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Proton-Translocating Inorganic Pyrophosphatase in Red Beet (*Beta vulgaris* L.) Tonoplast Vesicles[1]

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ABSTRACT

The substrate and ionic requirements of ATP and inorganic pyrophosphate (PPi) hydrolysis by tonoplast vesicles isolated from storage tissue of red beet (*Beta vulgaris* L.) were compared with the requirements of ATP- and PPi-dependent proton translocation by the same material. Both ATP hydrolysis and ATP-dependent proton translocation are most stimulated by Cl^- and inhibited by NO_3^-. NaCl and KCl support similar rates of ATP hydrolysis and ATP-dependent proton translocation while K_2SO_4 supports lesser rates for both. PPi hydrolysis and PPi-dependent proton translocation are most stimulated by K^+. KCl and K_2SO_4 support similar rates of PPi hydrolysis and PPi-dependent proton translocation but NaCl has only a small stimulatory effect on both. Since PPi does not inhibit ATP hydrolysis and ATP does not interfere with PPi hydrolysis, it is inferred that the two phosphohydrolase and proton translocation activities are mediated by different tonoplast-associated enzymes. The results indicate the presence of an energy-conserving proton-translocating pyrophosphatase in the tonoplast of red beet.

The primary active transport process across the plasma and vacuolar membranes of plant cells is considered to be the ATP-dependent electrogenic translocation of protons (19, 24). By extension of the chemiosmotic hypothesis (17), the proton electrochemical potential difference generated by proton translocation is thought to provide the requisite motive force for the secondary transport of a wide range of chemical species.

While the primacy of ATP for the energization of transport across the membranes of plant cells is not in dispute, the involvement of other phosphoesters, PPi in particular, has yet to be thoroughly evaluated. Walker and Leigh (27) have demonstrated the presence of a Mg^{2+}-dependent PPase[2] associated with vacuoles isolated from storage tissue of red beet (*Beta vulgaris* L.), and Karlsson (11) has partially purified a PPase with qualitatively similar kinetics from the 25,000 to 30,000*g* membrane fraction of sugar beet (*Beta vulgaris* L.) roots and cotyledons. Although Churchill and Sze (8) and Bennett *et al.* (4) have shown that PPi will support proton translocation by tonoplast vesicles isolated from oat and beet roots, respectively, it is not known if PPi-stimulated proton translocation results from the activity of the PPase or whether the tonoplast ATPase is capable of utilizing PPi as substrate. The different detergent-solubilities of the PPase and ATPase from tulip vacuolar membranes (26) and their different cation and anion requirements (11, 27) suggest that at least a proportion of the membrane-associated PPase activity is distinct from ATPase-mediated phosphohydrolysis, but it is not clear if PPi-stimulated proton translocation is mediated by the PPase *per se* or whether it simply reflects the ability of the ATPase to utilize PPi as well as ATP as an energy source.

In this paper we show the correspondence between the substrate, mineral ion and effector sensitivities of both inorganic pyrophosphohydrolysis and PPi-dependent proton translocation by red beet tonoplast vesicles and in so doing demonstrate that the tonoplast PPase and ATPase mediate two distinct proton translocation events.

MATERIALS AND METHODS

Plant Material. Fresh red beet (*Beta vulgaris* L.) with leaves intact were purchased commercially, stored at 4°C, and used within 1 week of purchase.

Isolation of Tonoplast Vesicles. The method of Poole *et al.* (20) was employed except that the compositions of the homogenization and suspension media were based on the recommendations of Scherer and Morré (22). The homogenization medium consisted of 53% (v/v) sterilized coconut milk or 10 mM glycerophosphate, 0.65 M ethanolamine (adjusted to pH 8.0 with concentrated H_2SO_4), 0.28 M choline chloride, 26 mM K-metabisulfite, 2 mM salicylhydroxamic acid, 0.2% (w/v) BSA (fraction, V, essentially fatty acid-free), 10% (w/v) insoluble PVP, 5 mM DTT, 0.5 mM butylated hydroxytoluene, and 1 mM nupercaine buffered to pH 8.0 with 70 mM Tris-Mes. The suspension medium consisted of 1.1 M glycerol, 1 mM Tris-EDTA, 1 mM nupercaine, 0.5 mM butylated hydroxytoluene, and 5 mM DTT buffered to pH 8.0 with 5 mM Tris-Mes. Choline and ethanolamine were included in the homogenization medium to minimize membrane degradation by phospholipase D while nupercaine and coconut milk, glycerophosphate, or glycerol were added to inhibit phospholipase A and phosphatidic acid phosphatase activity, respectively (22).

The KI solution, employed to diminish contamination of the tonoplast vesicles with acid phosphatase (20), and the sucrose density gradient solutions were prepared in suspension medium.

Sucrose Density Centrifugation. For the routine preparation of tonoplast vesicles, the 0.25 M KI-treated pellet from 300 to 350 g beet was resuspended in 12 ml suspension medium and 3 ml volumes were layered onto each of four step gradients of 5 ml 10% (w/w) and 5 ml 23% (w/w) sucrose. After centrifugation at 80,000*g* for 2 h, the membranes at the 10/23% sucrose interface were removed with a Pasteur pipette. The membranes were diluted 10- to 20-fold with the desired experimental solution, sedimented at 80,000*g* and resuspended in 1 to 2 ml of the same solution.

