

Proton Pumping Kinetics and Origin of Nitrate Inhibition of Tonoplast-type H⁺-ATPase

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A tonoplast-type vesicle preparation, substantially free from other subcellular membranes, was obtained from corn roots by equilibrium sucrose density gradient centrifugation. At pH 6.5 and in the presence of chloride ions, the tonoplast-type ATPase activity as measured by P_i release, was inhibited by nitrate ions. The ATPase activity was insensitive to molybdate and vanadate, indicating a minimum nonspecific phosphatase and plasma membrane contamination. The vesicles exhibited an ATP hydrolysis-supported proton uptake which was measured by the absorption change of acridine orange. The ATP hydrolysis supported uptake and the subsequent perturbant-induced release of protons (decay) was described by a kinetic model which was previously developed to evaluate the coupling between proton pumping and the primary energy yielding process for other biomembranes. The proton pumping activity was more sensitive to nitrate ions than was ATP hydrolysis. The differential effect and the kinetic analysis of nitrate inhibition led us to suggest that (i) the coupling between P_i release and proton pumping was indirect in nature and (ii) the primary inhibitory effect of nitrate ion was originated from an interaction with a protogenic protein domain which is functionally linked to the ATPase in the tonoplast-type membrane. © 1987 Academic Press, Inc.

The tonoplast-type membrane and plasma membrane isolated from microsomal fraction of plant root cells are now known to contain H⁺-ATPases (1-4). The properties of plasma membrane ATPase, in many regards, are similar to that of fungal plasma membrane H⁺-ATPase (5), e.g., formation of a phosphorylated intermediate with a 100 kDa polypeptide (6, 7). The catalytic mechanisms of these plasma membrane ATPases share striking similarities with E₁E₂-type ion translocating ATPases found in animal cells (5, 8). However, there appear to be some major func-

tional differences between corn root plasma membrane ATPase and fungal plasma membrane ATPase (9, 10). Partially purified root plasma membrane ATPase has been reconstituted in phospholipid vesicles and the resulting systems exhibited an ATP hydrolysis-supported electrogenic proton pumping activity (11). Thus, it is likely that the root plasma membrane ATPase may play a central role in the transport processes occurring between the soil-root interface (12).

On the other hand, the tonoplast-type H⁺-ATPase may be important in regulating intracellular pH values and transport processes (3). Plant tonoplast membrane which has a lower density (1.0-1.13 g/cc) than that of the plasma membrane (1.15-1.18 g/cc) contains a H⁺-ATPase which shows a unique sensitivity to nitrate ions. Unlike plasma membrane ATPase, the

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tonoplast ATPase is not inhibited by vanadate, suggesting a noninvolvement of a phosphorylated intermediate in the reaction mechanism (3, 4). The proton pumping and the ATP hydrolysis of tonoplast vesicles also show a similar anion dependence. The proton pumping of tonoplast vesicles has been followed by various techniques which include measuring changes of absorption of indicator dyes (13), emission of fluorescence probes (14, 15), or pH changes in the external solution (15). From the distribution of ^{14}C -labeled methylamine and thiocyanate, the direction of proton movement (inward) and the polarity of the membrane potential (positive inside) of isolated tonoplast vesicles have been determined (2). To account for the inhibition of nitrate, different mechanisms have been proposed in the literature. Based on the similar sensitivity of both ATP hydrolysis and its associated proton pumping in oat root tonoplast vesicles, a direct interaction of nitrate with ATPase was suggested (16). On the other hand, based on a kinetic study, nitrate inhibition was found not to be involved in substrate binding of red beet tonoplast ATPase (17). In fact, in red beet tonoplast vesicles, a proton-nitrate symport process was proposed to explain the greater sensitivity of proton pumping to nitrate ions (18, 19). This discrepancy and the lack of knowledge of the coupling mechanism between ATP hydrolysis and proton pumping in the tonoplast system, prompted us to undertake the present study.

In the present study, tonoplast-type vesicles obtained from the microsomal fraction of corn root cells were centrifuged to equilibrium density to minimize cross contamination with other low density membranes such as Golgi or endoplasmic reticulum. The ATP-driven proton uptake of isolated vesicles was found to obey a kinetic scheme which could be used to evaluate the nature of the coupling between ATP hydrolysis and proton movement. Based on the kinetic responses of the enzyme activities to nitrate ions, an indirect coupling model for tonoplast-type H^+ -ATPase reaction was proposed. Using this model, the primary inhibitory effect of nitrate was

assigned to an interaction with a protogenic protein domain which is functionally linked to the ATPase activity in the membrane.

MATERIALS AND METHODS

Plant materials. Corn seeds (*Zea mays* L. cv. FRB73, Illinois Foundation Seeds)³ were germinated and harvested as described previously (20). Approximately 60 to 80 g fresh wt of excised roots was homogenized with a chilled pestle in a precooled mortar at 4°C in 0.3 M sucrose containing 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithioerythritol (DTE)⁴, 5 mM 2-mercaptoethanol, and 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.7. The crude homogenate was filtered through four layers of cheesecloth before centrifugation.

Membrane fractionation. Subcellular membranes were initially isolated by differential centrifugation. The crude homogenate was successively centrifuged at 1000g for 5 min; 6000g for 20 min; 80,000g for 35 min; and 120,000g for 35 min. For equilibrium density gradient centrifugation, the 6000g to 80,000g pellet was suspended in grinding medium and overlaid on a 15 to 45% (w/w) linear sucrose gradient buffered with 5 mM Hepes-Mes (4-morpholineethanesulfonic acid), pH 7.7, plus 1 mM DTE and centrifuged at 4°C for 16 to 18 h at 84,000g (average *g*) in a SW-28 rotor. This centrifugation step was different compared to a previously reported procedure (21) in which 1 mM dithioerythritol and 1 mM EDTA (pH 7.2) were included in linear sucrose gradients centrifuged under nonequilibrium conditions. The gradients were fractionated as previously described (22). The ATP hydrolysis-supported proton uptake activity (inward movement) of each fraction was assayed.

