

Temperature Dependence and Mercury Inhibition of Tonoplast-Type H⁺-ATPase

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The effects of changing temperature on ATP hydrolysis and proton pumping associated with the H⁺-ATPase of tonoplast membrane vesicles isolated from the maize root microsomal fraction were determined. In the range 5 to 45°C, the maximal initial rate of ATP hydrolysis obeyed a simple Arrhenius model and the activation energy determined was ~14 kcal/mol. On the other hand, the initial proton pumping rate showed a bell-shaped temperature dependence, with maximum activity around 25°C. Lineweaver-Burke analysis of the activities showed that the K_m of ATP hydrolysis, unlike that of proton pumping, was relatively insensitive to temperature changes. Detailed kinetic analysis of the proton pumping process showed that the increase in membrane leakage to protons during the pumping stage constituted a major reason for the decreased transport. Nitrate-sensitive ATPase activities of the tonoplast vesicles were found to be inhibited by the presence of micromolar concentrations of Hg²⁺. The proton pumping process was more sensitive to the presence of Hg²⁺. Double-reciprocal analysis of kinetic data indicated that Hg²⁺ was a noncompetitive inhibitor of proton pumping but was an uncompetitive inhibitor of ATP hydrolysis. Further kinetic analysis of Hg²⁺ effects revealed that the lower proton transport did not result from enhanced membrane leakage but rather from reduced coupling between H⁺ pumping and ATP hydrolysis. © 1988 Academic

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It is generally accepted that the low-density (~1.08 – 1.12 g/cm³) membranes obtained from plant microsomal fractions contain a H⁺-ATPase that prefers Mg-ATP as the substrate and can be specifically inhibited by nitrate ions (1-4). The properties of this H⁺-ATPase, e.g., vanadate insensitivity, anion dependence, and generation of proton electrochemical potential, are similar to those of the ATPase associated with isolated vacuoles (5-8). This similarity suggests that the low-density membranes of the microsomal fractions may originate from the tonoplast membrane.

ATPase activity-induced proton pumping in isolated tonoplast membrane vesicles has been measured by changes in absorption of indicator dyes (9), emission of fluorescence probes (10), pH of external solutions (11), and uptake of ¹⁴C-labeled compounds (2). The inward direction of proton movement and the generation of a positive internal membrane potential (positive inside) indicate that most of the isolated tonoplast vesicles retain the right-side-out membrane orientation. Thus, the isolated vesicles may be an ideal working system for investigation of the functions of this enzyme in plant cells.

Because of its electrogenic proton pumping activity, the tonoplast H⁺-ATPase is thought to play an important role in intracellular pH regulation and transport pro-

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cesses (3). It was recently demonstrated that the transport of Ca^{2+} (12) in oat root tonoplast vesicles and the transport of NO_3^- (13) in red beet tonoplast vesicles were probably related to the proton pumping of the H^+ -ATPase. While considerable advances have been made in the biochemical characterization of the H^+ -ATPase (3), few reports are available to delineate the relationship between ATP hydrolysis and coupled proton pumping at a molecular level.

In general, there are two possible mechanisms for vectorial proton movement coupled to the energy release of ATP hydrolysis in tonoplast vesicles. If the process leading to proton movement shares part of the molecular pathway responsible for the ATP hydrolysis, a direct coupling mechanism would be operative. An example of this direct coupling mechanism is the redox-loop concept for electron transfer-coupled proton movement in mitochondria (14). Alternatively, the primary energy-yielding process, i.e., ATP hydrolysis, could be only indirectly linked to proton movement, in the sense that certain intermediate steps, whether chemical or conformational, are required for the coupling to the vectorial proton movement (15, 16). The observation that the tonoplast ATPase of red beet microsomal membranes exhibited a fixed stoichiometry of $2\text{H}^+/\text{ATP}$ at pH 6.13 supported the direct coupling mechanism (10). On the other hand, it was found that the proton movement was more sensitive to nitrate than was ATP hydrolysis in red beet tonoplast vesicles (17). Recently, we have analyzed the nitrate inhibition to the tonoplast vesicles of corn roots (18). Based on a detailed kinetic model for the proton movement, we proposed that the coupling between ATP hydrolysis and proton pumping, at least in corn root tonoplast vesicles, could be best described by an indirect coupling mechanism, and nitrate inhibition would originate from an interaction with the protogenic domain in the membrane.