For the experiment described in Table I, where plasma membrane as well as tonoplast vesicles were required for comparative purposes, the step gradients consisted of 2 ml 40% (w/w), 3 ml

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[2] PPase, inorganic pyrophosphatase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HK, hexokinase; YIP, yeast inorganic pyrophosphatase.

34% (w/w), 3 ml 23% (w/w), and 3 ml 10% (w/w) sucrose. Plasma membrane was collected from the 34/40% sucrose interface as described by Bennett *et al.* (4). Otherwise the conditions were identical to those outlined for the routine preparation of tonoplast vesicles.

Enzyme Assays. ATPase activity was either measured as the rate of liberation of Pi from ATP or as the rate of ADP-dependent NADH oxidation in a coupled system containing NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (ATP → ADP + Pi; ADP + PEP → ATP + pyruvate; pyruvate + NADH → lactate + NAD^+).

Pi liberation was measured in a 0.6 ml reaction volume containing 5 to 10 μg membrane protein, 50 mM Tris-Cl, or 50 mM KCl, 3 mM Tris-ATP, 3 mM $MgSO_4$, and 40 mM Tris-Mes buffer, pH 8.0. The reaction was initiated by the addition of membranes and allowed to proceed for 30 min at 37°C. Pi was measured by the Ames method (2).

The rate of ADP formation was measured in a 1 ml reaction volume containing 5 to 10 μg membrane protein, 50 mM KCl, 0 to 0.6 mM Tris-ATP, 3 mM $MgSO_4$, 30 mM Tris-Mes (pH 8.0), 3 mM phosphoenolpyruvate, 0.184 mM NADH, 1 unit pyruvate kinase (EC 2.7.1.40 from rabbit muscle), and 1 unit lactate dehydrogenase (EC 1.1.1.27 from hog muscle). The rate of ADP-dependent NADH oxidation was measured as the decrease in $A_{340\,nm}$ with time.

PPase activity was measured as the rate of liberation of Pi from PPi in reaction volume of 0.6 ml by the Ames method (2). The assay medium consisted of 5 to 10 μg membrane protein, 5 mM K-Mes or 50 mM KCl, 3 mM Tris-PPi, 3 mM $MgSO_4$ and 40 mM Tris-Mes buffer, pH 8.0. PPase activity was calculated as half the rate of Pi liberation (= μmol PPi consumed/unit time) since the hydrolysis of 1 mol of PPi yields 2 mol of Pi.

Variations in the assay pH, salt, or effector concentration are indicated in the text and legends.

Fluorescence Assays. The formation of inside-acid pH gradients across the membranes of the tonoplast vesicles was measured as the rate of fluorescence quenching of the permeant amine dye, acridine orange (20). Tonoplast vesicles (50–160 μg membrane protein), 30 mM Tris-Mes buffer (pH 8.0), 3 mM Tris-ATP or 3 mM Tris-PPi, 50 mM of the appropriate salt, 250 mM sorbitol, and 5 μM acridine orange were added to the sample cell to give a final volume of 1.0 ml. Proton translocation was initiated by the addition of 3 mM $MgSO_4$ and the fluorescence decrease with time was measured at 25°C with a Perkin Elmer spectrofluorimeter model LS-5 at excitation and emission wavelengths of 493 and 540 nm, respectively, and a slitwidth of 5 nm for both emission and excitation.

Protein. Protein was measured by a modification of the dye-binding method of Bradford (7) in which membrane protein was partially solubilized with 0.15% (w/v) Na-deoxycholate before the addition of the dye reagent concentrate (Bio-Rad Laboratories, Canada, Ltd). BSA (fraction V, essentially fatty acid-free) was employed as the protein standard.

Chemicals. General laboratory reagents were obtained from BDH Chemicals, Canada, and Sigma Chemical Co. Pyruvate kinase, lactate dehydrogenase, and hexokinase (EC 2.7.1.1) were purchased from Boehringer Mannheim, Montreal, Quebec, Canada and soluble inorganic PPase (EC 3.6.1.11 from yeast) was bought from Sigma. Na_2ATP and Na_4PPi were purchased from Boehringer and converted to their corresponding Tris salts by cation-exchange with Dowex 50W (Tris-form; 100–200 mesh).

RESULTS

Purity of Tonoplast Vesicles and Localization of K^+-Stimulated PPase Activity. Tonoplast vesicles were prepared routinely by density gradient centrifugation of the resuspended KI-treated microsomal pellet in a step gradient of 10% (w/w) and 23% (w/w) sucrose (20). The purity of the membrane fraction taken from the 10/23% sucrose interface with respect to phosphohydrolysis is indicated by the results in Table I.