Electron microscopy. The fraction exhibiting proton uptake activity (19 to 22% sucrose) was diluted with grinding medium and repelleted at 84,000g for 1 h. The pellet was suspended in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, and fixed for 1 h in a cellulose nitrate tube. The fixed suspension was centrifuged at 84,000g for 35 min in a SW-28.1 rotor. The pellet was postfixated in 1% OsO_4 , dehydrated with acetone, embedded in Epon, thin sectioned, and viewed with a Zeiss EM 10 B electron microscope.

³ Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

⁴ Abbreviations used: DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N'*-tetraacetic acid; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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Measurement of ATPase activity. The average and initial ATPase activity of linear sucrose density gradient fractions was assayed by the direct measurement of inorganic phosphate released and coupled to enzymatic NADH oxidation, respectively, at 22°C as previously described (10). The appearance of inorganic phosphate was determined in a volume of 2.2 ml of the basal solution containing 50 mM KCl, 5 mM MgSO₄, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 7.5 μM acridine orange, 1% sucrose, 17.5 mM Mes-Tris, pH 6.45, and an appropriate amount of membrane fractions. The reaction was started by the addition of ATP and was terminated by the addition of 1 ml of ice-cold 5% trichloroacetic acid at appropriate time intervals. The amount of inorganic phosphate released was determined by the malachite green-molybdate complex formation (10) or by the modified Fiske and Subbarow method (1). Alternatively, the initial rate of ATP hydrolysis was determined in the same assaying solution also containing suitable amounts of pyruvate kinase (54 units), lactate dehydrogenase (75 units), 0.4 mM phosphoenolpyruvate, and 0.3 mM NADH. The induced oxidation of NADH by the presence of ATP in the medium was used to measure the ATPase activity.

Proton pumping. The ATP hydrolysis-supported proton uptake of sealed membrane vesicles was monitored by the absorbance change of acridine orange at 492 nm (13) with a Beckman DU-7 spectrometer. Membrane fractions (50–200 μl) were incubated in a final volume of 2.2 ml of the basal solution for 10 min at 22°C before the addition of 20 μl of 0.3 M ATP buffered at pH 6.5.

Assays of membrane markers. Various subcellular membrane markers were used to assess the possible cross contamination in fractions of tonoplast vesicles. The tonoplast and plasma membranes were identified by their differential ATPase inhibition with 50 mM KNO₃ and 0.2 mM vanadate. The addition of 0.2 mM molybdate was used to estimate the possible contribution of phosphatase activity to total ATP hydrolysis. The activity of antimycin A-insensitive NADH-cytochrome *c* reductase (endoplasmic reticulum marker) and detergent-stimulated UDPase (Golgi marker) activities in the fractions were determined as described in a previous report (22).

Protein determination. The Coomassie blue binding method (23) or modified Lowry method (20) was used for protein concentration estimation. All the chemicals used were of analytical grade.

RESULTS

Isolation and purification of proton pumping vesicles. The results from differential centrifugation experiments demonstrated that the microsomal membrane

fraction pelleted between 6000*g* and 80,000*g* contained more than 80% of ATP hydrolysis-supported proton uptake under the assay conditions employed. The distribution, as shown in Fig. 1, was similar to a previous report (21). Such a distribution, however, was unusual since most microsomal components (Golgi, plasma membrane, endoplasmic reticulum) normally distribute fairly evenly between crude mitochondrial and crude microsomal fractions (22). The 80,000*g* to 120,000*g* pellet and 120,000*g* supernatant contain very little or no proton uptake activity (Fig. 1).

The proton pumping activity of the membrane fraction collected between 6000*g* and 80,000*g* could be inhibited by either 50 mM KNO₃ (~60%) or 0.2 mM vanadate (~40%) indicating the presence of both tonoplast-type and plasma membrane H⁺-ATPases (data not shown). Since this fraction contained the highest yield and highest specific activity for proton uptake, it was used for further purification in linear sucrose density gradients. Following isopycnic centrifugation, the gradient was fractionated and assayed for ATPase activity and ATP-dependent proton pumping

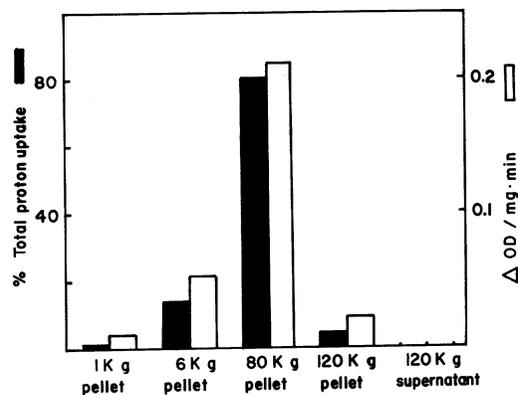


FIG. 1. The distribution of ATP hydrolysis-induced proton uptake activity in corn root fractions separated by differential centrifugation. Corn roots were fractionated as described and the proton pumping activity of each fraction was sequentially determined throughout the isolation procedure. The fraction volume and protein concentration were used to calculate the total and specific activities, respectively. The specific activity was expressed in terms of the absorbance decrease of acridine orange at 492 nm.

in the presence and absence of vanadate or nitrate. A typical distribution of these activities is shown in Fig. 2A. Most of the total ATPase activity equilibrated in the middle of the gradient (peak in fraction 15). This activity was only partially inhibited by vanadate indicating the presence of the plasma membrane and other ATPase-containing fragments e.g., ER and Golgi. The inclusion of 0.2 mM molybdate in the as-

saying solution, decreased the ATPase activity of the major peak by $\sim 20\%$ but had no effect on the activity associated with fractions 3-6.