In the present report, we studied the effects of temperature and mercuric ion on the kinetics of the coupled events. We found that the response of ATP hydrolysis,

but not proton pumping, to temperature changes followed a simple Arrhenius model. We also demonstrated that the presence of micromolar concentrations of Hg^{2+} effectively inhibited the activities of the tonoplast ATPase. Furthermore, the pattern of inhibition of ATP hydrolysis was different from that of proton pumping. These results were interpreted in terms of an indirect coupling mechanism previously suggested (18).

MATERIAL AND METHODS

Membrane preparation. The crude microsomal fraction of corn roots (*Zea mays* L. cv. FRB73, Illinois Foundation Seeds,² Champaign) was obtained by differential centrifugation as previously described (19). The fraction was suspended in a medium consisting of 0.3 M sucrose, 5 mM EDTA, 5 mM dithioerythritol (DTE),³ 5 mM 2-mercaptoethanol, and 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.7, and then overlaid on a 15 to 45% (w/w) linear sucrose gradient buffered with 5 mM Hepes-Mes (4-morpholinoethanesulfonic acid), pH 7.7, with 1 mM DTE. After centrifugation for 16 to 18 h at 84,000g in a Beckman SW28 rotor, the gradient was fractionated as previously described (20). Morphologically homogeneous tonoplast membrane vesicles, which were shown to be essentially free from other subcellular contamination (18), were collected between fractions with densities equivalent to 18–23% of sucrose. All isolation procedures were performed at 4°C.

Measurement of coupled ATPase activities. ATP hydrolysis and its supported proton pumping activities were measured according to a previously described procedure (18). The coupled activities were measured in 2 mL of a basal solution containing 50 mM KCl, 5 mM MgSO_4 , 1 mM ethylene glycol bis- β -aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 7.5 μM acridine orange, tonoplast membrane (20–50 μg of protein), 17.5 mM Mes with pH adjusted to 6.450 by solid Tris, and other additions (e.g., Hg^{2+} in the form of HgSO_4) as indicated. After the selected temperature was

² 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.

³ Abbreviations used: DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholinoethanesulfonic acid; EGTA, ethylene glycol bis(α -aminoethyl ether) *N,N'*-tetraacetic acid; BSA, bovine serum albumin; ER, endoplasmic reticulum; DCCD, *N,N'*-dicyclohexylcarbodiimide; TCA, trichloroacetic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

reached in a thermally controlled cuvette, a small aliquot of ATP (final concentration 0 to 5 mM) was added to initiate the reactions. The time course of the absorbance change at 492 nm was used to determine the transmembranous proton movement (9). After the absorbance change reached a steady state, a small aliquot of the solution was rapidly added to ice-cold TCA and the total amount of P_i released was determined by malachite green-molybdate complex formation (19). In the absence of Hg^{2+} , ATP hydrolysis could also be measured by a coupled enzyme system. In the basal solution (with the omission of acridine orange), 54 units pyruvate kinase, 75 units L-lactic acid dehydrogenase, 0.4 mM phosphoenol pyruvate, and 0.3 mM NADH were added. The kinetics of the absorbance change at 340 nm as induced by the addition of ATP was used to calculate the rate of ATP hydrolysis. As discussed later, within the specified time range (0 to a few minutes after proton pumping reached steady state), the rates of ATP hydrolysis and proton pumping determined by these two methods were essentially identical.

The basal solution was normally prepared at 25°C. The corresponding pH values of the solution at 5, 10, 15, 20, 30, 35, 40, 45, and 50°C were measured as 6.493, 6.487, 6.469, 6.477, 6.429, 6.414, 6.394, 6.375, and 6.361, respectively. Since the temperature coefficient of the buffer solution was small, the amount of KOH or HCl needed to readjust the pH back to 6.450 at other temperatures would not cause any significant change in the ionic composition of the solution.