The ATPase activity of the 10/23% interface is 72% inhibited by KNO_3 but inhibited only 14% by Na-molybdate, 13% by Na-vanadate, and 9% by NaN_3. Since inhibition by NO_3^- is a feature of higher plant tonoplast ATPase (1) whereas inhibition by molybdate, vanadate, and N_3 are characteristic of nonspecific phosphatase (13), plasma membrane ATPase (20), and mitochondrial F1 ATPase (6), respectively, the activity at the 10/23% sucrose interface is predominantly derived from the tonoplast (also see Ref. 20). The ATPase activity at the 34/40% interface is only 15% inhibited by NO_3^- whereas vanadate causes 66% inhibition. Since molybdate and N_3 exert little or no inhibition, the principal activity at this interface is vanadate-sensitive, presumably plasma membrane-derived ATPase (4, 20).

PPase activity is greatest at the 10/23% sucrose interface. The 10-fold greater specific activity of PPase at the 10/23% interface *versus* the 34/40% interface and its cosedimentation with NO_3^--sensitive ATPase confirms that PPase activity is associated with the tonoplast not plasma membrane. The lack of inhibition by molybdate or N_3 shows that nonspecific phosphatase and mitochondrial ATPase do not contribute to inorganic pyrophosphohydrolysis in this system.

Nitrate Sensitivity. The tonoplast PPase is not subject to inhibition by NO_3^- (Tables I and II). PPase activity is the same with either 50 mM KCl or 50 mM KNO_3, whereas the ATPase is 2.7-fold less active with 50 mM KNO_3 *versus* 50 mM KCl (Table II). Although 100 mM KCl causes a small but statistically significant stimulation of PPase activity over that seen with 50 mM KCl or KNO_3, 50 mM KCl plus 50 mM KNO_3 gives a similar rate to that seen with 100 mM KCl alone. The stimulatory effects of KCl and KNO_3 therefore appear to interact in a simple additive manner. One hundred mM KNO_3 is however 8% less stimulatory than 100 mM KCl and 6% less stimulatory than 50 mM KCl plus 50 mM KNO_3. If NO_3^- does have an inhibitory action on the PPase, it is very small and only seen at concentrations greater than 50 mM.

pH Activity Profiles. Since the tonoplast ATPase is strongly inhibited by 50 mM KNO_3, whereas the PPase is unaffected by NO_3^- but stimulated 5- to 8-fold by 50 mM K^+ (Tables I, II, and III), ATPase activity may be measured specifically by assaying NO_3^--sensitive ATP hydrolysis and PPase activity by assaying K^+-stimulated inorganic PPi hydrolysis (Fig. 1).

Both the total and NO_3^--sensitive components of ATPase activity show a pH optimum of 6.5 units. The total and K^+-stimulated components of PPase activity, on the other hand, approach a maximum at pH 7.5 to 8.0 and retain more than 83% of the maximal activity in the pH range 8.0 to 9.5. Similar pH activity profiles have been obtained with the PPase of isolated red beet vacuoles (26) and the microsomal pellet of sugar beet (11).

Substrate Concentration Dependence and Substrate Specificity. The different pH activity profiles and NO_3^- and K^+ sensitivities of the tonoplast ATPase and PPase suggest that they are different enzymes; their substrate concentration dependencies and specificities support this (Fig. 2).

The activity of the tonoplast ATPase approximates Michaelis-Menten kinetics with respect to ATP concentration. A double-reciprocal plot yields a straight line relationship ($r = 0.998$ by Least Squares Method) and K_m and V_{max} values of 0.12 mM and 67.6 μmol/mg·h, respectively. Since the inclusion of 1.2 mM PPi in the assay medium does not change the relationship between reaction velocity and ATP concentration, the tonoplast ATPase seems to have little or no affinity for PPi even when the latter is present at concentrations 10-fold greater than the apparent K_m of the enzyme for ATP.

Table I. *Purity of Tonoplast Vesicles (10/23% Sucrose Interface) and Localization of K^+-Stimulated PPase*

Membranes from a step gradient of 10, 23, 34, and 40% (w/w) sucrose were collected from the 10/23 and 34/40% interfaces and assayed. Fifty mM KCl was included in all of the assay media except where indicated to the contrary (−KCl). KNO_3 (50 mM), Na-molybdate (170 μM), Na-vanadate (90 μM), or NaN_3 (2 mM) were added as indicated. Five μM gramicidin-D was included in all of the assay media to ensure that hydrolysis was not rate-limited with respect to proton-cation equilibration (20). The assays were performed at pH 8.0.

Sucrose Interface	Effector	PPase Activity		ATPase Activity	
		μmol PPi/mg·h	*% control*	*μmol Pi/mg·h*	*% control*
10/23%	None	12.9	100	51.0	100
	KNO_3	13.1	102	14.4	28
	Na-molybdate	13.8	107	43.9	86
	Na-vanadate	13.7	106	44.1	87
	NaN_3	12.7	98	46.4	91
	−KCl	2.5	19		
34/40%	None	1.3	100	4.0	100
	KNO_3	1.5	117	3.4	85
	Na-molybdate	1.5	115	4.1	101
	Na-vanadate	1.3	106	1.5	38
	NaN_3	1.4	108	3.9	96
	−KCl	0.7	58		

Table II. *Influence of KCl and KNO_3 on Tonoplast PPase and ATPase Activities*

The assays were performed as described in Table I except that KCl was only included in the media where indicated. Values shown are mean ± SE for $n = 6$ (PPase assays) and $n = 4$ (ATPase assays). An ANOVA test of the PPase data shows that there is no significant difference beween treatments A and B and treatments C and E but that treatments A (or B) and C (or E) and treatments C (or E) and D differ significantly at the 0.1% and 5% levels, respectively, by Student's *t* test.

