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Effects of pH on proton transport by vacuolar pumps from maize roots

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Protons pumps of the tonoplast may be involved in the regulation of cytosolic pH, but the effects of pH on the coupled activities of these transporters are poorly understood. The effects of pH on the activities of the H^+-translocating pyrophosphatase (PP_iase) and vacuolar-type H^+-translocating adenosine triphosphatase (H^+-ATPase) from maize (*Zea mays* L. cv. FRB 73) root membranes were assessed by changes in acridine orange absorbance, and the data were analyzed according to a model that simultaneously considers proton transport by the pump and those processes that reduce net transport. The addition of either pyrophosphate or ATP to either microsomal or tonoplast membranes generated a pH gradient. The pH gradient generated in the presence of both substrates was not the sum of the gradients produced by the two substrates added separately. When membranes were separated by sucrose density gradient centrifugation, pyrophosphate (PP_i)-dependent proton transport was associated with light density membranes having tonoplast H^+-ATPase activity. These results indicate that some portion of the PP_iase was located on the same membrane system as the tonoplast ATPase; however, tonoplast vesicles may be heterogeneous, differing slightly in the ratio of ATP- to PP_i-dependent transport. Proton transport by both the PP_iase and ATPase had maximal activity at pH 7.0 to 8.0. Decreases in proton transport by the ATPase at pH above the optimum were associated with increases in the processes that reduce net transport. Such an association was not observed at pH values below the optimum. These results are discussed in terms of in situ regulation of cytoplasmic pH by the two pumps.

Key words – ATP, corn, leakage, pyrophosphate, tonoplast, *Zea mays*.

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Introduction

Cytoplasm pH of plant cells must be regulated within a relatively narrow range to maintain cellular viability (Smith and Raven 1979). Therefore, the actions of proton pumps localized on plasma membrane and tonoplast are suspected to have a role in the regulation of cytoplasmic pH. Two of these proton pumps utilize adenosine triphosphate (ATP) as the substrate, while the third uses pyrophosphate (PP_i). The two H^+-ATPases belong to two distinct classes of transport proteins, commonly referred to as the vacuolar- and plasma membrane-type ATPase (Nelson and Taiz 1989, Sze 1984). This designation is derived from their relative enrichment in vacuolar-type membranes and the plasma membrane. These two classes of H^+-ATPases can be distinguished biochemically by their differential sensivity to nitrate and vanadate, formation of a covalent phosphorylated intermediate and polypeptide composition (Sze 1984). These two types of transport ATPases are also different from H^+-ATPases that synthesize ATP in organelles (Nelson and Taiz 1989). The PP_i-dependent proton pump is also localized on vacuolar-type membranes (Chanson and Pilet 1988, Macri and Vianello 1987, Marquardt and Lüttge 1987) and can be separated from the H^+-ATPase after Triton X-100 solubilization by size-exclusion chromatography (Bremberger et al. 1988, Rea and Poole 1985).

Cytoplasmic pH may be regulated by the tonoplast proton pumps if the pH dependence of these enzymes varies appreciably within the normal range of cytoplasmic pH. Induction of Crassulacean acid metabolism is associated with a shift in the pH optimum of the tonoplast ATPase towards more alkaline pH values (Struve and Lüttge 1987). This alteration in pH optimum may reflect a coordination between changes in cytoplasmic pH and fluxes across the tonoplast membrane. The pH optimum reported to date for the tonoplast ATPase varies widely with species and tissue, ranging from pH 6.5 to over 8.0 (Admon et al. 1981, Aoki and Nishida 1984, DuPont et al. 1982, Jochem and Lüttge 1987, Kakinuma et al. 1981, Struve and Lüttge 1987). The activity of the PP_i-dependent pump appears to have a broader pH optimum, between 6.7 and 8.5 (Chanson and Pilet 1988, Macri and Vianello 1987). Most of the previous studies have examined the effects of pH on either substrate hydrolysis or proton transport. Little is known of how pH affects proton transport and the coupling between ATP hydrolysis and pumping, except in the case of leaf tonoplast vesicles from the Crassulacean acid metabolism plant, *Mesembrythemum crystallium* L. (Struve and Lüttge 1987). In this case, changes in proton transport were approximately proportional to changes in ATP hydrolysis. The present study examines the effects of pH on the ATP- and the PP_i-dependent proton transport and the coupling between activities of the tonoplast ATPase. Between pH 5.5 and 6.45, changes in proton transport were not proportional to changes in ATP hydrolysis.