A detailed method for the analysis of proton pumping kinetics in the tonoplast vesicles was previously developed (18). In that scheme, the net proton flux or unidirectional pumping is given by (18, p. 6)

$$d\delta/dt = mR_{ATP} - k_i\delta \quad [1]$$

where δ is the net amount of protons transported across the membrane at time t after the initiation of proton pumping. This quantity may be conveniently expressed as the net absorbance change of acridine orange at the selected wavelength. Under the experimental conditions employed (membrane potential minimized by the presence of 50 mM KCl), the ATP hydrolysis rate (R_{ATP}) has been shown to remain unchanged during the period needed to establish the steady-state value of δ_s (10, 18). Thus, R_{ATP} (initial) is equal to R_{ATP} (steady state) in the tonoplast system during the specified period. The first-order rate constant, k_i , represents the total inhibition of the active pumping process. This quantity consists mainly of membrane proton leakage during the pumping stage (18). At steady state, the net proton flux approaches zero and therefore

$$mR_{ATP}(\text{steady state}) \simeq mR_{ATP}(\text{initial}) \simeq k_i\delta_s \quad [2]$$

where δ_s is the steady-state value. This steady-state approximation reduces Eq. [1] to

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

The integrated form of Eq. [3]

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

Since δ and δ_s can be accurately estimated from the absorbance change of acridine orange, the rate constant, k_i , is easily determined. Under a given set of experimental conditions, m is a constant, so that Eq. [1] may also be written as

$$d\delta/dt = R_0 - k_i\delta \quad [5]$$

where R_0 is the initial proton pumping rate. The steady-state approximation leads to

$$R_0 = k_i\delta_s \quad [6]$$

Equation [6] provides an accurate method to calculate the initial proton transport rate without the risk of subjective errors from drawing a tangent line through the initial portion of a pumping curve.

Protein determination. The Coomassie blue binding method (21) or modified Lowry method (22) was used for protein concentration estimation with bovine serum albumin (BSA) as the standard.

Materials. Adenosine 5-triphosphate; vanadium-free disodium salt (ATP); adenosine 5-diphosphate, sodium salt (ADP); fatty acid-free BSA; nicotinamide adenine dinucleotide, reduced form (NADH); phosphoenol pyruvate; L-lactic acid dehydrogenase, salt-free powder; pyruvate kinase, salt-free powder; Hepes; Tris; EDTA; EGTA; DTE; 2-mercaptoethanol; and acridine orange were obtained from Sigma Chemical Company. All other chemicals used were of analytical grade.

RESULTS

Effects of temperature change on the apparent activities of tonoplast ATPase. The tonoplast membrane-enriched fraction of corn roots has been characterized in detail as previously described (18). This morphologically homogeneous preparation was estimated to be at least 95% tonoplast in origin (based on the extent of inhibition of the ATP hydrolysis by nitrate ions) and was free from plasma membrane, mitochondria, Golgi, and ER markers.

The effects of temperature changes on tonoplast H^+ -ATPase activities are shown in Fig. 1. By keeping the concentration of ATP at 4 mM, the initial ATP hydrolysis rate increased as the temperature increased. On the other hand, the coupled net proton flux exhibits a bell-shaped dependence, with the maximum located between

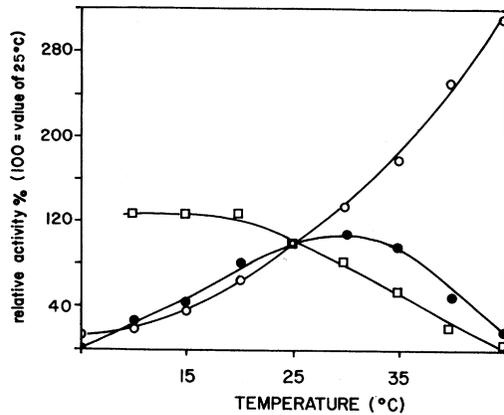


FIG. 1. Temperature dependence of tonoplast ATPase activities. Proton pumping and ATP hydrolysis activities (measured as initial rates) were determined at different temperature as described under Materials and Methods. ATP hydrolysis rate (○) was determined by the coupled-enzyme method and proton pumping rate (●) was determined by the absorbance change of acridine orange. The ratio of proton pumping to ATP hydrolysis was also calculated (□). Proton pumping, ATP hydrolysis, and the ratio at 25°C were 1.86 $\Delta A/\text{min} \cdot \text{mg}$, 1055 nmol $P_i/\text{mg} \cdot \text{min}$ and $1.76 \times 10^{-3} \Delta A/\text{nmol } P_i$, respectively, and were arbitrarily assigned as 100%.