The peak activities for the ER marker (antimycin A-insensitive NADH-cytochrome *c* reductase; Fig. 2C) and Golgi marker (detergent-stimulated UDPase; data not shown) were both coincidental with the major peak of ATPase activity and were essentially absent in the minor peak of ATPase activity (fractions 4 to 6). Although Golgi, ER, and plasma membrane from certain corn varieties could be separated in linear gradients (22), there was an apparent aggregation phenomena in other corn varieties such as the one used by DuPont *et al.* (4, 21) and also in this study. The aggregation was to our advantage since the proton pumping vesicles equilibrated between 20 to 21% sucrose (1.085 g/cc) and were free from other microsomal membranes.

A single proton pumping fraction (1.085 g/cc) was coincident with a nitrate-sensitive, vanadate-insensitive Mg-ATPase activity (Fig. 2). The proton pumping peak did not contain any significant phosphatase activity (24) as indicated by a lack of molybdate sensitivity (data not shown). No other membrane system from root cells has a reported density as low as the proton pumping vesicles. The observed low density of these vesicles, lack of ER, Golgi, and plasma membrane associated marker activity, and the coincidence of proton pumping activity with a nitrate-sensitive but vanadate-resistant ATPase activity all indicate that these membrane vesicles were tonoplast in origin. At its peak, the proton pumping was enriched 7- to 10-fold in the linear gradient when compared to the crude overlay. The pooled fractions (19-23% sucrose) contained an average 5-fold enrichment and were used for further experiments.

Electron microscopy of proton pumping vesicles. The fractions containing the peak of proton pumping activity were pooled and processed for electron microscope observation. This was necessary because very few morphological examinations of tonoplast-type vesicles have been reported and

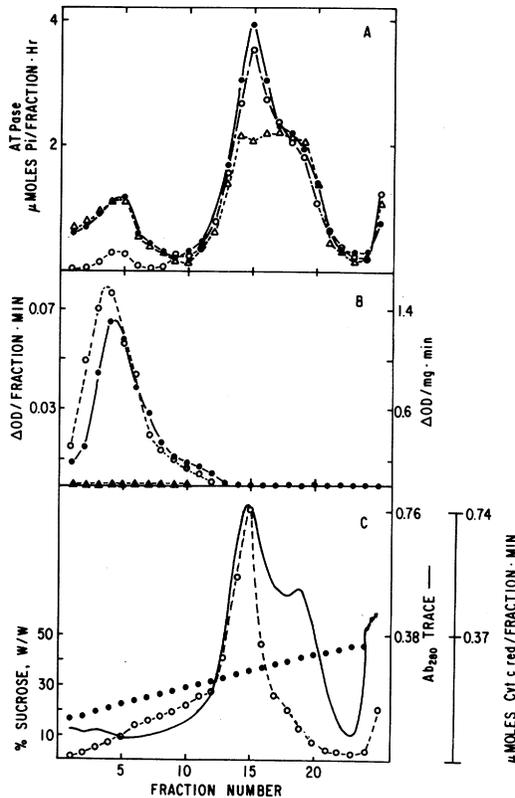


FIG. 2. The distribution of enzymatic activities in a linear sucrose density gradient. The microsomal fraction obtained between 6000g and 80,000g was further separated by a linear sucrose density centrifugation. At the end of centrifugation, the gradient was fractionally collected and certain physical and biochemical properties were determined. (A) The distribution of total ATPase activity (average rate) in the absence (●) and presence of (Δ) of 0.1 mM vanadate or 50 mM KNO_3 (○). (B) The distribution of proton pumping activity in the absence (total activity = ●; specific activity = ○) and presence of 50 mM KNO_3 (Δ). (C) The distribution of protein (—), antimycin A-insensitive NADH-cytochrome *c* reductase activity (○), and sucrose gradient (●).

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none for tonoplast-type vesicles from corn roots. As shown in Fig. 3, the pooled sample contained a small vesicle population of fairly uniform size. This uniformity may explain the rather sharp single proton pumping peak shown in Fig. 2. No Golgi cisternae were observed and the electron dense, amorphous, nonvesicular, particulate materials usually present in plasma membrane fractions in sucrose gradients were also absent in these membrane vesicles.

Mg-ATP hydrolysis-linked proton pumping. The tonoplast-type H^+ -ATPase catalyzes hydrolysis of Mg-ATP which is the preferred substrate (2, 3) and the energy released may be conserved in the form of a transmembranous proton electrochemical potential (5, 25). A typical ATP hydrolysis-induced proton uptake and the subsequent proton release caused by the ad-

dition of a protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or nitrate ions are shown in Fig. 4A. In order to quantitatively describe the proton movement, a kinetic scheme which was used to account for energy-dependent proton pumping in chloroplast (26), mitochondrial (27), and purple membrane (28) systems was employed. First, it was assumed that the initial proton pumping rate was related to the catalyzed hydrolysis of added ATP. The validity of this assumption is shown in Fig. 5. Clearly both the release of P_i (5B-2) and the proton pumping (5B-1) associated with the hydrolysis of ATP may be adequately described by simple Michaelis-Menten kinetics. The K_m 's of ATP hydrolysis and proton pumping processes are 0.55 and 0.82 mM at pH 6.5 and 22°C, respectively. The K_m of tonoplast ATPase obtained here is within the re-