Treatment	PPase Activity	ATPase Activity
	μmol PPi/mg·h	*μmol Pi/mg·h*
(A) 50 mM KCl	11.5 ± 0.3	50.7 ± 0.8
(B) 50 mM KNO_3	11.5 ± 0.2	19.1 ± 0.5
(C) 100 mM KCl	13.1 ± 0.2	53.2 ± 0.6
(D) 100 mM KNO_3	12.1 ± 0.4	16.2 ± 0.9
(E) 50 mM KCl + 50 mM KNO_3	12.9 ± 0.2	19.0 ± 0.6

The reciprocal experiment in which the effect of ATP on PPase activity is measured indirectly demonstrates that ATP has little or no affinity for the PPase (Fig. 2B). The inclusion of ATP in the PPase assay medium yields a rate of Pi liberation equivalent to the sum of the rates obtained with ATP alone and PPi alone. The concentration dependence data for PPi alone and those for different concentrations of PPi and a fixed concentration of 1.25 mM ATP are superimposable. For Figure 2B, the rate of liberation of Pi is assumed to be the same in the presence as in the absence of PPi, as shown by Figure 2A, so that its contribution to the overall rate of phosphohydrolysis can be corrected by simple subtraction over the entire PPi concentration range.

The relationship between PPase activity and PPi concentration is sigmoid rather than rectangular hyperbolic. Half maximal velocity is obtained at 20 μM PPi.

Ionic Requirements. It is established that the tonoplast ATPase of higher plants is most stimulated by halides (1, 4, 14, 20) and the results in Table III confirm this. K-Mes accelerates ATP hydrolysis by 4.1-fold over that found with $MgSO_4$ alone, whereas Tris-Cl, KCl, and NaCl yield accelerations of 6.2, 6.4, and 5.7-fold, respectively. By contrast, inorganic pyrophosphohydrolysis is accelerated 7.3-fold by K-Mes, unaffected by Tris-Cl, and only 1.3-fold stimulated by NaCl. The similar PPase stimulations obtained with K_2SO_4, KCl, and K-Mes suggest that K^+ is the determining factor. The stimulations observed probably correspond to direct steric effects on the enzymes concerned rather than indirect effects on $\Delta\psi$ or ΔpH across the membranes of the tonoplast vesicles because all of the assays were performed in the presence of 5 μM gramicidin-D, a membrane pore-former which dissipates both $\Delta\psi$ or ΔpH (4, 20).

Because of the relative inability of Na^+ to substitute for K^+ in pyrophosphohydrolysis and the relative interchangeability of KCl and NaCl for ATP hydrolysis, the effects of NaCl on PPase activity were investigated further (Fig. 3). The maximum stimulation achieved with NaCl alone is only 13% of that achieved with KCl alone, but the stimulation exerted by KCl is 75% inhibited by 41.7 mM NaCl. Although KCl and NaCl evidently interact negatively, the precise mode of interaction is not known since the data yield nonlinear plots when transformed by the method of Hanes (9).

PPi-Dependent Proton Translocation. ATP- and PPi-dependent proton translocation should be distinguishable according to four criteria on the basis of the inhibitor sensitivities and substrate and ionic requirements of the tonoplast ATPase and PPase delineated above. ATP-dependent proton translocation should be: (a) stimulated by Cl^- but not to the same extent by monovalent cations; (b) similar whether Cl^- is in the form of KCl or NaCl; (c) inhibited by NO_3^-; and (d) abolished by the addition of hexokinase and D-glucose which deplete the medium of ATP (ATP + D-glucose → glucose-6-phosphate + ADP). By contrast, PPi-dependent proton translocation should be: (a) stimulated by K^+ but not by Cl^-; (b) stimulated by KCl but only slightly by NaCl; (c) unaffected by NO_3^-; and (d) abolished by exogenous soluble inorganic PPase which depletes the medium of PPi (PPi → 2Pi).