25 and 30°C. The ratio of proton pumping to ATP hydrolysis, expressed as m , remains nearly constant with temperatures below 25°C. However, above this temperature, m starts to decrease continually. To obtain certain insights into the molecular basis and possible significance of the observation, further experiments were performed.

Kinetic parameters of ATP hydrolysis. ATP hydrolysis catalyzed by tonoplast ATPase was previously shown to obey simple Michaelis-Menten kinetics (13, 18, 23). Over a relatively wide temperature range (5 to 45°C), this kinetic model appeared to be valid. As shown in Table I, the K_m of the catalytic process is only slightly affected by the temperature change. Since the concentration of the substrate (Mg-ATP) used in Fig. 1 was in large excess over K_m at all temperatures tested, the observed ATPase activity should be close to the V_{max} or

$$V(T) \simeq V_{\text{max}}(T) = k_2(T)[E]_0 \quad [7]$$

where $V(T)$, $V_{\text{max}}(T)$, $k_2(T)$, and $[E]_0$ repre-

sent the observed activity (Fig. 1), the maximum activity, the catalytic rate constant, and the total enzyme concentration at the given temperature T , respectively. By keeping $[E]_0$ constant, as in Fig. 1, the activation energy, E_a , of the enzyme-substrate breakdown process may be evaluated by simple Arrhenius plots [$\log V(T)$ versus $1/T$]. This approach has been used to determine the activation energy of corn root plasma membrane ATPase (24) and its suitability for the tonoplast system is demonstrated in Fig. 2. The activation energy of the tonoplast ATPase so determined was ~ 14 kcal/mol, which was comparable to that of the plasma membrane ATPase (24).

Origin of temperature effects on proton pumping. The proton pumping activity associated with the tonoplast ATPase at room temperature was also shown to obey simple Michaelis-Menten kinetics at room temperature and the K_m of Mg-ATP related to proton pumping was higher than that associated with ATP hydrolysis (18). It was found that the kinetic scheme was also valid for proton pumping except at temperatures $> 40^\circ\text{C}$ or $< 10^\circ\text{C}$. The K_m values obtained at different temperatures

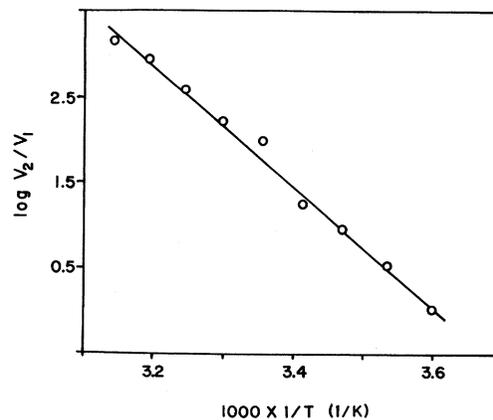


FIG. 2. Arrhenius plot of tonoplast ATPase activity. The temperature dependence of the ATP hydrolysis rate described in Fig. 1 was analyzed by use of the Arrhenius equation. The ATP hydrolysis rate obtained at 5°C was assigned as the reference rate (V_1) in the calculation. The activation energy (E_a) determined from the slope is 14.1 kcal/mol \pm 4%.

TABLE I
 K_m OF THE TONOPLAST ATPase ACTIVITIES AT
DIFFERENT TEMPERATURES

Temperature (°C)	ATP hydrolysis ^a	Proton pumping ^b
10		0.26 mM ± 10%
15	0.14 mM ± 10%	
20		0.47 mM ± 5%
25	0.18 mM ± 10%	
30		0.77 mM ± 5%
35	0.16 mM ± 10%	
40		1.32 mM ± 5%

^a The initial rate of ATP hydrolysis was determined by the coupled-enzyme system. The concentration range of ATP tested was 0 to 3 mM.