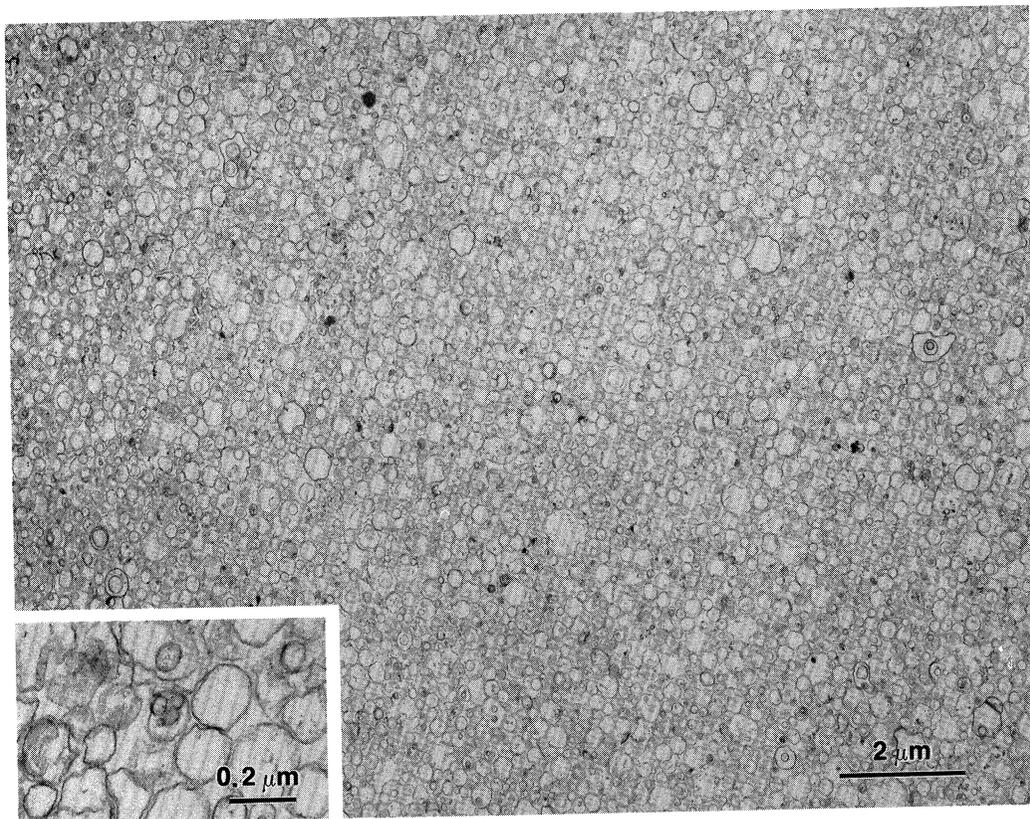


FIG. 3. The electron micrograph of proton pumping tonoplast vesicles. The peak proton pumping fractions were pooled and processed as described in the text.

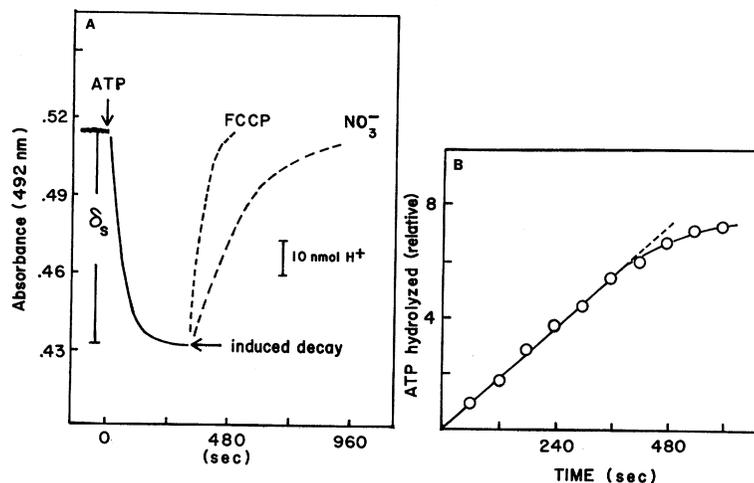


FIG. 4. (A) The ATP hydrolysis-induced proton uptake of tonoplast vesicles. The purified tonoplast membrane vesicles, containing about 50 μg of protein, were incubated in the basal medium (2 ml in volume) for 10 min before the addition of a 20- μl aliquot of 0.3 M ATP. After reaching a steady state (δ_s), the proton uptake measured by the absorbance decrease of acridine orange at 492 nm was discharged by the addition of a 5- μl volume of 20 μM FCCP in alcohol or a 20- μl aliquot of 5 M KNO_3 . For an estimation of the extent of proton translocation, 10 nmol of either NaOH or HCl was added after δ_s was reached. The alkalization or acidification-induced absorbance change was marked to the right of the proton movement trace. (B) The time course of ATP hydrolysis. The extent of ATP hydrolysis of an identical tonoplast suspension (without the addition of FCCP or nitrate ions) was determined at different time intervals. The measured amount of ATP hydrolyzed (by average rate method) at the end of first minute was assigned as 1.0.

ported range of 0.1 to 0.6 mM (3, 16, 29). It is interesting to note that the cytosolic concentration of mobile ATP in corn root cells determined by ^{31}P NMR (30, 31) is about 0.5 to 0.7 mM. This concentration range would allow a more sensitive response of the ATPase activity to the concentration change of ATP in corn roots.