Abbreviations – BTP, bis-tris-propane; k_L, rate constant for proton leakage after the substrate utilization by the pump has been inhibited; k_p, rate constant for the processes that reduce net proton transport; m, coupling stoichiometry or ratio of the initial rate of proton transport divided by the initial rate of substrate utilization; R, rate of ATP hydrolysis; R_H, initial rate of proton transport.

Materials and methods

Preparations of membrane fractions

Corn seeds (*Zea mays* L. cv. FRB 73) were germinated on filter paper moistened with 0.1 m*M* $CaCl_2$ for 3 days at 28 to 30°C and harvested as described previously (Nagahashi and Baker 1984). Microsomes and highly purified tonoplast vesicles were purified by differential and sucrose density centrifugation, respectively, essentially as described previously (Tu et al. 1987). Microsomes from roots of this cultivar have little if any vanadate-sensitive, ATP-dependent proton transport (Brauer and Tu 1991). Based on inhibition by nitrate and vanadate, more than 95% of the proton transport and ATP hydrolysis by purified tonoplast vesicles can be attributed to the vacuolar H^+-ATPase (Tu et al. 1987). Purified tonoplast vesicles exhibit no proton transport in the presence of ADP or Mg-ADP (Tu et al. 1987). Protein concentration was determined after precipitation by trichloroacetic acid in the presence of deoxycholate by the Lowry method (Bensadoun and Weinstein 1976).

Assay for proton pumping and phosphate appearance

Proton transport by the vacuolar H^+-ATPase was assayed by changes in acridine orange absorbance at 492.5 nm as described previously (Brauer et al. 1988). Vesicles were diluted to 2.0 ml with assay medium containing 17.5 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) titrated to pH 6.45 with bis-tris-propane (BTP), 0.2 m*M* ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA), 2.5 m*M* $MgSO_4$, 7.5 μ*M* acridine orange and 50 m*M* KCl. After incubation at 18 to 24°C for 5 min, proton transport was initiated by the addition of 20 μl of 0.2 *M* ATP titrated to pH 6.45 with BTP. To measure PP_i-dependent proton transport, the reaction was conducted in a similar fashion except transport was initiated by the addition of 20 μl of 0.02 *M* Na-PP_i. The collapse of the steady-state pH gradient was induced by the rapid removal of Mg by the addition of 0.5 *M* ethylenediaminetetraacetate (EDTA) (neutralized with BTP) to a final concentration of 7.5 m*M* (Brauer et al. 1989). In experiments in which the pH of the assay medium was varied, 20 m*M* BTP was titrated to various pH values between 5.0 and 9.0 with MES. ATP and PP_i hydrolysis catalyzed by the pumps was assayed by the direct measurement of inorganic phosphate release (Tu et al. 1987). Each experiment contained at least three replicates and results from at least duplicate experiments are reported. The magnitude of the standard error of the means was smaller than the size of the data symbol.

The initial rate of proton transport was determined by analyzing the time course of quenching of acridine orange absorbance by a model that considers net proton transport as the difference between the rate of pumping by the transport and the summation of the inhibitory processes including proton leakage and pump slippage as described previously (Tu et al. 1987). The model is based on the equation:

$$d\delta/dt = m\,R - k_p\,\delta \qquad (1)$$

where δ is the amount of transported protons, m is the coupling stoichiometry between proton transport and substrate utilization, t is time after the initiation of transport and k_p is a rate constant which quantifies the processes that reduce net proton transport. Values for k_p were obtained from:

$$\ln\,(1-A_t/A_s) = k_p\,t \qquad (2)$$

where A is the change in absorbance of acridine orange at time t and at steady state, s (Tu et al. 1987). The initial rate of proton transport, R_H, was obtained from:

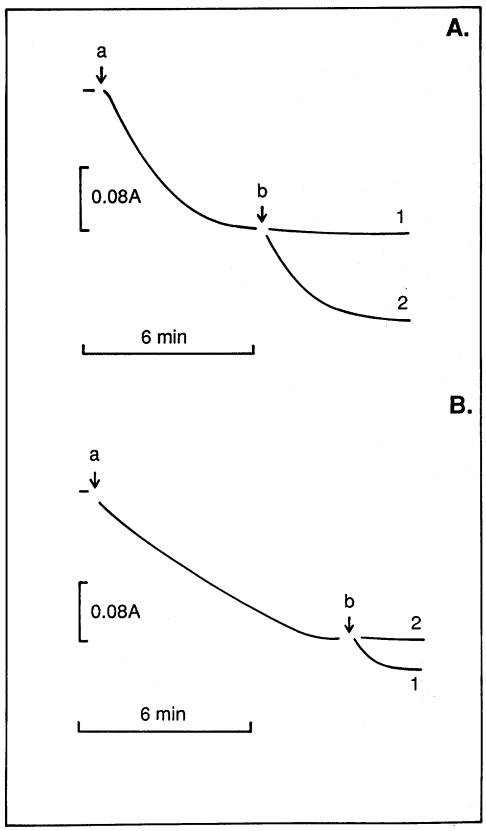

Fig. 1. Effects of successive additions of either PP_i or ATP on proton transport by microsomes. Proton transport by microsomes (1.1 mg of protein) after the addition of either ATP (panel A, point a) or PP_i (panel B, point a) was monitored at pH 6.45 by changes in acridine orange absorbance. After a steady-state gradient was established, the second substrate was added at point b and formation of an additional pH gradient was monitored. Lines 1 and 2 monitor changes in pH induced by the addition of ATP and PP_i, respectively.

$$R_H = k_p A_s \quad (3)$$

The initial rate of proton transport is the rate of flux while δ and $k_p \delta$ are approximately zero.

Assays for marker enzymes

Reduced *b*-nicotinamide adenine dinucleotide (NADH)-dependent cytochrome *c* reductase and cytochrome *c* oxidase were assayed as described by Hodges and Leonard (1974), except 0.03% (w/v) Triton X-100 was substituted for digitonin in the oxidase assay and 25 μM antimycin A was included in the reaction medium for the reductase. Vanadate-sensitive ATP hydrolysis was determined as described by Brauer et al. (1988). Triton-stimulated inosine 5′-diphosphatase (IDPase) was assayed by the procedure of Nagahashi and Nagahashi (1982). The amount of inorganic phosphate release was determined by the malachite green assay as described previously (Tu et al. 1987).

Results and discussion

Localization of PP_i-proton pump

When microsomes were supplied with either ATP or PP_i, a decrease in acridine orange absorbance was detected, which is consistent with the formation of acidic intravesicular pH gradient (Fig. 1). ATP-dependent proton transport was totally abolished by the addition of 50 mM KNO_3 and unaffected by 0.2 mM vanadate (data not shown), indicating the presence of only the vacuolar ATPase. PP_i-dependent proton transport was unaffected by either 50 mM nitrate or 0.2 mM vanadate (data not shown). In the presence of ATP, the initial rate of proton transport was 0.082 ± 0.006 A (mg protein)$^{-1}$ min^{-1} with a rate constant of 0.52 ± 0.02 min^{-1}

Tab. 1. Characteristics of proton transport by microsomes in the presence of 2 mM ATP and 0.2 mM PP_i. Proton transport was initiated by the addition of either ATP or PP_i and after attaining a steady-state pH gradient, the second substrate was added. The time course of changes in acridine orange absorbance were analyzed as described previously to determine the initial rate of proton transport, R_H, the rate constant for the inhibitory processes, k_p, and the magnitude of the steady-state gradient, A_s. Values show means ± SE (n = 9).

Assay conditions	R_H A (mg protein)$^{-1}$ min^{-1}	k_p min^{-1}	A_s A (mg protein)$^{-1}$
ATP alone	0.082 ± 0.006	0.52 ± 0.02	0.16 ± 0.02
PP_i alone	0.024 ± 0.003	0.13 ± 0.01	0.18 ± 0.02
ATP first, PP_i second			
ATP component	0.076 ± 0.006	0.50 ± 0.02	0.15 ± 0.02
PP_i component	0.068 ± 0.007	0.76 ± 0.03	0.09 ± 0.01
PP_i first, ATP second			
PP_i component	0.026 ± 0.003	0.15 ± 0.02	0.17 ± 0.02
ATP component	0.045 ± 0.004	0.80 ± 0.05	0.04 ± 0.01
ATP, PP_i together	0.059 ± 0.006	0.81 ± 0.04	0.24 ± 0.0

for k_p, a quantitative measure of the inhibitory processes (Tab. 1). In the presence of PP_i, the initial rate of proton transport and the value for k_p were less than those found with ATP. However, the magnitude of steady-state pH gradient with PP_i was approximately equal to that observed with ATP.