^b The initial proton pumping rate was calculated using the kinetic equation $R_0 = k_i \delta_s$ described in the text.

are listed in Table I. Clearly, in contrast to ATP hydrolysis, the K_m of proton pumping increased considerably as the temperature increased. This result indicated that certain molecular processes of kinetic importance contributing to transmembranous proton movement are not directly involved in the pathway leading to the hydrolysis of ATP.

The data in Fig. 1 show a bell-shaped temperature dependence of the proton pumping rate of tonoplast ATPase. However, as previously described (18) and as already mentioned, a complete description of the pumping process requires a more thorough kinetic analysis. Thus, the proton pumping data obtained at different temperatures were further analyzed, and the results are shown in Table II. It is evident that k_i , which measures mainly the proton leakage of the membrane during pumping, increases as the temperature increases in the tested range. This increase in k_i coupled with the decrease in initial net flux (R_0) above 30°C, constituted the basis of the dramatic decrease in δ_s at higher temperature. Since the proton pumping activity was measured in the presence of sufficient chloride ions (50 mM) to minimize the membrane potential (11), the steady-state δ_s would closely represent the energy of

ATP hydrolysis conserved in transmembranous proton movement.

The effect of temperature change on the coupling, as measured by the ratio of initial rates of proton pumping and ATP hydrolysis, is shown as m in Fig. 1. Clearly, the coupling, which represents the efficiency of utilizing the energy derived from ATP hydrolysis to support the transmembranous proton movement, remains relatively constant between 10 and 20°C. Above 20°C, the coupling decreased as the temperature increased. Below 10°C, proton movement was not measured. It should be mentioned that the value of m was estimated in a previous report (18) as 1.45 H⁺/ATP at 25°C under identical conditions.

Effects of nitrate and mercuric ions on ATPase activity. The tonoplast ATPase has been reported to be sensitive to nitrate ions (3, 25). In a previous report (18), we showed that nitrate ions preferentially inhibited the proton pumping activity of corn root tonoplast ATPase. The presence of ~1 mM nitrate ions inhibited ~50% of proton pumping but had a negligible effect on ATP hydrolysis. In fact, the ATP hydrolysis rate was not affected by this concentration of nitrate ions in the temperature range 5 to 45°C (data not shown). Consequently, the activation energy associated with the rate constant (k_2) was not significantly affected by the presence of 1 mM ni-

TABLE II
EFFECT OF TEMPERATURE ON PROTON
PUMPING KINETICS^a

Temperature (°C)	δ_s^b (ΔA)	k_i (min ⁻¹)	R_0^c ($\Delta A/\text{min}$)
10	0.0372	0.285	10.6×10^{-3}
20	0.0860	0.349	30.0×10^{-3}
30	0.0816	0.493	40.2×10^{-3}
40	0.0220	0.755	16.6×10^{-3}

^a Proton pumping activity of the tonoplast vesicles (20 μg protein) was measured in the presence of 4 mM ATP.

^b The net absorbance change of acridine orange at 492 nm observed at the steady state was used.

^c R_0 was calculated as described in Table I.

trate ions. This result supported our previous claim that the primary action of nitrate did not involve an interaction with the catalytic site but rather interfered with the linkage between the catalytic site and the protogenic domain of the tonoplast membrane (18). The nature of nitrate inhibition was also quite different from that of *N,N'*-dicyclohexylcarbodiimide (DCCD), which was known to covalently modify the protein subunit of tonoplast ATPase (3). The inhibition of ATP hydrolysis (50%) became evident only when the nitrate concentration (~ 20 mM) was considerably higher than that needed for inhibiting proton pumping.