Kinetic model of the proton pumping. As the proton gradient starts to build up, the active proton pumping as well as the ATP hydrolysis may be decreased by the generated electrochemical potential through a back-pressure effect in a tightly coupled system (32, 33). This decrease, however, may also be accounted for by pump slipping which represents a certain number of failures of molecular events that results in no proton movement in a loosely coupled system (34, 35). In addition, the back diffusion of protons due to leakage also tends to decrease the net pumping. The mathematical treatment of these proton pumping inhibitory processes in reconstituted purple

membrane system has been discussed in our previous reports (28, 36). By analogy, in the tonoplast system, the net proton pumping rate at a given time in the gradient growth phase may be represented by

$$(d\delta/dt) = mR - (k_1 + k_2)\delta = mR - k_2\delta, \quad [1]$$

in which δ is the net amount of protons transported (nmol H^+ /mg membrane protein) across the membrane at time t after the addition of ATP. This quantity may be qualitatively expressed as the net absorbance change of acridine orange or semi-quantitatively estimated (37), as shown in Fig. 4A, by relating this change to the acidification or alkalization of the sample solution with standard HCl or NaOH. R is the ATP hydrolysis rate and m is the ratio of proton pumping rate (in the absence of inhibitory processes) to ATP hydrolysis rate. In essence, m is a measure of the coupling between proton pumping and ATP hydrolysis or simply a stoichiometric ratio of proton transported to ATP hydrolyzed.

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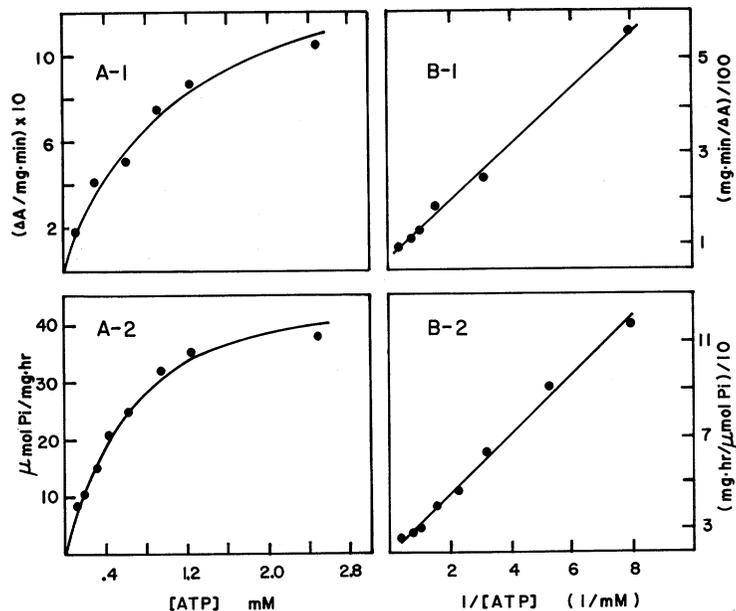


FIG. 5. V vs S and $1/V$ vs $1/S$ plots of ATP hydrolysis-induced activities. The initial rates of proton pumping (A-1) and P_i release (A-2) associated with the tonoplast vesicles ($20 \mu\text{g}$ of proteins) were determined in 2-ml aliquots of the basal solution containing different concentrations of ATP. The corresponding Lineweaver-Burk plots for proton pumping (B-1) and P_i release (B-2) were used to determine K_m values.

The first-order rate constants, k_1 and k_2 , represent the membrane leakage at the pumping stage and the back-pressure effect (or pump slipping), respectively. The total inhibition of the buildup of a gradient is the sum of k_1 and k_2 , or k_i .

At steady state, the net proton pumping rate, $(d\delta/dt)$ approaches zero and therefore,

$$mR_s = k_i \delta_s,$$

where R_s and δ_s are steady-state values. As shown in Fig. 4B, the ATP hydrolysis rate remains unchanged when the proton pumping already reaches a steady state. Thus, R_s may be substituted by the initial ATP hydrolysis rate, R_0 , which can be accurately determined by the coupled enzyme assay. The application of this steady-state approximation to the tonoplast system yields

$$mR_s = mR_0 = mR = k_i \delta_s. \quad [2]$$

This treatment reduces Eq. [1] to

$$(d\delta/dt) = k_i(\delta_s - \delta). \quad [3]$$

Upon integration of Eq. [3], the following result is obtained:

$$\log(1 - \delta/\delta_s) = -k_i t. \quad [4]$$

The apparent insensitivity of ATP hydrolysis to the buildup of H^+ gradient observed in this study (Fig. 4) and in a previous report (14) suggests that the back-pressure effect may not be a dominant factor in these tonoplast systems. The eventual decrease of ATP hydrolysis rate shown in Fig. 4B may be, at least partially, due to the generation of ADP which is a known inhibitor of tonoplast ATPase (29). In the event that the "pump slipping" is independent of δ , the value of m will be lowered by a constant value and k_i will then be the same as k_1 .

The established proton gradient may be dissipated by the addition of either protonophores or ATP hydrolysis inhibitors. The return of the transported protons, δ , may be assumed to obey simple first-order kinetics,

$$(d\delta/dt) = -k_3\delta, \quad [5]$$

in which the first-order rate constant k_3 represents the membrane leakage in the presence of protonophores or inhibitors. The integrated form of Eq. [5] is

$$\log(\delta/\delta_s) = -k_3t, \quad [6]$$

in which the (δ/δ_s) represents the fraction of δ_s remaining at time t after the addition of perturbants. As shown in Fig. 6, the proton movement across the tonoplast vesicles can be adequately described by the kinetic scheme developed (linear fitting better than 99%, in all cases). It is interesting to note that an increase of sucrose concentration from 1 to 5%, which would reduce the vesicle volume osmotically, significantly changes the rate constant k_i .