The results of the fluorimetric assays of proton translocation are shown in Figures 4 and 5. Both ATP and PPi elicit quenching of the fluorescence of acridine orange with initial rates (calculated as per cent decrease total fluorescence/min) of 64% and 5%, respectively. The observed decreases in fluorescence require the addition of Mg^{2+} in accord with the enzymological data which show that Mg·ATP and Mg·PPi are the primary substrates for both reactions (4, 20, 27). That uncouplers (CCCP) and ionophores such as gramicidin-D, which mediates proton-cation equilibration, and monensin, which mediates electroneutral Na^+-

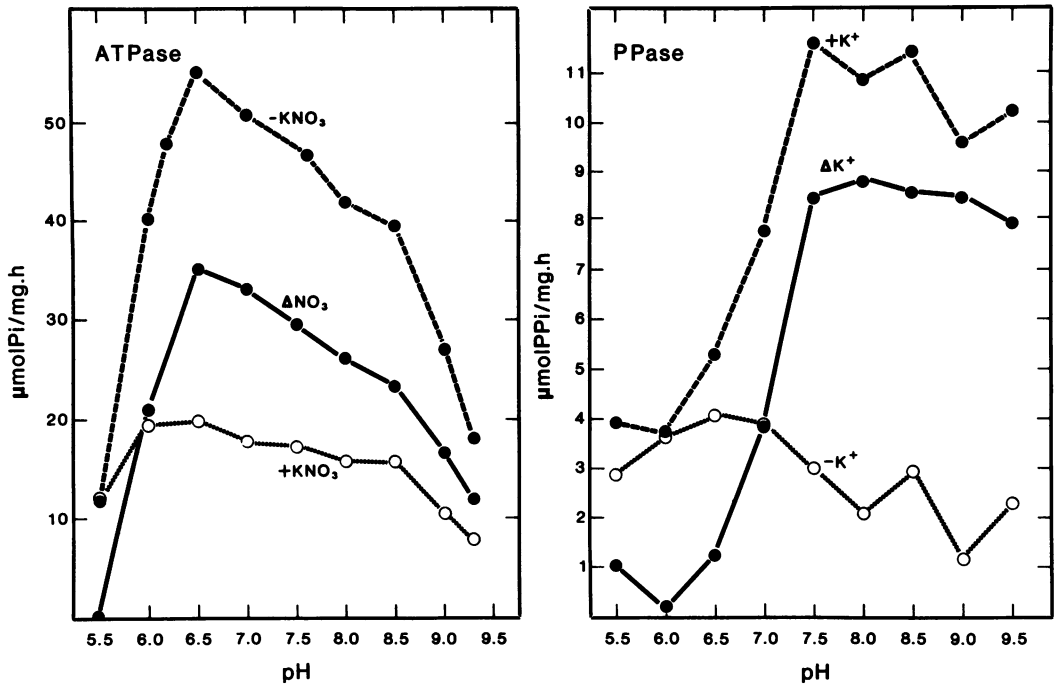

FIG. 1. pH activity profiles of tonoplast ATPase and PPase. ATPase activity was measured in the presence of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM Tris-Cl, and 5 μM gramicidin-D while PPase activity was assayed in the presence of 3 mM Tris-PPi, 3 mM $MgSO_4$, and 5 μM gramicidin-D. NO_3^--sensitive ATPase activity (●——●) was calculated from the difference between the activities measured in the absence (●- - -●) and presence (○——○) of 50 mM KNO_3. K^+-stimulated PPase activity (●——●) was calculated from the difference between the activities measured in the presence (●- - -●) and absence (○——○) of 50 mM K-Mes. Tris-Mes buffers were employed throughout.