In searching for an inhibitor that may interact closer to the catalytic site of the tonoplast ATPase, we have tested the effects of Hg^{2+} ions on the coupled activities of the enzyme. Reagents that react with thiols, such as *p*-hydroxymercuribenzoate, *p*-chloromercurisulfonate, and *N*-ethylmaleimide, are known to inhibit the tonoplast ATPase of the latex of the rubber tree, *Hevea brasiliensis* (26). However, the possible effects of Hg^{2+} on the tonoplast H^+ -ATPase of corn roots have not been systematically investigated. As shown in Fig. 3, the concentrations of Hg^{2+} needed to inhibit 50% of the ATP hydrolysis and proton pumping are ~ 100 and 60 μM , respectively. Furthermore, complete inhibition of both activities was achieved with less than 400 μM Hg^{2+} . Comparison of the data shown in Fig. 3 with the NO_3^- inhibition data previously reported (18) clearly indicates that Hg^{2+} was a more potent inhibitor of the tonoplast H^+ -ATPase. Similar to the effects of nitrate ions, Hg^{2+} also exerted a preferential inhibition of pumping activity. It should be mentioned that *p*-hydroxymercuribenzoate had inhibition properties similar to those of Hg^{2+} for the coupled activities of corn root tonoplast ATPase (data not shown). Finally, because of the presence of no more than 2.5 μM DTE in the final assay medium (resulting from the addition of vesicle suspension obtained by sucrose density gradient centrifugation), the actual concentrations of Hg^{2+} required to produce the observed inhibition

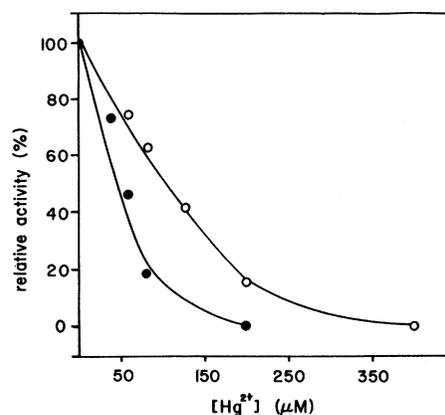


FIG. 3. Differential inhibition of proton pumping and ATP hydrolysis activities of corn root tonoplast ATPase by Hg^{2+} . H^+ -ATPase activities were measured in the presence of 0 to 400 μM Hg^{2+} at 25°C . The tonoplast vesicles, in the assay medium, were incubated with Hg^{2+} for 10 min before the addition of ATP (final concentration, 4 mM). ATP hydrolysis rate was determined by the malachite green-molybdate method. The activities of proton pumping (1.80 $\Delta A/\text{min} \cdot \text{mg}$) and ATP hydrolysis (982 $\text{nmol } P_i/\text{min} \cdot \text{mg}$) obtained in the absence of Hg^{2+} were assigned as 100. Effects on ATP hydrolysis (○) and proton pumping (●) are shown.

may be somewhat lower than those shown in Fig. 3.

Effects of Hg^{2+} on enzyme kinetics. The mode of inhibition by Hg^{2+} of proton pumping and ATP hydrolysis was determined by kinetic analysis at 30°C . As shown in Fig. 4, the apparent patterns of inhibition of the two processes were quite different. In Fig. 4A, Lineweaver-Burk analysis showed that Hg^{2+} inhibition of proton pumping was similar to that of a noncompetitive inhibitor. On the other hand, as shown in Fig. 4B, Hg^{2+} inhibition of ATP hydrolysis was similar to that of an uncompetitive inhibitor. Since Hg^{2+} generally interacts with proteins in a near-covalent manner, the results in Fig. 4 do not necessarily describe the actual mode of Hg^{2+} inhibition, but rather indicate the different effects of the ion on the coupled activities of the tonoplast ATPase. The differential effects of Hg^{2+} on proton pumping and ATP hydrolysis would also be explained by the presence of both cou-