Coupling between P_i release and proton pumping. The mechanism of energy-driven proton movement across biological membranes is often a subject of controversy. For example, the coupling between electron transfer and proton pumping in mitochondria has been suggested as either direct (loop or cycle concept; Ref (38)) or indirect (membrane Bohr effect; Ref. (39)) in nature. In essence, the indirect coupling

mechanism suggests that the primary energy release step is only conformationally linked to proton translocation. To test the nature of the coupling between ATP hydrolysis and proton movement in tonoplast vesicles, the sensitivity of these two events to anions was evaluated. By keeping the total K^+ concentration and the ionic strength constant, while changing the ratio of chloride to nitrate ions (Cl^- from 75 to 25 mM, NO_3^- from 0 to 50 mM) in the assay medium, the initial proton pumping and ATP hydrolysis rates of tonoplast vesicles were determined. As shown in Fig. 7, the proton pumping activity shows a more pronounced sensitivity to the mole fraction of nitrate ions compared to ATP hydrolysis. This differential effect suggests that the coupling is indirect in nature.

The differential effect presented in Fig. 7 could also be accounted for by other alternatives. The observed lower sensitivity of ATP hydrolysis may be attributed to the presence of a nonspecific phosphatase activity in the membrane preparation. However, as mentioned in Fig. 2, the ATP hydrolysis catalyzed by the tonoplast fraction is insensitive to molybdate, indicating little or no phosphatase activity in the mem-

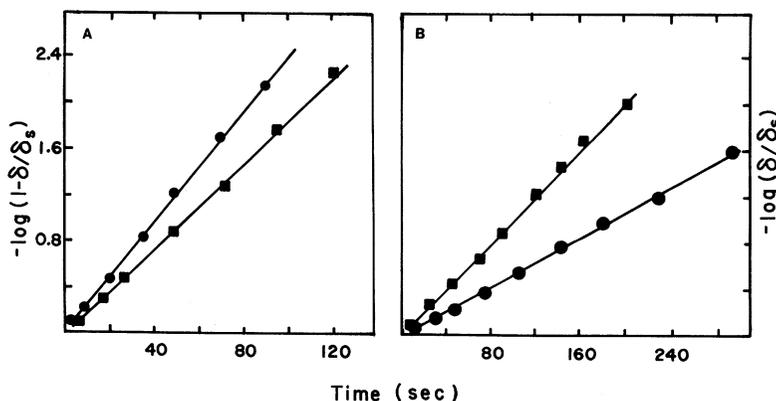


FIG. 6. The growth and decay of ATP hydrolysis-induced proton uptake. The proton translocation of tonoplast vesicles measured with the procedure described in Fig. 4 was analyzed by the kinetic scheme mentioned in the text. The growth curves obtained in the basal (■) and modified basal (●; 5% instead of 1% sucrose) solutions are shown in (A). The induced decay by the addition of 5 mM (●) and 45 mM (■) nitrate are shown in (B). The extents of proton translocation at the steady state (δ_s), as measured by the absorbance decrease, were 3.36 and 7.17 $\Delta A/mg$ protein in the basal and modified basal solutions, respectively. The k_i values obtained in (A) were 1.164 and 1.416 min^{-1} for the basal and modified basal solutions, respectively. The induced leak rate constants (k_3) as shown in (B), were 0.336 and 0.618 min^{-1} in the presence of 5 and 45 mM nitrate, respectively.

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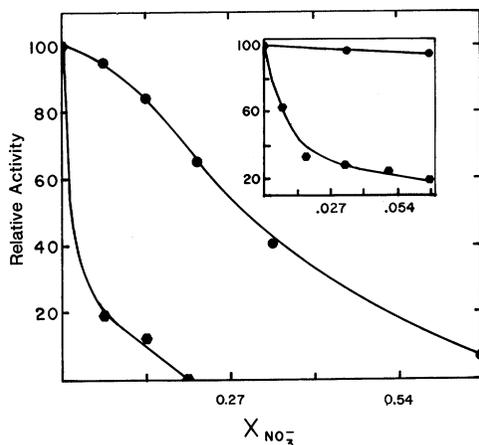


FIG. 7. Differential inhibition of P_i release and proton uptake. The tonoplast membrane vesicles were incubated in the basal solution, except that the chloride ions were replaced by nitrate ions as indicated. After 10 min incubation, ATP was added. The initial rates of P_i release and proton uptake (measured by the absorbance change of acridine orange) obtained in the absence of nitrate ions were assigned as 100%. The mole fraction of nitrate $X_{NO_3^-}$ is defined as $[NO_3^-]/([NO_3^-] + [Cl^-])$. The changes of proton pumping rate and P_i release rate as nitrate ions increased, are shown by (●) and (●), respectively. The differential inhibition at low $X_{NO_3^-}$ (0 to 0.067) is shown in the insert. The representation of the abscissa and ordinate of the insert is the same as the larger figure. When 0.02% Triton X-100 was included in the medium, the inhibition of P_i release by nitrate ions was not significantly affected.

brane fraction. The contamination of NO_3^- -insensitive ATP hydrolyzing enzymes of non-tonoplast origin was used to account for an apparent preferential inhibition of proton pumping in a corn root tonoplast vesicle enriched fraction obtained by step sucrose gradient centrifugation (40). However, unlike the ATPase activity of the membrane fraction used in (40) which was only about 60% inhibited even in the presence of 100 mM NO_3^- , the ATPase activity of tonoplast vesicles used in this study was about 95% inhibited by 50 mM NO_3^- . In addition to the tonoplast, the only other nitrate-sensitive ATPase was Golgi associated. However, the inhibition of Golgi ATPase by nitrate was considerably less than with tonoplast ATPase (41). Furthermore, the tonoplast-type vesicles fraction

used in this study did not contain detergent-activated UDPase activity, which is the marker for Golgi membranes or antimycin A-insensitive NADH-cytochrome *c* reductase activity which is a marker for ER. Thus, the contamination of other known low density subcellular membranes cannot be a factor for the observed differential inhibition between proton pumping and P_i release.