An additional pH gradient was generated by the addition of a second substrate (Fig. 1). However, the characteristics of the generation of this additional pH gradient were different from the build-up of the pH gradient in the absence of a steady-state pH gradient. When vesicles were supplied with ATP first and allowed to attain a steady-state pH gradient, the rate of proton transport after the addition of PP_i was a value intermediate between that observed for PP_i- and ATP-dependent transport (Tab. 1). The rate constant for the inhibitory processes was 0.76 ± 0.03 min^{-1}, a value greater than that observed for either substrate alone. When vesicles were initially supplied with PP_i and allowed to attain a steady-state gradient, the addition of ATP produced a very small change in the pH gradient (Tab. 1). The rate constant for the inhibitory processes, k_p, was rather high. When both substrates were added simultaneously to vesicles, the initial rate of proton transport, R_H, and the rate constant for the inhibitory processes, k_p, were similar to those found after the addition of the second substrate for the experiments in Fig. 1.

It is clear from these results that the characteristics of proton transport in the presence of both substrates were different from those obtained in the presence of only one substrate. The sum of the steady-state change in proton transport in the presence of either PP_i or ATP alone was 0.34 A (mg protein)$^{-1}$, far greater than the steady-state gradient in the presence of both substrates, 0.21 to 0.25 A (mg protein)$^{-1}$. Therefore, the actions of one pump have effects on transport by the other pump. Such an interaction by the two pumps is possible if both transporters are on the same membrane system. The possibility that either pump was inhibited by the products of the other pump seemed unlikely, since the addition of either 0.2 m*M* phosphate or 0.2 m*M* ADP had no effect on PP_i or ATP-dependent proton transport (data not shown).

Previous research from this laboratory demonstrated that the membranes from roots of cv. FRB 73 are resolved on linear sucrose density gradient as two protein peaks (Tu et al. 1987). The lighter density peak contained tonoplast vesicles with nitrate-sensitive ATPase activities. The lower density peak was devoid of NADH cytochrome *c* reductase, Triton-stimulated IDPase, vanadate-sensitive ATP hydrolysis and cytochrome *c* oxidase, which were used as marker enzymes for endoplasmic reticulum, golgi, plasma membrane and mitochondria, respectively (Nagahashi 1985, Quail 1979). The above four enzyme markers are unresolved and associated with the higher protein peak. Similar distributions for the four marker enzymes were obtained from experiments to localize the PP_i-dependent proton transport (data not shown). PP_i-dependent proton transport was associated with the lighter density peak (Fig. 2), substantiating its localization to the tonoplast vesicles. However, the distribution of the PP_i-dependent proton transport activity was slightly different from that of the ATP-dependent activity. The PP_i-proton transport activity exhibited a broader, more symmetrical peak than the tonoplast ATPase activity. The discrepancy could mean that the tonoplast vesicles are heterogeneous, differing slightly in the ratio of PP_i- to ATP-dependent pumps. The possibility of such a heterogeneity among tonoplast vesicles is a project of ongoing research in our laboratory. The recovery of PP_i-catalyzed transport among the fractions from the sucrose gradient was approximately 75% of the activity in crude microsomes (data not shown). A similar recovery of ATP-dependent proton transport was found (data not shown).