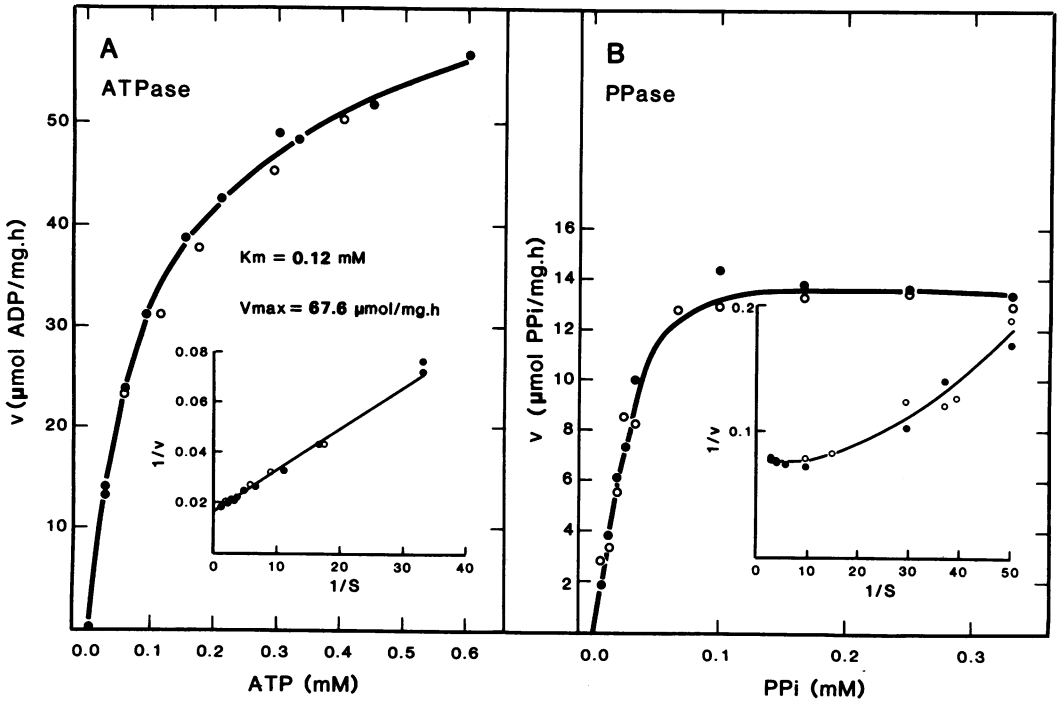

FIG. 2. A, Concentration dependence of tonoplast ATPase with respect to ATP and influence of 1.2 mM PPi on relationship. Rate in the absence (●) and presence of 1.2 mM PPi (○). ATPase activity was measured in a medium containing 50 mM KCl, 0.0 to 0.6 mM Tris-ATP, 3 mM $MgSO_4$, and 30 mM Tris-Mes (pH 8.0). Reaction velocity (= v = μmol ADP produced/mg·h) was measured as the steady state rate of formation of ADP in a coupled system containing NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase. B, Concentration dependence of PPase with respect to PPi and influence of 1.25 mM ATP on relationship. PPase activity was assayed as the rate of liberation of Pi in a medium containing 50 mM KCl, 0.00 to 0.35 mM Tris-PPi, 3 mM $MgSO_4$, and 30 mM Tris-Mes (pH 8.0). Rate in the absence (●) and presence of 1.25 mM ATP (○). The rate in the presence of ATP was estimated by subtracting the rate of Pi liberation obtained with ATP alone from that obtained with PPi plus 1.25 mM ATP.

Table III. *Ionic Requirements of Tonoplast PPase and ATPase*

K-Mes, KCl, Tris-Cl, and NaCl were added to give final concentrations of 50 mM. K_2SO_4 was added to give a final concentration of 25 mM. The assays were otherwise performed as described in Table I.

Salt	PPase Activity		ATPase Activity	
	μmol PPi/mg·h	*% control*	*μmol Pi/mg·h*	*% control*
$MgSO_4$ alone	2.3	100	8.0	100
K-Mes	18.1	787	32.9	411
KCl	18.2	791	51.1	639
K_2SO_4	16.9	735	32.2	403
Tris-Cl	2.3	100	49.6	620
NaCl	3.2	139	45.6	570

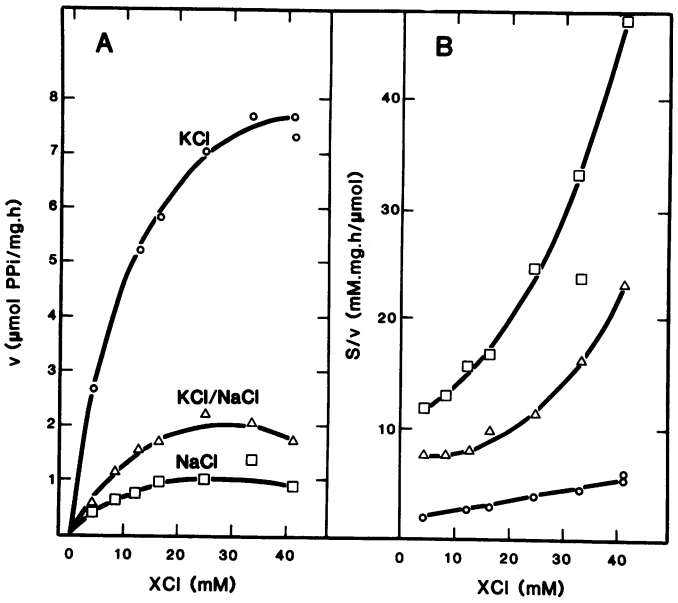

FIG. 3. Influence of KCl and NaCl on PPase activity. Influence of KCl alone (O), NaCl alone (□), and KCl in the presence of 41.65 mM NaCl (Δ). Zero activity is the activity measured in the absence of both KCl and NaCl. A, Arithmetic plot (μmol PPi/mg·h *versus* XCl mM). B, Hanes plot (mM XCl·mg·h/μmol PPi *versus* XCl mM). Incubation conditions otherwise as for Figure 1.