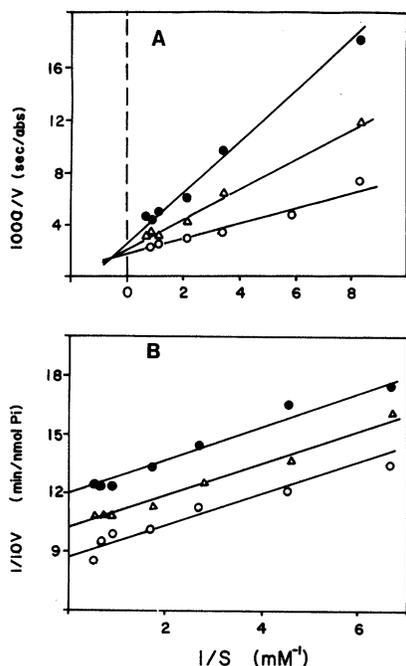


FIG. 4. Effects of Hg^{2+} on enzyme kinetics. Tonoplast vesicles were incubated with Hg^{2+} at the indicated concentrations in the assay medium for 10 min at 30°C before the addition of various concentrations of ATP. (A) Double-reciprocal plots of the initial proton pumping rate versus substrate concentration. (B) Similar plots for ATP hydrolysis. Data were obtained in the presence of 0 (O), 10 (Δ), and 50 (\bullet) μM Hg^{2+} .

pled and uncoupled (damaged) tonoplast vesicles. The damaged vesicles would still contribute to the hydrolysis of ATP but be silent in proton pumping (leaky vesicles). The results mentioned in Fig. 3 could be accounted for if the ATPase of uncoupled vesicles was less sensitive to Hg^{2+} . However, we found that the presence of $0.1 \mu\text{M}$ CCCP, which effectively uncoupled the tonoplast system, increased the rate but did not change the observed sensitivity (Fig. 3) of ATP hydrolysis to Hg^{2+} inhibition. Thus, this alternative was not considered further.

Kinetic origin of Hg^{2+} effect on H^+ pumping. When the kinetics of Hg^{2+} inhibition of proton pumping were further analyzed, the results presented in Table III were obtained. By assigning the value of 100 to the parameters obtained in the absence of

Hg^{2+} or NO_3^- , the relative effects of adding inhibitors became evident. The presence of $4 \mu\text{M}$ Hg^{2+} did not significantly affect any of the parameters. At $50 \mu\text{M}$, Hg^{2+} substantially decreased both proton pumping and ATP hydrolysis rates. However, the membrane proton leakage during the pumping stage, as measured by the value of k_i , appeared to remain unchanged. This result implies that Hg^{2+} does not significantly change the arrangement of phospholipids in the tonoplast membrane under the experimental conditions employed. Thus, the effect of Hg^{2+} on the catalytic step of ATP hydrolysis was most likely the result of a direct interaction with the region involved in the breakdown of the substrate at the catalytic site.

DISCUSSION

Both the tonoplast membrane ATPase and the plasma membrane ATPase act in the transport of protons out of the cytoplasm of plant root cells. The transmembranous proton electrochemical potentials generated are believed to be the primary

TABLE III

EFFECTS OF Hg^{2+} ON THE KINETIC PARAMETERS OF PROTON PUMPING

Experiment ^a	δ_s	k_i	R_0	R_{ATP}	m
Control	100	100	100	100	100
+4 μM Hg^{2+}	104	93	94	97	97
+50 μM Hg^{2+}	44	103	45	74	61
+0.6 mM NO_3^-	57	84	48	99	48

^a The proton pumping activity of the tonoplast vesicles was measured in assay medium containing $20 \mu\text{g}$ protein at 30°C . The concentration of ATP used to initiate proton pumping was 4 mM. The numerical values of the kinetic parameters obtained in the control sample were arbitrarily assigned as 100. To test the effects of Hg^{2+} or NO_3^- , the vesicles were first incubated with the additions at the given concentrations for 10 min before ATP was added. The obtained kinetic parameters were then expressed in relative terms with respect to the control ones. The values of δ_s , k_i , R_0 , R_{ATP} , and m of the control samples are $0.0816 \Delta A$, 0.493 min^{-1} , $40.20 \times 10^{-3} \Delta A/\text{min}$, $28.3 \text{ nmol } P_i/\text{min}$, and $1.43 \times 10^{-3} \Delta A/\text{nmol } P_i$, respectively.

