium gluconate. Quinacrine dihydrochloride (5 μM) or oxonol V (0.5 μM) was added, and fluorescence quenching was initiated with the addition of 0.1 mM sodium pyrophosphate or 0.5 mM ATP (Tris salt) at 20 °C.

For ATP synthesis, the vacuolar membranes (15 μg/ml) were suspended in 20 mM MES-Tris buffer, pH 7.2, containing 75 mM KCl (or other salt), 150 mM sucrose, 5 mM $MgSO_4$, and 50 μM Ap_5A. The ATP synthesis at 10 °C was initiated by the simultaneous addition of 0.1 mM sodium pyrophosphate, 0.2 mM ADP, and 1 mM potassium phosphate. Aliquots (100 μl) taken at various times were mixed with 900 μl of 100

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[1] The abbreviations used are: V-ATPase, vacuolar H^+-ATPase; Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FITC, fluorescein isothiocyanate; MES, 2-(*N*-morpholino)ethanesulfonic acid; V-PPase, vacuolar H^+-translocating pyrophosphatase; ΔpH, proton chemical gradient; $\Delta\mu H$, electrochemical proton gradient; $\Delta\Psi$, membrane potential.

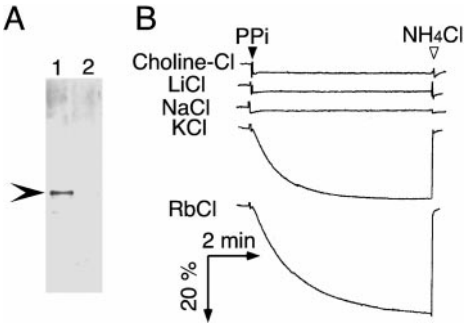

FIG. 1. **Expression of V-PPase in yeast.** *A*, *A. thaliana* V-PPase expressed in yeast. Vacuolar membranes (2 μg protein) from yeast cells harboring pKT10-*N*-myc-AVP3 (V-PPase expression plasmid, *lane 1*) or pKT10 (control plasmid, *lane 2*) were subjected to immunoblotting with anti-V-PPase antibodies. Signals were detected with an ECL immunoblotting detection kit (Amersham Pharmacia Biotech). A 66-kDa protein was detected (*lane 1, arrowhead*). *B*, cation-dependent proton transport by V-PPase expressed in yeast. ΔpH formation was assayed in buffer A containing 10 μg/ml vacuolar membranes. Quinacrine fluorescence quenching was followed in the presence of various salts and 0.1 mM sodium pyrophosphate (*PPi*). Reversal of the quenching was observed on the addition of 5 mM NH_4Cl.

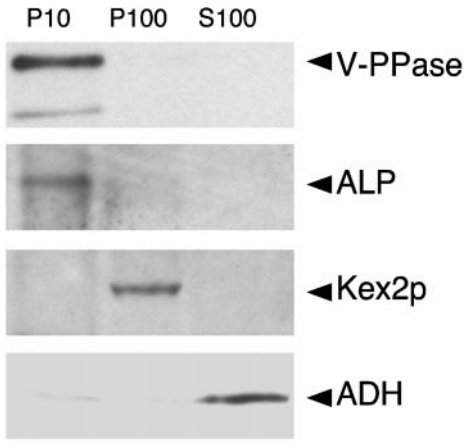

FIG. 2. **Subcellular fractionation of V-PPase expressed in yeast.** Spheroplasts harboring pKT10-*N*-myc-AVP3 (V-PPase expression plasmid) were lyzed, and the lysate was centrifuged at 10,000 × *g* for 15 min to obtain a pellet (*P10*) after removing unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 1 h, and pellet (*P100*) and supernatant (*S100*) fractions were obtained. Each fraction was subjected to polyacrylamide gel electrophoresis and then immunoblotting using specific antibodies against V-PPase, alkaline phosphatase (*ALP*, a vacuolar membrane marker), Kex2p (a late Golgi marker), and alcohol dehydrogenase (*ADH*, a cytosol marker).

mM HCl on ice and then the ATP concentrations were determined immediately with a bioluminescent assay mixture (Sigma) and a luminometer (Berthold LB9501). The solution for determination of ATP was diluted to maintain the inorganic pyrophosphate concentration at lower than 0.2 μM to avoid any effect on the luciferin/luciferase assay.