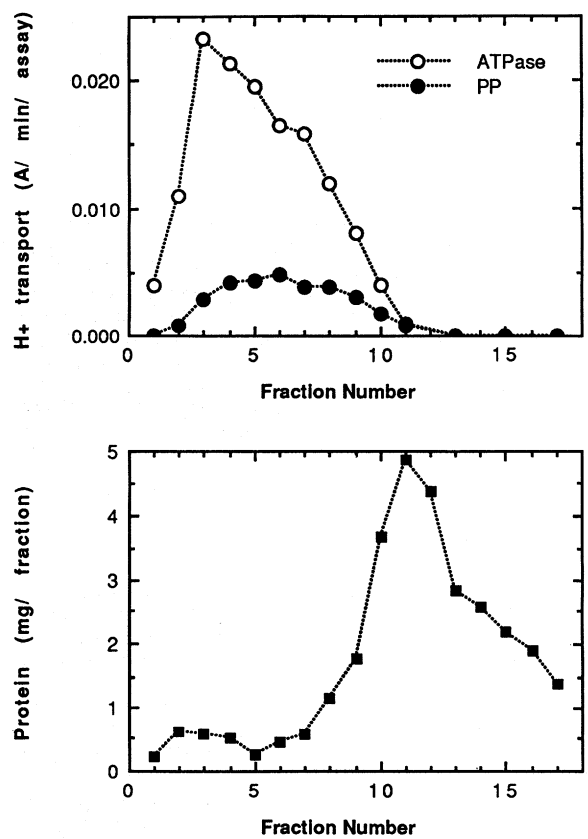

Fig. 2. Distribution of protein, and vacuolar ATPase-catalyzed and PP_i-dependent proton transport among fractions after isopycnic sucrose density centrifugation. Resuspended microsomes were layered over a 15 to 45% (w/w) sucrose gradient and centrifuged for 18 h in an SW 28 rotor at 82 000 *g*. The gradient was then fractionated into 1.8 ml aliquots. Aliquots were assayed for ATP and PP_i-dependent proton transport (○ and ●, respectively) as well as protein (■) as described in Materials and methods.

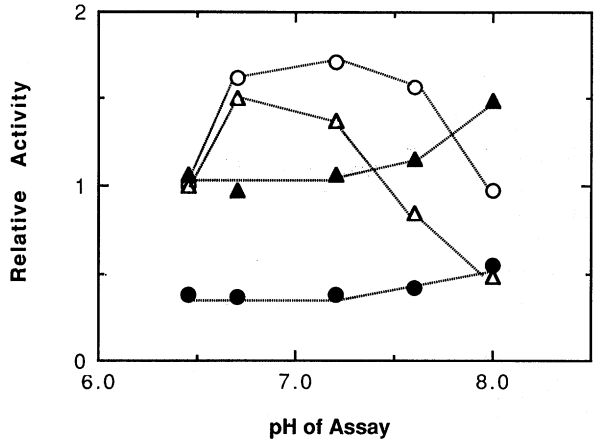

Fig. 3. Effects of assay pH on ATP- and PP_i-dependent proton transport by microsomes. Proton transport catalyzed by either ATP or PP_i was assayed in reaction media containing BTP titrated to various pH values between 6.45 and 8.0 with MES. The time course of proton transport was analyzed according to the kinetic model in order to determine initial rate of transport (△ and ○ for ATP and PP_i, respectively) and the rate constant (min^{-1}) for the inhibitory processes, k_p (▲ and ● for ATP and PP_i, respectively). The data for the initial rate of proton transport are plotted relative to the values obtained at pH 6.45 which averaged 0.082 ± 0.003 and 0.023 ± 0.001 A (mg protein)$^{-1}$ min^{-1} for the initial rate of ATP- and PP_i-dependent transport, respectively. Data for k_p are plotted relative to the value obtained for ATP-dependent transport at pH 6.45, 0.55 min^{-1}.

Effects of pH on tonoplast pump activities with microsomes

Proton transport by both the PP_i-dependent pumps was affected by the pH of the assay solution (Fig. 3). The initial rate of transport by the ATPase rapidly increased by about 50% as pH was increased from 6.45 to 6.7. Activity decreased slightly as pH was increased from 6.7 to 7.2. Between pH 7.2 and 8.0, activity continued to decrease. Maximum rates of proton transport were associated with minimal values for the rate constant for inhibitory processes, k_p. The decrease in activity between pH 7.2 and 8.0 was associated with a substantial increase in k_p. From pH 7.2 to 8.0, proton transport declined by over 60%, as k_p increased over 50%.

The pH optimum for PP_i-dependent proton transport had a broader range (Fig. 3). Values for the initial rate of proton transport catalyzed by PP_i varied less than that observed for the ATPase. Activity increased by about 60% as pH increased from 6.45 to 6.7, and remained at this maximum until the pH was increased beyond 7.6. Between pH 7.6 and 8.0, initial rate of proton transport declined to a level approximately the same as that found at pH 6.45. As reported above, values for the rate constant for the inhibitory processes in the presence of PP_i were less than half the values observed with the ATPase. The rate constant was slightly greater at pH values above 7.2, as found with the ATPase.