Based on the sensitivity to NO_3^- , we conclude that the membrane fraction used in our present work was about 95% tonoplast in origin. It is not possible, from the data obtained, to evaluate whether all of the vesicles contribute to the observed proton pumping. Leaky vesicles are completely silent in proton pumping but still contribute to the total ATPase activity. However, it has been shown that the nitrate inhibition can be reversed by simple dilution (18), indicating a noncovalent interaction with the membrane. Thus, it is not likely that the sensitivity of proton pumping of sealed vesicles to a noncovalent interaction caused by NO_3^- , can be significantly affected by the presence of leaky vesicles. On the other hand, the ATPase of leaky vesicles might exhibit a different sensitivity to NO_3^- . To test this possibility, the experiments for the ATPase activity measurement mentioned in Fig. 7 were repeated in the presence of 0.02% Triton X-100. The addition of Triton X-100 caused about 50% increase of ATPase activity with the mole fraction of NO_3^- as zero. However, the relative inhibition (percentage) of the ATPase activity caused by the increase of NO_3^- was essentially unaffected by the presence of Triton X-100. These observed Triton X-100 effects were in agreement with the findings mentioned by others (Table II of (19)). Thus, the ATPase in the leaky tonoplast vesicles does not require a higher concentration of NO_3^- to reach the same extent of inhibition.

Since the membrane preparation may also contain sealed vesicles with inverted orientation (the catalytic site of the ATPase on the inside surface of the vesicles), the observed differential effects would be expected if the membrane permeability of ATP is significantly greater

than that of NO_3^- . However, there is no information available to support the existence of such an efficient ATP transporter in tonoplast systems (3). Furthermore, the observed Triton X-100 stimulation of the ATPase activity without changing the inhibition pattern of NO_3^- does not support its existence in the membrane. It should be mentioned that 25 mM Cl^- is adequate to provide a maximum stimulation of the tonoplast ATPase activity (15).

Origin of nitrate inhibition. Since nitrate ions specifically inhibit the tonoplast ATPase, an understanding of the inhibition mechanism is needed to account for the tonoplast energy transduction process. The approaches mentioned in Figs. 4 and 6 allow a qualitative assessment on the nature of the inhibition. According to the kinetic model present in this study, the slower proton uptake rate and the lower steady-state extent of transport (δ_s) observed when a low concentration of NO_3^- (≤ 2 mM) was added before the initiation of proton pumping, could be either due to a decrease of m or an increase of k_i , or both. The proton pumping kinetics were analyzed and results are summarized in Table I. The observed proton pumping inhibition without significant effect on ATPase activity caused by low concentration of nitrate in red beet

tonoplast vesicles was attributed to a proton-nitrate symport movement in the direction opposite to that of pumping (18, 19). Such a mechanism would predict an increase of membrane proton leakage during ATP-induced pumping stage (higher k_i values in the presence of nitrate). However, the results shown in Table I demonstrate that with the presence of NO_3^- , the proton pumping inhibition, as measured by k_i , was actually lowered. With both the ATPase activity and proton pumping completely inhibited by the addition of 45 mM NO_3^- after reaching the steady state, the proton leakage measured as k_3 was even lower. On the other hand, protonophores (e.g., FCCP) which increase the proton conductance of biomembranes (42), do indeed increase the shuttling of protons in the tonoplast membrane as evident from a threefold increase of proton leakage rate constant (comparing the values of k_i and k_3 in Table I). Thus, the symport movement of proton and nitrate may not be a major mechanism for the apparent lowering of proton pumping in the tonoplast vesicles used in this study.

Therefore, the nature of nitrate inhibition to the pumping is quite different from that caused by protonophores. The data shown in Fig. 7 indicate that the P_1 release remains essentially unchanged in the

TABLE I
KINETIC PROPERTIES OF NITRATE INHIBITION ON PROTON PUMPING

Experiment ^a	Rate constant (1st order, min^{-1})	δ_s		R_0^b (nmol P_1 / $\text{mg} \cdot \text{min}$)	m	
		$\Delta A/\text{mg}$	nmol H^+/mg		$\Delta A/\text{nmol } P_1$	H^+/P_1
1. Control	1.164 (k_i)	3.36	1317	908	4.31×10^{-3}	1.45
2. + 1 mM NO_3^-	1.116 (k_i)	2.20	862	858	2.86×10^{-3}	1.01
3. + 2 mM NO_3^-	0.984 (k_i)	1.10	431	818	1.32×10^{-3}	0.53
4. + 50 nM FCCP	3.216 (k_3)			952		
5. + 45 mM NO_3^-	0.618 (k_3)			85		

^a The ATP hydrolysis-induced proton uptake of tonoplast vesicles (50 μg) was measured as described in Figs. 4 and 5. The rate constants were determined by the kinetic model mentioned in the text. For experiments 2 and 3, the vesicles were incubated in the basal solution containing nitrate before the addition of ATP. For experiments 4 and 5, the proton uptake was allowed to reach steady state as in experiment 1 and then discharged by the addition of either FCCP or KNO_3 . The values of δ_s and m were calculated by the net absorbance change at 492 nm directly or related to alkalization/acidification of the samples as mentioned in Fig. 4A.

^b The hydrolysis rate of ATP (R_0) was determined by the coupled enzyme method in the same solution used for proton pumping experiment as described under Materials and Methods.