Other Assays and Chemicals—V-PPase was assayed by measuring the release of phosphate at 25 °C in the buffer used for assaying the formation of ΔpH except that the fluorescent probe was omitted. One unit of V-PPase activity was defined as the amount of enzyme hydrolyzing 1 nmol of inorganic pyrophosphate in 1 min. Phosphate (21) and protein concentrations (22) were determined by the established methods. Reagents were obtained from commercial sources. Bafilomycin A_1 and concanamycin A were from Wako Chemicals Co.; ADP (K^+ salt) was from Roche Molecular Biochemicals; ATP (Tris salt), oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and Ap_5A (sodium salt) were from Sigma. All other chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Expression of Arabidopsis V-PPase in Saccharomyces cerevisiae—We introduced a multicopy expression plasmid, pKT10-*N*-myc-AVP3, carrying the cDNA (*AVP3*) for *A. thaliana* V-PPase downstream of a yeast constitutive *TDH3* promoter, into yeast cells to express a plant V-PPase. Vacuolar membranes isolated from the transformants contained a protein with apparent molecular mass of 66 kDa recognized by anti-V-PPase antibodies (Fig. 1*A*). Essentially the same results were obtained using anti-myc-antibodies. The pyrophosphatase activities of vacuolar membranes from cells harboring pKT10-N-myc-AVP3 and pKT10 (control plasmid) were 240 and 70 units/mg protein, respectively, giving the activity due to V-PPase 170 units/mg. Upon the addition of sodium pyrophosphate, ΔpH across the vacuolar membrane was generated (Fig. 1*B*). The pyrophosphate-dependent H^+ pump required potassium or rubidium ions; essentially no acidification was observed in the absence of either cation or in the presence of choline chloride, LiCl, or NaCl. The cation dependence was similar to that observed for plant vacuolar membranes (23). This pyrophosphate-driven acidification was not observed in the membranes prepared from the wild type yeast transformed with the vector used for cloning (data not shown). These results indicated that the plant V-PPase functions as a proton pump in yeast vacuoles.

Plant V-PPase Was Co-localized with V-ATPase in Vacuolar Membranes—Although pyrophosphatase activity was found in the vacuolar membrane fraction, it was still possible that V-

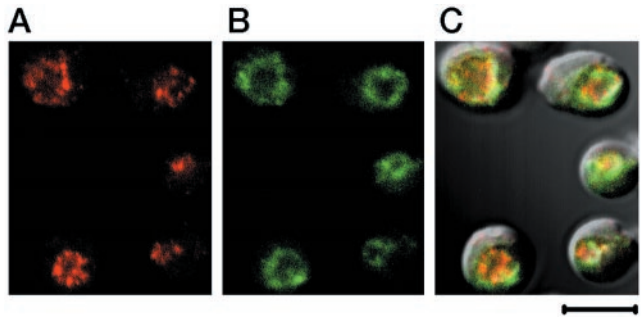

FIG. 3. **Co-localization of V-PPase and V-ATPase in vacuolar membranes.** Spheroplasts harboring pKT10-*N*-myc-AVP3 (V-PPase expression plasmid) were labeled with rabbit anti-V-PPase and the mouse monoclonal anti-yeast vacuolar H^+-ATPase 60-kDa subunit and then probed with Cy3-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG, respectively. Cells were viewed under a laser scanning confocal microscope. *A*, Cy3 fluorescence; *B*, FITC fluorescence; and *C*, superimposed Cy3 and FITC images with a differential interference contrast image. *Bar*, 5 μm.

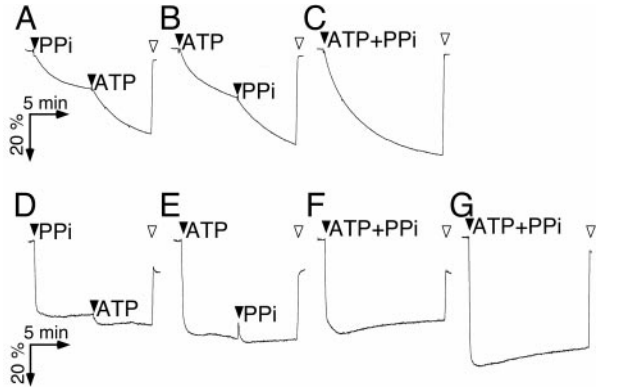

FIG. 4. **Electrochemical proton gradient formation by V-PPase and V-ATPase.** ΔpH formation (*A-C*) and ΔΨ formation (*D-G*) were assayed by means of quinacrine and oxonol V fluorescence quenching, respectively. Vacuolar membranes (5 μg/ml) from cells expressing V-PPase were suspended in buffer A containing 75 mM KCl (*A-F*) or potassium gluconate (*G*). At the indicated times (closed triangles), 0.5 mM ATP and/or 0.1 mM sodium pyrophosphate (*PPi*) were added. Quenching was reversed on the addition of 5 mM NH_4Cl (*A-C*) or 25 μM CCCP (*D-G*) (*open triangles*).