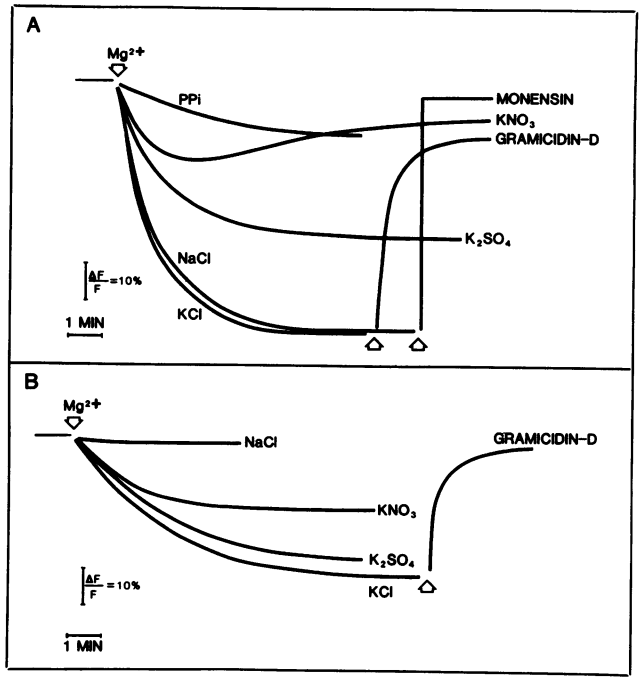

FIG. 4. ATP-dependent (panel A) and PPi-dependent (panel B) quenching of acridine orange fluorescence by tonoplast vesicles. Panel A, 48 μg membrane protein per assay. Panel B, 125 μg membrane protein per assay. Mg^{2+} (as $MgSO_4$) was added to give a final concentration of 3 mM. KCl (50 mM), K_2SO_4 (25 mM), NaCl (50 mM), KNO_3 (50 mM), gramicidin-D (5 μM), and monensin (2 μM) were added as indicated. Tris-ATP and Tris-PPi concentrations of 3 mM were employed throughout. F = total fluorescence; ΔF = change in fluorescence. The line labeled 'PPi' in panel A represents the rate of fluorescence quench elicited by 3 mM PPi in the presence of 50 mM KCl and is shown to enable direct comparison between the rates of PPi-dependent and ATP-dependent proton translocation by the same concentration of tonoplast vesicles (48 μg membrane protein).

proton exchange (21), cause a rapid recovery of the fluorescence implies that the quenches derive from intravesicular acidification.

Coupling is implied by the stimulatory effects of ionophores on ATP and PPi hydrolysis (Table IV). ATPase activity is stimulated 1.5-, 1.2-, and 1.6-fold by gramicidin-D, CCCP, and a mixture of CCCP and valinomycin, respectively, whereas the corresponding PPase stimulations are 1.4-, 1.3-, and 1.4-fold. Valinomycin, alone, which equilibrates K^+ with respect to $\Delta\psi$ has negligible effect on both ATP and PPi hydrolysis (Table IV) and the extent of ATP-dependent and PPi-dependent fluorescence quenching (Fig. 5) suggesting that the steady state ATPase and PPase activities are not rate limited by $\Delta\psi$. Valinomycin, however, stimulates the initial rate of ATP-dependent fluorescence quenching by more than 2-fold in the presence of KCl but has negligible effect on PPi-dependent quenching. Since valinomycin should relieve electrical constraints on proton transport in vesicles equilibrated with K^+ (4), the results may reflect a genuine regulatory difference between the two translocation mechanisms. Alternatively, the magnitude of the inside-positive $\Delta\psi$ developed with PPi is smaller because the net rate of proton translocation is lower.

Criteria 'a', 'b', and 'd', above, are satisfied. KCl and NaCl yield similar rates and extents of fluorescence quenching whereas K_2SO_4 is less stimulatory when ATP is the substrate (Fig. 4). PPi-dependent fluorescence quenching is, however, similar in rate and extent with KCl or K_2SO_4 but low in the presence of NaCl. Connection between phosphohydrolysis and proton translocation (criterion 'd') is implied in that exogenous hexokinase plus D-glucose and exogenous soluble yeast PPase reverse the fluorescence quenching elicited by ATP and PPi, respectively (Fig. 5). ATP (not ADP) and PPi (not Pi) are the active substrates.

Criterion 'c', above, is not satisfied. The initial rates of ATP- and PPi-dependent fluorescence quenching are decreased 2.9- and 1.6-fold by 50 mM KNO_3, whereas the corresponding decreases in the extents of fluorescence quenching (calculated as per cent decrease in total fluorescence at steady state) are 6.3- and 1.9-fold (Fig. 4). These results indicate that NO_3^- has effects on proton translocation auxiliary to its effects on ATP and PPi hydrolysis and recent experiments in our laboratory confirm this (Discussion). The alternative explanation that the NO_3^- sensitivity of PPi-dependent proton translocation reflects the participation of the NO_3^--sensitive ATPase is not borne out by the NO_3^- sensitivities of PPi and ATP hydrolysis (Table II), the kinetic data in Figure 2, and the observation that NaCl does not support PPi-dependent proton translocation but supports ATP-dependent proton translocation.

DISCUSSION

The results indicate that the tonoplast-associated ATPase and PPase activities of red beet storage tissue are functionally distinct

Table IV. *Effect of Ionophores on PPase and ATPase Activity*

All of the assays were performed in a medium containing 50 mM KCl, 3 mM Tris-ATP, or 3 mM Tris-PPi, 3 mM $MgSO_4$, and 40 mM Tris-Mes buffer, pH 8.0. The ionophores were added at concentrations of 5 μM.