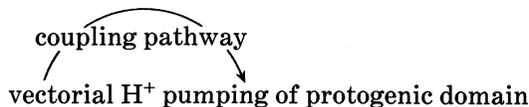
driving force for secondary ion transport processes (3, 27). Despite their similarity in function, the two ATPases nevertheless possess fundamental differences in structure and reaction mechanism. The plasma membrane ATPase contains only one type of polypeptide ($M_r \sim 100,000$) which forms a phosphorylated intermediate during catalysis (28, 29). On the other hand, the tonoplast ATPase is composed of at least three different peptide subunits and does not involve phosphorylation of the enzyme during the reaction cycle (3, 30, 31). However, surprisingly, the catalysis of ATP hydrolysis by both enzymes was temperature dependent, when the data in this report were compared with to previous results from this laboratory (24). As mentioned, the K_m values of both enzyme systems were relatively insensitive to temperature change in the range 10 to 40°C and the forward breakdown of enzyme-substrate complexes leading to the formation of products, ADP and P_i , was about the same (14 to 15 kcal/mol) for both enzymes. The insensitivity of K_m to temperature changes suggests that the rate of substrate binding also increased with temperature to compensate for the faster breakdown (sum of both forward and reversel processes) of the enzyme-substrate complexes.

The inhibition of the tonoplast ATPase activities by Hg^{2+} , in a general sense, may have originated from its interaction with -SH or -S-S- groups of the protein (32). While it was not possible to identify the exact molecular origin of these -SH or -S-S- groups, the results in Figs. 3 and 4 and the effects on the activation energy associated with the rate constant k_2 (Eq. [7]) support

the idea that these groups are likely involved in maintenance of the integrity of the catalytic domain of the ATPase. Since the proton pumping process was inhibited to a greater extent than ATP hydrolysis, these Hg^{2+} -interacting sites should also be involved in the process of energy transduction from the catalytic domain to the protogenic domain.

As described in our previous report (18) and mentioned in this work, the constant k_i in Eq. [1] represents mainly the membrane leakage under the experimental conditions employed. The differential effects cited in Fig. 1, Table II, and Table III may be discussed in relation to this leakage. As shown in Fig. 1, the coupling between ATP hydrolysis and proton pumping, as expressed by m , remains nearly constant between 10 and 20°C. However, the data in Table II indicate that the membrane leakage increases from 0.285 min^{-1} at 10°C to 0.349 min^{-1} at 20°C. Similarly, as shown in Table III, 50 μM Hg^{2+} and 0.6 mM NO_3^- decrease the value of m by 39 and 52%, respectively. Yet, the leakage either remains constant or decreases slightly. Thus, the observed decrease in the coupling by various treatments is not a simple consequence of increasing membrane leakage.

Although there is little information to firmly establish the coupling mechanism between proton pumping and ATP hydrolysis in the tonoplast system, the different temperature sensitivities and the differential inhibition patterns of Hg^{2+} and NO_3^- suggest that these two events are only indirectly linked. To qualitatively describe these effects, a minimal working mechanism may be used as follows:



Here E, E^* , and \cdot represent free enzyme, activated enzyme, and noncovalent interaction, respectively. In essence, this reaction scheme is similar to the indirect coupling mechanism proposed for mitochon-

drial ATPase (33). Since there is no phosphorylated intermediate in the tonoplast ATPase, the energy released from the hydrolysis of ATP could first be stored in the protein structure (E^*), e.g., comfor-

mational strains, before the release of ADP and P_i . The activated enzyme can then transmit this energy to the protogenic domain through a series of molecular events to induce the vectorial proton movements. The step linking the ATP hydrolysis and proton pumping may involve the conformational interactions among subunits of the ATPase and other membrane components. According to this hypothesis, the interaction site of Hg^{2+} should be involved in the catalytic breakdown of $ADP \cdot E^* \cdot P_i$ as well as the initial step(s) of the coupling pathway. On the other hand, nitrate ions exert their effects by interacting mainly with the protogenic domain or latter steps of the pathway which are closer to the activation of the protogenic domain. It should be mentioned that the exact order of releasing ADP and P_i from the enzyme was not addressed in the proposed reaction scheme. Experimental evidence either supporting or disproving this hypothesis is our current research objective.

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