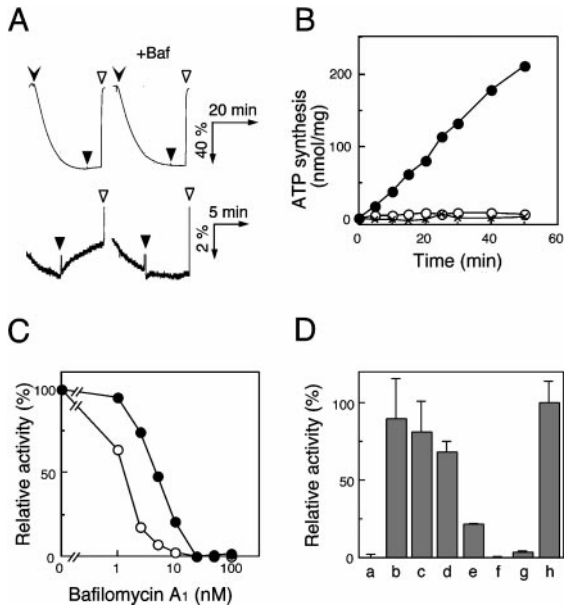

FIG. 5. **ATP synthesis by V-ATPase using an electrochemical proton gradient generated by V-PPase.** *A*, the reversal of pyrophosphate-dependent quinacrine quenching on the addition of ADP (*lower traces*, expanded scale). Vacuolar membranes (15 μg/ml) from yeast cells harboring pKT10-*N*-myc-AVP3 were incubated in buffer A with (+*Baf*) or without 100 nM bafilomycin A_1 in the presence of 75 mM KCl and 1 mM potassium phosphate at 10 °C. Quinacrine quenching was started by the addition of 0.1 mM sodium pyrophosphate (*arrowheads*). After 23 min of incubation, about 80% quenching was observed, and 0.2 mM ADP was added (*closed triangles*). At the indicated times, 5 mM NH_4Cl was added (*open triangles*). Low bafilomycin A_1-sensitive reversal of quenching was observed. *B*, time course of ATP synthesis by vacuolar membranes from yeast cells harboring pKT10-*N*-myc-AVP3 (V-PPase expression plasmid). ATP synthesis was assayed in the presence of 0.2 mM ADP and sodium pyrophosphate (*closed circles*). No ATP synthesis was observed in the absence of sodium pyrophosphate (*open circles*) or in the presence of 25 μM CCCP (×). Vacuolar membranes from cells harboring pKT10 (control plasmid) did not show ATP synthesis. *C*, effects of bafilomycin A_1 on V-ATPase proton pumping (*open circles*) and pyrophosphate-dependent ATP synthesis (*closed circles*). Proton pumping activity was assayed with 15 μg/ml vacuolar membranes, and the initial rate of quinacrine quenching was titrated with bafilomycin A_1. *D*, pyrophosphate-dependent ATP synthesis was measured in the presence of ATPase inhibitors or various salts. Concentrations of ATPase inhibitors were as follows: *a*, 25 nM concanamycin A; *b*, 100 μM sodium vanadate; *c*, 500 μM sodium azide; *d*, 10 μM oligomycin. KCl (*h*) was substituted by KNO_3 (*e*), LiCl (*f*), or NaCl (*g*).