It was not possible to determine reliably the rates of substrate hydrolysis by these two pumps in crude microsomes because of the presence of other phosphate-producing enzymes. Therefore, it was not possible to determine if the decreases in proton transport by these two pumps resulted from a decrease in substrate utilization by the pump or from uncoupling of substrate hydrolysis from transport.

Effects of pH with purified tonoplast vesicles

Effects of pH on the coupled activities of the ATPase were examined further with tonoplast vesicles purified from the microsomal preparation by continuous sucrose density centrifugation. The effect of pH on the initial rate of proton transport by the ATPase in isolated tonoplast differed slightly from that observed with microsomal preparations (Figs. 3 and 4). The magnitude of the changes in initial rate of transport with varying pH was greater with purified tonoplast vesicles than with microsomes. Activity approximately doubled between pH 6.45 and 7.0, and remained at this level until the pH exceeded 8.0. Initial rates declined by over 75% between pH 8.0 and 9.0. Thus, the optimum pH for initial rate of transport was broader in purified vesicles than in microsomes. Changes in proton transport between pH 6.45 and 8.0 were not associated with a change in the K_m for ATP. The K_m for ATP was 0.21, 0.26 and 0.22 m*M* at pH 6.45, 7.5 and 8.0, respectively (data not shown).

Rates of ATP hydrolysis by the pump varied less than rates of proton transport as a function of pH (Fig. 4). There was very little change in ATP hydrolysis between 6.0 and 8.0. As the pH was decreased from 6.0 to 5.0, rate of ATP hydrolysis declined by about 50% as compared to complete inhibition for proton transport. As

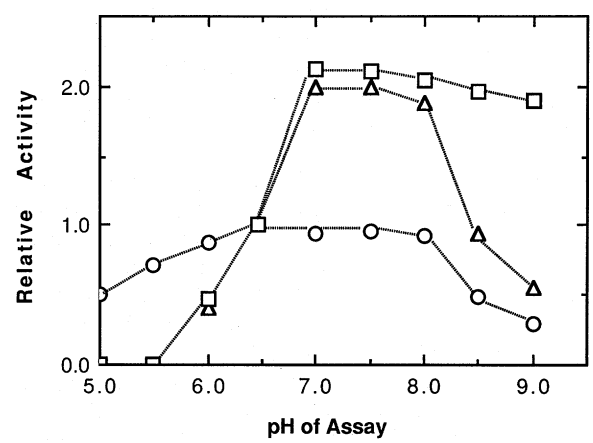

Fig. 4. Effect of assay pH on the coupled activities of the H^+-ATPase in isolated tonoplast vesicles. ATP hydrolysis (○) and proton transport (△) were assayed as described in Materials and methods and the data are plotted relative to the activity at pH 6.45, which averaged 1.61 ± 0.05 A (mg protein)$^{-1}$ min^{-1} and 1300 ± 60 nmol P_i (mg protein)$^{-1}$ min^{-1}, respectively. The coupling factor (□) was determined by dividing the relative initial rate of proton transport by the relative rate of ATP hydrolysis, and thus is a relative measurement with the units A (nmol P_i)$^{-1}$, not H^+ per ATP hydrolyzed.

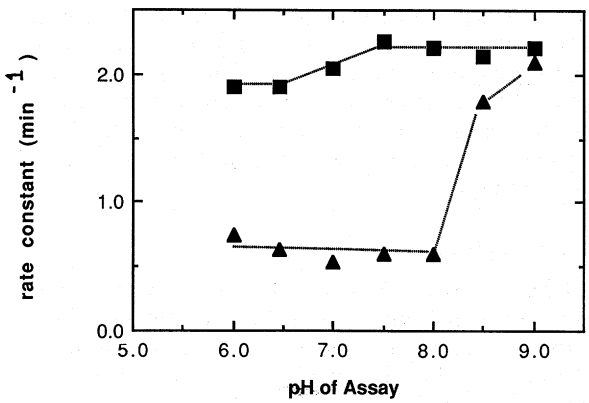

Fig. 5. Effects of assay pH on the rate constants for the processes that reduce net proton transport and proton leakage with isolated tonoplast vesicles. Time course of proton transport was analyzed according to a kinetic model to determine k_p (▲), rate constant for the processes that reduce net proton transport (27), as described in Materials and methods. After a steady-state pH gradient was attained, ATP utilization by the pump was rapidly inhibited by the addition of EDTA and the time course of the collapse of the proton gradient was analyzed to determine the rate constant for proton leakage, k_L (■), as described in Materials and methods.