Ionophore	PPase Activity		ATPase Activity	
	μmol PPi/mg·h	*% control*	*μmol Pi/mg·h*	*% control*
None	13.0	100	34.1	100
Gramicidin-D	18.2	140	52.8	155
CCCP	17.4	134	40.5	119
Valinomycin	13.3	102	34.9	102
CCCP + valinomycin	18.6	143	53.8	158

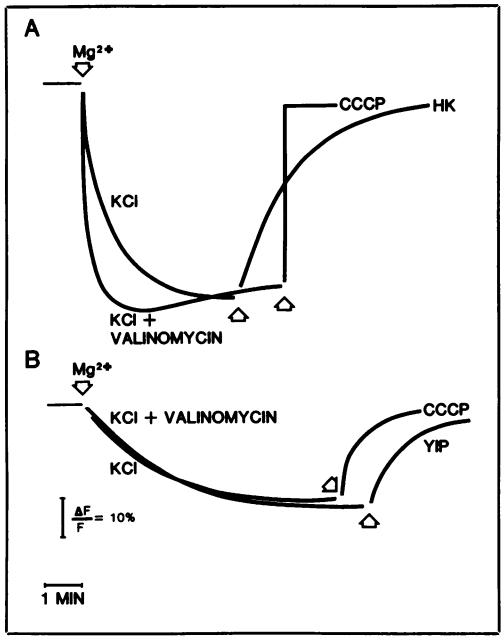

FIG. 5. ATP-dependent (panel A) and PPi-dependent (panel B) quenching of acridine orange fluorescence by tonoplast vesicles. Panel A, 52 μg membrane protein per assay. Panel B, 156 μg membrane protein per assay. KCl, valinomycin, and CCCP were added to give final concentrations of 50 mM, 5 μM, and 2 μM, respectively. Hexokinase (HK; EC 2.7.1.1 from yeast), yeast inorganic pyrophosphatase (YIP; EC 3.6.1.11), and D-glucose were added to give final concentrations or activities of 10 units/ml, 10 units/ml, and 2 mM, respectively. Conditions otherwise as for Figure 4.

and that the proton translocation events mediated by each are distinguishable on the basis of their substrate and mineral ion requirements. One element of doubt does remain: the apparent susceptibility of both translocation events to inhibition by NO_3^-. However, recent studies in our lab show that NO_3^- has a dissipating effect on ΔpH across the tonoplast which is independent of its effect on ATPase activity. ΔpH is diminished by NO_3^- concentrations which are insufficient to inhibit the ATPase. The dissipation of ΔpH by low concentrations of NO_3^- is accompanied by the stimulation (uncoupling) of ATPase activity, when measured in the absence of ionophores. Evidently, inhibition of proton accumulation by NO_3^- is not suitable as a criterion for distinguishing ATP-dependent from PPi-dependent proton translocation.

The data presented here are consistent with two models for PPi-dependent intravesicular acidification. In the first model, the tonoplast PPase is considered to span the membrane and translocate protons inwards. In the second model, the tonoplast PPase is considered to be located on the inside of the tonoplast and catalyze intravesicular acidification by the conversion of PPi to Pi. Although measurements of ΔpH do not enable discrimination between these two schemes, we now know that PPi-dependent intravesicular acidification is electrogenic and associated with the development of an inside-positive $\Delta\psi$ (data to be published elsewhere). Since the simple hydrolysis of PPi to Pi is electroneutral, unless there is transmembrane charge separation, the first model is supported.

It is assumed that the PPi formed in the pyrophosphohydrolytic reactions of polynucleotide, polypeptide, polysaccharide, and coenzyme synthesis and fatty acid activation is hydrolyzed by soluble inorganic PPases to shift the equilibria of the reactions concerned in favor of net synthesis (12). While formally correct, there are a number of studies which indicate that this may be too restrictive an interpretation of PPi in biological energy transduction. First, several authors have shown that not only ATP but also PPi can be formed during the transfer of reducing equivalents down membrane-bound electron transfer chains. Net synthesis of PPi from Pi has been demonstrated in chromatophores of *Rhodospirillum rubrum* (3), the mitochondria of lower (15) and higher heterotrophic organisms (16), and in the chloroplasts of algae and higher plants (3). The enzymes concerned appear to be functionally and structurally distinct from the membrane-bound ATPase complexes of these systems (3). PPi hydrolysis by the chromatophores of *Rhodospirillum* induces changes in carotenoid absorbance and a decrease in the acidity of the bathing medium (18) suggesting electrogenic proton translocation. Second, thermodynamic considerations imply that energy-conserving PPi-dependent reactions may be of greater quantitative importance than is generally thought. From experimentally determined values for the concentrations of Pi, PPi, and Mg^{2+} in rat liver and ameba, the free energies of hydrolysis of PPi in these tissues have been estimated to be −17 and −25 kJ/mol, respectively (10). Consequently, there is no thermodynamic obstacle to the conservation of the energy of hydrolysis of PPi in esterification and other reactions. Substrate concentrations of PPi in various tissues of pea and corn (23) suggest that the same may be true of higher plants.

The universality of PPi-dependent proton translocation across vacuolar membranes has yet to be established. Our observations and those of Walker and Leigh (27), Churchill and Sze (8), and Bennett *et al.* (4) suggest that PPi-dependent proton translocation may be a feature common to the tonoplast of higher plants but it is not known if similar mechanisms apply to other types of vacuolate cells. We are therefore investigating the phenomenon in yeast to determine if PPi-dependent proton translocation operates in this system also.

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