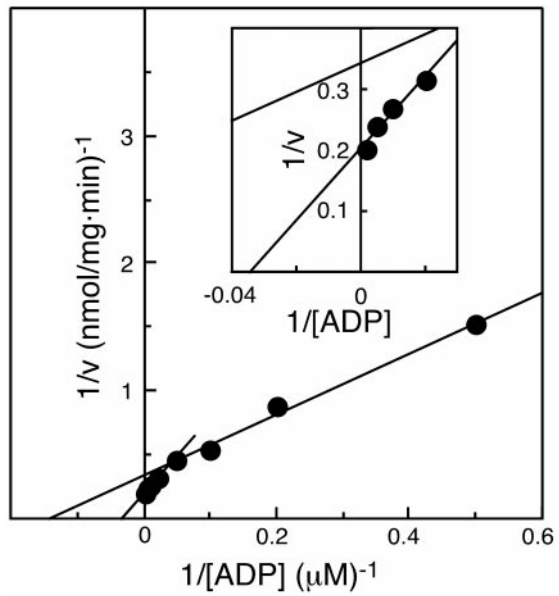

FIG. 6. **Effect of the ADP concentration on pyrophosphate-dependent ATP synthesis in yeast vacuolar membranes.** Lineweaver-Burk plots (*1/[ADP] versus 1/v*) of pyrophosphate-dependent ATP synthesis are shown. The ADP concentration was varied in the range of 2–500 μM. *Inset*, plots in the higher ADP concentration range. Apparent K_m values for ADP of 6.9 (for the low substrate range) and 29 μM (for the high substrate range) were obtained; corresponding V_{max} values were 2.9 and 4.9 nmol/mg/min, respectively.

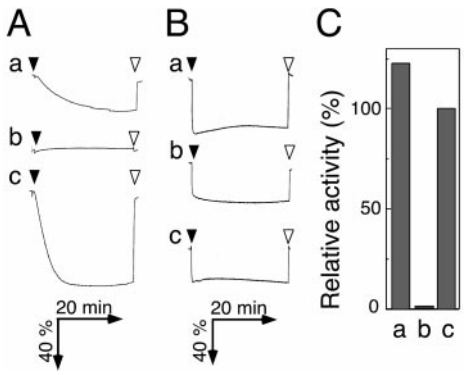

FIG. 7. **Effects of the electrochemical gradient on ATP synthesis by V-ATPase.** Formation of ΔpH (*A*), ΔΨ (*B*), and ATP synthesis (*C*) dependent on pyrophosphate were examined at 10 °C in the presence of 75 mM potassium gluconate (*a*), 75 mM KCl and 50 nM nigericin (*b*), or 75 mM KCl (control, *c*). For the measurement of ΔpH and ΔΨ formation, vacuolar membranes (15 μg/ml) were incubated in the assay medium used for ATP synthesis (*C*) containing 0.2 mM ADP and 1 mM potassium phosphate, and reaction was started by the addition of 0.1 mM sodium pyrophosphate (*closed triangles*). Quenching was reversed with the addition of 5 mM NH_4Cl (*A*) or 25 μM CCCP (*B*) (*open triangles*).

PPase was localized in a compartment different from that of V-ATPase. This possibility was examined by subcellular fractionation and immunofluorescence microscopy. Yeast cells expressing V-PPase were lyzed and then fractionated by differential centrifugation (Fig. 2). V-PPase was co-fractionated with an endogenous vacuolar marker protein, alkaline phosphatase, but separated from a late Golgi marker protein, Kex2p, and a cytosol marker, alcohol dehydrogenase. The localization of V-PPase was studied more directly by immunofluorescence microscopy. Antibodies against V-PPase (Fig. 3*A*) and the vacuolar H^+-ATPase 60-kDa subunit (Fig. 3*B*) essentially stained the same region of the vacuoles. These results confirmed that V-PPase and V-ATPase were localized in the same compartment.

V-PPase- and V-ATPase-dependent Formation of an Electrochemical Proton Gradient—It was not known whether the proton pumping of V-PPase and V-ATPase was regulated by an electrochemical proton gradient. We examined the formation of ΔpH and ΔΨ using quinacrine and oxonol V, respectively. The addition of pyrophosphate increased the ATP-dependent quenching of quinacrine fluorescence, indicating that the vacuolar membranes were hyper-acidified by V-ATPase and V-PPase (Fig. 4*A*). Similarly, the addition of ATP further increased the pyrophosphate-dependent acidification of vacuoles (Fig. 4*B*). Furthermore, the steady state quenching observed with ATP alone (Fig. 4*B*) was essentially the same as the ATP-dependent quenching induced by the addition of ATP after ΔpH formation by V-PPase (Fig. 4*A*). The addition of both substrates at the same time also led to hyper-acidification (Fig. 4*C*). These observations suggested that the steady state ΔpH formation by V-ATPase and V-PPase may not be strictly regulated, although stimulation of V-ATPase and V-PPase activity by protonophore (24, 25) has been observed.

In contrast to ΔpH, the formation of ΔΨ was not increased by the successive or simultaneous addition of proton pump substrates (Fig. 4, *D*, *E*, and *F*). These results were not due to the limited response of oxonol V used for assays, because greater fluorescence quenching was observed when chloride was sub-

stituted for gluconate (Fig. 4, *F* and *G*). The lack of an additive effect of the two proton pumps on ΔΨ formation may be due to the mechanism maintaining a constant ΔΨ across vacuolar membranes. The ion transporting systems for cations and anions in vacuolar membrane (5, 26, 27) may be responsible for such regulation.