the pH was increased from 8.0 to 9.0, rates of ATP hydrolysis declined sharply, falling about 75%.

The relative efficiency of coupling between ATP hydrolysis and proton transport was followed by changes in the ratio of the initial rate of proton transport to the rate of ATP hydrolysis. This ratio was relatively constant between pH 7.0 and 9.0 (Fig. 4). Therefore, the declines in proton transport and ATP hydrolysis were proportional at pH values above 8.0, indicative of a decrease in energy utilization by the ATPase.

Decreases in proton transport and ATP hydrolysis were not proportional at pH values below 6.45. The ratio of proton transport to that of ATP hydrolysis decreased as the pH was reduced from 6.45 to 5.0, indicating that either proton transport or the coupling mechanism was affected more than ATP hydrolysis. The differential effects of assay pH on proton transport and ATP hydrolysis are consistent with an indirect coupling mechanism previously proposed for the maize root tonoplast H^+-ATPase (Tu et al. 1987, 1990). According to this proposal, the maize root tonoplast ATPase has three functionally distinct mechanisms. The two mechanisms catalyzing ATP hydrolysis and proton transport do not share a common intermediate. Rather, the reactions of these two pathways are coordinated by a distinctly separate coupling mechanism. Therefore, if proton transport is affected more than ATP hydrolysis by assay pH, the primary site of action must be either the proton transport of coupling mechanism.

Decreases in proton transport above pH 8.0 were associated with increases in the rate constant for the processes that reduce net transport (Fig. 5). Between pH 6.0 and 8.0, k_p averaged between 0.5 and 0.7 min^{-1}. The value increased to approximately 2 when the pH was increased from 8.0 to 9.0. The rate constant for the inhibitory processes at high pH values approached that of k_L, the rate constant for the dissipation of the pH gradient after the pump had been inhibited by the depletion of substrate. Values for k_L were relatively constant throughout the pH range of 6.0 to 9.0 with a slight increase (20%) at higher pH values. Therefore, the decrease in the coupling between proton transport and ATP hydrolysis by the H^+-ATPase between pH 6.0 and 6.45 was not associated with significant increases in either the inhibitory processes, k_p, or the leakage of protons in the absence of an active proton pump.

The large increase in k_p as the pH was increased above 8.0 was not associated with a change in coupling between proton transport and ATP hydrolysis. Such a result is plausible when one closely considers the kinetic model by which the data were analyzed. The value for k_p considers those inhibitory processes that are related to the amount of protons transported (i.e., $k_p\delta$). Inhibitory processes that affect proton transport and are not related to the number of transported protons are reflected by changes in the coupling stoichiometry, m. The stoichiometry between proton transport and ATP hydrolysis in Fig. 4 were calculated from initial rates of these two processes. The initial rate of proton transport is the rate of proton transport while the number of transported protons is negligible, i.e., $k_p\delta$ is approximately zero. Therefore, rates of initial proton transport are not affected by those processes dependent on the amount of protons transported. Such a rationale explains how the coupling stoichiometry and k_p can vary independently.