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presence of a minimal amount of nitrate ($X_{\text{NO}_3^-} \leq 0.067$). However, under the same conditions the initial proton pumping rate already shows a 80% decrease. This result implies that the energy released from the hydrolysis of ATP becomes unavailable to sustain the transmembranous proton pumping. In terms of the kinetic model, the effect of nitrate is to decrease the value of m which may be regarded as a measure of the link between proton pumping and P_i release. Indeed, as qualitatively shown in Table I, m does decrease when nitrate is increased.

It should be mentioned that the relatively low stimulation of ATPase activity by FCCP mentioned in Table I was, in part, due to the low concentration of FCCP used (50 nM). A fourfold stimulation of red beet tonoplast ATPase activity was caused by 10 μM FCCP at pH 8.0 (19). However, in a tonoplast fraction isolated from corn tips, only about 50% stimulation of the ATPase activity was induced by 10 μM FCCP at pH 6.75 (4). We have tested the efficiency of FCCP enhancement of the tonoplast ATPase activity under the experimental conditions mentioned in Table I. The results (not shown) indicated a near bell-shaped concentration dependence with a maximum enhancement (about 65%) positioned at about 1.1 μM which was 20 times more than that used in Table I. The exact molecular basis for the difference in sensitivity to FCCP between red beet and corn root tonoplast vesicles is unknown. The lesser stimulation of the ATPase activity by FCCP may suggest the presence of a significant amount of leaky tonoplast vesicles in our samples. However, as described before, this situation does not invalidate that data analysis method used in this study.

DISCUSSION

In intact plant root cells, plasma membrane ATPase is believed to induce a proton extrusion outward (3). Thus, it is likely that the proton uptake of plasma membrane in a crude microsomal fraction is due to the presence of inverted vesicles. Since the employed acridine orange assay can measure only the proton uptake of sealed ves-

icles, plasma membrane vesicles with preserved *in vivo* orientation, would be proton pumping silent because the catalytic site of ATPase is inaccessible to the substrate. The presence of leaky plasma membrane vesicles with an inverted orientation would also escape the detection of proton uptake. While the microsomal fraction of corn root cells contains proton pumping activities of both tonoplast-type and plasma membrane ATPases, further separation by linear sucrose density centrifugation, as reported here (Fig. 2), yielded only one fraction (tonoplast) exhibiting an ATP-dependent proton uptake (4, 15). Whether prolonged linear density centrifugation (17 h) enhances the damage of the plasma membrane vesicles by protease and/or phospholipase activities remains to be determined.

The observed H^+ -ATPase properties of the low density tonoplast-type membrane vesicles reported by other researchers (1-4) as well as in this study, in many regards, e.g., vanadate insensitivity, anion dependence, proton electrochemical potential generation, are similar to the ATPase associated with isolated vacuoles from many different plant tissues (43-46). This similarity suggests that (A) the low density membrane vesicles of microsomal fraction may originate in large part from the tonoplast membrane and (B) the possible involvement of ATP-driven, proton movement-mediated, chemiosmotic-type transport processes between plant cell cytoplasm and vacuoles (3, 12). However, based on the lack of substrate-specific ATPase activity in intact vacuoles isolated from the protoplasts of cultured tobacco cells, non-energy requiring processes, e.g., exchange diffusion and Donnan phenomenon, have also been proposed as the major transport mechanisms (47). Thus, further investigation is needed to determine the relative physiological significance of these different transport mechanisms in intact plant cells. From the data shown in Figs. 1 and 2, only 0.59% of total pelleted membrane protein (0g to 120,000g) or 1.3% of the total microsomal protein is recovered by equilibrium centrifugation in the proton pumping tonoplast-type fraction. It is in-

teresting to note that the isolated vacuole vesicles contain less than 1% of the total protein from intact protoplasts (47).

Among the subcellular membrane ATPases in plant root cells, the reaction mechanism of tonoplast-type ATPase is probably the one least understood. For tonoplast-type ATPase, some of its properties, e.g., no detectable formation of phosphorylated intermediate (3, 15), are similar to the mitochondrial ATPase. Although there is little information to firmly establish the molecular coupling mechanism between ATP hydrolysis and its associated proton pumping in the tonoplast-type system, the differential inhibition reported here suggests that proton pumping and ATP hydrolysis may be indirectly coupled; in the sense that at least some intermediate steps, whether chemical or conformational, are required for the coupling between these two events. Using the indirect coupling model proposed for mitochondrial ATPase (48) and bacteriorhodopsin (28) as a conceptual guide, the effects of the chemical events occurring at the catalytic site in a protein domain (domain I) of the tonoplast-type ATPase may propagate through the link to trigger proton pumping of a "protogenic domain" (domain II) which may or may not be an integral structure part of the ATPase. The observed preferential inhibition of proton pumping suggests that nitrate ions may interact with the protogenic domain to cause it to be less responsive to ATP hydrolysis at the catalytic site. At higher concentration of nitrate ions, the link between the two domains is completely blocked. Consequently, the turnover of the enzyme cannot be completed and the result is an inhibition of ATP hydrolysis.

The coupling between P_i release and proton pumping may be expressed by the value of m which can be conveniently evaluated by Eq. [2] as demonstrated in Table I (experiments 1, 2, and 3). On the other hand, the basal membrane proton leakage in the absence of ATP hydrolysis cannot be easily determined. Although nitrate ions may arrest the ATPase activity, the needed concentration unavoidably causes significant composition changes in the assaying

solution. In this regard, the use of the developed kinetic scheme for a complete quantitative description of proton translocation awaits the availability of a potent and specific inhibitor of the tonoplast membrane ATPase.

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