ATP Synthesis by V-ATPase in Yeast Vacuolar Membranes—We were interested in whether V-ATPase can synthesize ATP from ADP and phosphate coupled with an electrochemical proton gradient. This possibility was predicted from the small reversal of the pyrophosphate-dependent fluorescence quenching on the addition of ADP (in the presence of inorganic phosphate) (Fig. 5*A*). This reversal was inhibited by bafilomycin A_1, a specific V-ATPase inhibitor (28). Consistent with the reversal, we observed pyrophosphate-dependent synthesis of ATP when engineered vacuolar membranes were incubated with phosphate, ADP, and pyrophosphate (Fig. 5*B*). An adenylate kinase inhibitor, Ap_5A, had no effect on the V-ATPase and V-PPase proton pumping activity (data not shown). A protonophore, CCCP, completely inhibited the ATP synthesis, and vacuolar membranes lacking V-PPase showed no activity. The pyrophosphate-dependent ATP synthesis was fully inhibited by 25 nM bafilomycin A_1 (Fig. 5*C*). It is of interest that proton transport is slightly more sensitive to bafilomycin A_1. The ATP synthesis was also sensitive to other V-ATPase inhibitors, concanamycin A (29) and nitrate (30), but not inhibited by vanadate, azide, or oligomycin (Fig. 5*D*). These results indicated that the ATP synthesis was coupled with an electrochemical proton gradient established by V-PPase and that V-ATPase is responsible for ATP synthesis.

As shown in Fig. 1, pyrophosphate-dependent proton transport requires potassium ions. Thus, no ATP synthesis was observed when KCl was substituted for LiCl or NaCl (Fig. 5*D*). This observation is consistent with the notion that ATP synthesis is coupled with $\Delta\mu H$ generated by V-PPase.

Although ATP synthesis in plant tonoplasts and chromaffin granules has been observed using luciferine/luciferase assay (31–33) and isotope exchange reaction (33, 34), previous studies were not conclusive as to the involvement of V-ATPase in the net ATP synthesis. In these experiments, an adenylate kinase inhibitor was not included in the assay mixture (31), and only slight inhibition was observed with a relatively high concentration of bafilomycin A_1 (1 μM) (32). ATP synthesis in tonoplast-enriched vesicles isolated from maize (34) may be due to the exchange between contaminating ATP and radioactive phosphate, because an adenylate kinase inhibitor was not included. Bafilomycin A_1 sensitivity of ATP synthesis in chromaffin granules was not examined previously (33). In this study we concluded that yeast V-ATPase is a reversible enzyme. It should be noted that our conclusion is based on the results of studies involving vacuolar membranes with or without plant V-PPase and assays in the presence of an adenylate kinase inhibitor. Furthermore, the sensitivities to known ATPase inhibitors and ionophore were consistent with the reversibility of V-ATPase.

Lineweaver-Burk plots of pyrophosphate-dependent ATP synthesis showed biphasic kinetics when the ADP concentration was varied. Two apparent K_m values for ADP, 6.9 and 29 μM (V_{max} values of 2.9 and 4.9 nmol/mg/min, respectively), were obtained (Fig. 6). The ATP synthesis by V-ATPase is consistent with the previous findings that archaebacterial A-type and eubacterial V-type ATPase synthesizes ATP (35–38). However, the low rate of ATP synthesis by yeast vacuoles strongly agrees with the physiological role in V-ATPase proton pumping; the V_{max} rate of ATP synthesis of mitochondrial membranes (39) was at least 200-fold higher than that of the vacuolar membranes by V-ATPase.

No ΔpH and ΔΨ were generated by V-PPase in the presence of 75 mM KCl and 20 μM valinomycin for unknown reasons. Thus, we could not test the effects of valinomycin on ATP synthesis. We tested whether ΔpH was essential for ATP synthesis by V-ATPase. Although ΔpH formation was reduced slightly and ΔΨ was increased in the presence of potassium gluconate (Fig. 7, *A* and *B*), pyrophosphate-dependent ATP synthesis was not affected (Fig. 7*C*). This result suggests that both ΔpH and ΔΨ contribute to the ATP synthesis, and the reduced ΔpH may be compensated by the increased ΔΨ. Nigericin (K^+/H^+ antiporter) did not affect ΔΨ formation, dissipated ΔpH (Fig. 7, *A* and *B*), and inhibited pyrophosphate-dependent ATP synthesis. These results suggest that ΔpH is essential for ATP synthesis.

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