The pH dependence of PP_i-dependent proton trans-

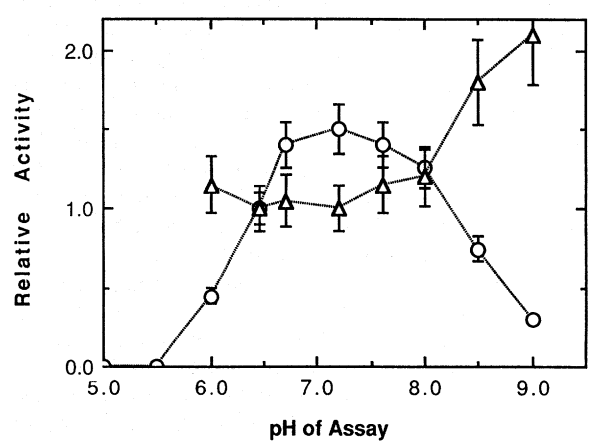

Fig. 6. Effects of assay pH on the characteristics of PP_i-dependent proton transport catalyzed by tonoplast vesicles. Time course of proton transport was analyzed according to a kinetic model to determine initial rate (○) and k_p (△), rate constant for the processes that reduce net proton transport (27), as described in Materials and methods. Data are plotted relative to the activity at pH 6.45, which averaged 0.42 ± 0.04 A (mg protein)$^{-1}$ (min)$^{-1}$ and 0.32 ± 0.05 min^{-1} for initial rate of transport and k_p, respectively.

port catalyzed by highly purified tonoplast vesicles was similar to that observed with microsomes (Fig. 6). The optimum pH for PP_i-dependent activity ranged from about 7.0 to 8.0. The initial rate of transport declined sharply above or below this optimum pH range. Changes in the initial rate of PP_i-dependent proton transport above pH 8.0 were associated with increases in k_p, similar to that observed with ATP-dependent proton transport. Values of k_p for PP_i-dependent transport were less than that found for ATP-dependent activity. Changes in substrate utilization by the PP_i-dependent pump, as followed by assaying the level of Mg-dependent PP_i hydrolysis, varied in a manner similar to that of proton transport activity (data not shown).

General discussion

The ability of the tonoplast membrane to be a barrier to protons varied with pH and the presence of substrates for two of the proton pumps localized on this membrane (Figs 3, 4 and 5). For the sake of this discussion, it is assumed that the values of k_p, the rate constant for the total inhibitory processes, are the maximum values for proton leakage during active proton transport, i.e. feedback inhibition and pump slippage are either negligible or reflected by changes in only the coupling ratio, m. Highest rates of proton leakage were found in the absence of an active proton pump, i.e., k_L was greater than the rate constant for inhibitory processes to net proton transport, k_p (Figs 4 and 5). Values for proton leakage from tonoplast vesicles after the PP_i-dependent proton transport was inhibited by the addition of EDTA to a final concentration of 7.5 m*M* averaged 2 min^{-1}, a value similar to those reported for H^+-ATPase in Fig. 5. Proton leakage across the tonoplast was next greatest when the ATP-dependent proton pump was active and least when only the PP_i-dependent pump was catalyzing transport.

Based on these in vitro measurements, the activity of either the ATP- or PP_i-dependent tonoplast proton pump would probably not be affected by variations in cytoplasmic pH. Cytoplasmic pH in viable maize root tips averages about 7.6 with only a small variation (Pfeffer et al. 1986). This pH range is within the optimal range of both proton pumps (Figs 2, 3 and 4).

Data from the present study and those obtained previously by in vivo ^{31}P-NMR form the basis for the proposal that the PP_i-dependent proton pump can maintain the trans-tonoplast pH gradient when cellular ATP is low. The PP_i-dependent pump is probably not active in aerobically respiring corn root tissue because of a lack of substrate. Results of ^{31}P-NMR indicate that there is little if any PP_i and ADP present in the cytosol of corn root tips during O_2 perfusion (Pfeffer et al. 1986). Compounds with a chemical shift corresponding to ADP and/or PP_i accumulate under anoxia (Pfeffer et al. 1986). Since it is not possible to resolve ^{31}P resonances originating from ADP and PP_i, we cannot be sure which of these compounds is being produced under anoxia. Therefore, the PP_i-dependent proton pump may be active under anoxia. The PP_i-dependent proton pump has characteristics that are suited to the role of maintaining the tonoplast pH gradient under anoxia. The relatively low k_p associated with PP_i-dependent transport would aid in the maintenance of the trans-tonoplast pH gradient when ATP concentrations have fallen below that needed to activate the tonoplast H^+-ATPase. The low values for the inhibitory processes, k_p, indicate that protons transported by the PP_iase would be more likely to contribute to the maintenance of pH gradient than proton leakage and thus favor the maintenance of the trans-tonoplast pH gradient under conditions of anoxia.

Acknowledgements – Mention of brand or firm names does not constitute an endorsement by the USDA over others of a similar nature not mentioned.

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