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 (\(\Delta \psi\)) 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 (\(\Delta \psi\)). 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 \(\Delta \psi\) established by V-PPase. The ATP synthesis was sensitive to bafilomycin A1 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 \(\Delta \psi\) but not \(\Delta \Psi\), suggesting that \(\Delta \psi\) 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) 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 and a proton translocating pyrophosphatase (V-PPase). 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 \(\Delta \psi\) generated by V-PPase and that proton translocation by the two pumps is not strictly regulated by \(\Delta \psi\).

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-m\(\mu\)m ori) (15). The resultant plasmid, pKT10-N-myc-AVP3 (AVP3) cDNA with the Myc epitope sequence, was introduced into strain WY12 (16). The in vitro amplified segments were verified by sequencing.

Preparation of Vacuolar Membranes—Yeast cells were grown up to \(2 \times 10^7\) 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% peptone). Vacuolar membranes were prepared (17), suspended in 5 mM MES-Tris buffer, pH 7.2, containing 400 mM sucrose, and then stored at \(-80^\circ\)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 ImmunoResearch 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 ImmunoResearch 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 \(\Delta \psi\) 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 \(\mu\)M) or oxonol V (0.5 \(\mu\)M) was added, and fluorescence quenching was initiated with the addition of 0.1 mM sodium pyrophosphate or 0.5 mM Tris salt at 20 °C.

For ATP synthesis, the vacuolar membranes (15 \(\mu\)g/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 \(\mu\)M ApA. 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 \(\mu\)l) taken at various times were mixed with 900 \(\mu\)l of 100 mM potassium phosphate and 1 mM MgSO\(_4\).
buffer A containing 10 mM ATP and/or 0.1 mM sodium pyrophosphate (PPi). Reversal of the quenching was observed on the addition of 5 mM NH₄Cl.

**FIG. 1. Expression of V-PPase in yeast.** A, A. thaliana V-PPase expressed in yeast. Vacular 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 (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 NH₄Cl.

**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).

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. Vacular membranes isolated from the transformants contained a protein with apparent molecular mass of 66 kDa recognized by anti-V-PPase antibodies (Fig. 1A). 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. 1B). 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 Vacular Membranes**—Although pyrophosphatase activity was found 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.

**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, contrast image.

**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. Vacular 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 NH₄Cl (A-C) or 25 mM CCCP (D-G).
After 23 min of incubation, about 80% quenching was observed, and 0.2 traces phate-dependent quinacrine quenching on the addition of ADP (lower Proton pumping activity was assayed with 15 mM and 1 mM potassium phosphate at 10 °C. Quinacrine quenching was bafilomycin A1. vacuolar membranes from yeast cells harboring pKT10-NB sal of quenching was observed. H KCl (100 mM sodium vanadate; 500 mM sodium azide; 10 mM CCCP (g)), or without 100 nM bafilomycin A1 in the presence of 75 mM KCl (control, c). For the measurement of pH 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 NH4Cl (A) or 25 mM 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. 3A) and the vacuolar H-ATPase 60-kDa subunit (Fig. 3B) 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 vacular membranes were hyper-acidified by V-ATPase and V-PPase (Fig. 4A). Similarly, the addition of ATP further increased the pyrophosphate-dependent acidification of vacuoles (Fig. 4B). Furthermore, the steady state quenching observed with ATP alone (Fig. 4B) was essentially the same as the ATP-dependent quenching induced by the addition of ATP after ΔpH formation by V-PPase (Fig. 4A). The addition of both substrates at the same time also led to hyper-acidification (Fig. 4C). 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-
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 involving vacuolar membranes with or without plant V-PPase were not conclusive as to the involvement of V-ATPase in the proton transport; the physiological role in V-ATPase proton pumping; the eubacterial V-type ATPase synthesizes ATP (35–38). However, with the previous findings that archaebacterial A-type and renotype antporter) did not affect ATP generation, dissipated ∆H and ∆Ψ was reduced at pH formation was reduced (Fig. 7, A and B). This result suggests that both ∆H and ∆Ψ contribute to the ATP synthesis, and the reduced ∆H may be compensated by the increased ∆Ψ. Nigericin (K+/H+ antiporter) did not affect ATP formation, dissipated ∆H (Fig. 7, A and B), and inhibited pyrophosphate-dependent ATP synthesis. These results suggest that ∆H is essential for ATP synthesis.

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No ∆H and ∆Ψ 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 ∆H was essential for ATP synthesis by V-ATPase. Although ∆H formation was reduced slightly and ∆Ψ was increased in the presence of potassium gluconate (Fig. 7, A and B), pyrophosphate-dependent ATP synthesis was not affected (Fig. 7C). This result suggests that both ∆H and ∆Ψ contribute to the ATP synthesis, and the reduced ∆H may be compensated by the increased ∆Ψ. Nigericin (K+/H+ antiporter) did not affect ∆Ψ formation, dissipated ∆H (Fig. 7, A and B), and inhibited pyrophosphate-dependent ATP synthesis. These results suggest that ∆H is essential for ATP synthesis.