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POLY(L-ASPARTATE) INHIBITS THE PROTON PUMPING OF TONOPLAST H⁺-ATPASE FROM CORN ROOTS

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ABSTRACT: Pre-incubation of the tonoplast vesicles from corn roots with poly(L-aspartate) (MW 14,000 Da) significantly inhibited the proton pumping associated with the ATP hydrolysis activity of the H⁺-ATPase. However, this polyanion only slightly affected the hydrolytic activity. The potency of poly(L-aspartate) to inhibit the proton pumping increased as the concentration of KCl or ionic strength decreased suggesting an electrostatic origin for the inhibition. When poly(L-aspartate) was added after the initiation of the ATPase activities, no significant effect on proton pumping was noted. The results suggest that certain -NH³⁺ groups on the cytoplasmic surface of the tonoplast membrane may electrostatically interact with poly(L-aspartate) to produce observed inhibition. Furthermore, the accessibility of these -NH³⁺ groups to poly(L-aspartate), appears to be regulated by the functional state of the H⁺-ATPase.

INTRODUCTION

Plant root cells contain tonoplast (v-type) H⁺-ATPase, which catalyzes the hydrolysis of Mg-ATP to support vectorial movements of protons across the vacuolar membranes (Sze, 1984). The resulting proton electrochemical potentials, $\Delta\mu_{\text{H}^+}$, serves as the driving force for the redistribution of nutrients and metabolites

between the cytoplasm and vacuole. Tonoplast H⁺-ATPase contains many different subunits and does not form a phosphorylated intermediate in its reaction pathway (Nelson and Taiz, 1989; Sze, 1984). The exact mechanism by which proton pumping links to ATP hydrolysis, remains to be established. However, to explain differential sensitivity of coupled activities to many treatments, we have proposed an indirect coupling mechanism (Tu et al, 1990, 1992).

In our previous study (Tu et al., 1990), we have shown that the -NH³⁺ groups on the surface of corn root tonoplast membranes, originating from phospholipids and proteins, can serve as the anchoring sites for derivatised fluorescamine structures to perturb the activities of the tonoplast H⁺-ATPase. However, experiments demonstrating the possible direct involvement of these -NH³⁺ groups in the control or regulation of the H⁺-ATPase activities, to our knowledge, has never been attempted. In our current work, we found that poly(L-aspartate), a highly negatively charged and membrane impermeable polypeptide, preferentially inhibited the proton pumping activity. The inhibitory efficacy of the polyanion increased as the ionic strength decreased. The results suggest that the electrostatic interaction between poly(L-aspartate) and clustered positive charges of the tonoplast membranes could change the efficiency of utilizing ATP hydrolysis energy to drive transmembrane proton movement.

MATERIAL AND METHODS

Isolation of Tonoplast Vesicles

Corn (*Zea mays* L. Cv. FRB 73) seeds were germinated on filter paper moistened with 0.1 mM CaCl₂ for three days at 28°C and harvested as previously described (Tu et al., 1987). Approximately 60 g of excised roots were homogenized with a mortar and pestle at 4°C in the isolation medium containing 0.3M sucrose, 5 mM ethyleneglycol-bis(β-aminoethylether-N,N'-tetraacetic acid) (EGTA), 5 mM β-mercaptoethanol, 5 mM dithiothreitol (DTT), and 0.1 m HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.7 (adjusted at 4°C). After being filtered through four layers of cheesecloth, the homogenate was subjected to differential centrifugation, 6,000 g for 20 min and 80,000 g for 40 min, to obtain a microsomal pellet. The microsomal pellet was suspended in isolation medium and centrifuged again at 80,000 g for 40 min. The washed microsomes were suspended in the isolation medium and layered over a 15 to 45% (w/w)

linear sucrose gradient buffered with 5 mM HEPES (titrated to pH 7.7 with MES, 2-[N-morpholino]ethanesulfonic acid) plus 1 mM DTT and centrifuged at 4°C for 18 h at 84,000 g. The tonoplast fractions between 19 to 23% sucrose were pooled and used for experiments. The protein concentration in the membrane was determined by a modified Lowry method using bovine serum albumin (BSA) as the standard (Bensadoun and Weinstein, 1976).

Media Used for the Measurement of Coupled Activities

The coupled activities of the tonoplast H⁺-ATPase were measured in media of different compositions. The standard assay medium contained 75 mM KCl, 17.5 mM MES (titrated to pH 6.45 with BTP, bis-tris-propane), 2.5 mM MgSO₄, 0.1 mM sodium vanadate, 7.5 mM acridine orange, and 5 mM glucose. To test the effects of changing K-salt concentration, isotonic solutions were used. The solutions contained the identical components described for the standard assay medium except that the 150 mM of KCl was replaced by various concentrations of sucrose. The total osmolarity of the replacement solutes was kept at 300 mM. Since osmotic pressure is determined by the number of particles in solution, K₂SO₄ and KCl are three and two times, respectively, more effective than sucrose in producing the pressure. The exact composition of the assay solutions in different experiments is described in the figure legends.

ATP Hydrolysis Measurement

ATP hydrolysis catalyzed by the H⁺-ATPase was assayed by the direct measurement of inorganic phosphate released. After the tonoplast vesicles were incubated in 2.2 mL of assay solutions for 10 to 20 min at room temperature, the reaction was initiated by the addition of 20 μL of 0.2M ATP. At least 90% of the total ATP hydrolysis was catalyzed by the tonoplast H⁺-ATPase based on the sensitivity to nitrate, bafilomycin A₁, vanadate, azide, and molybdate. The amount of phosphate released was determined by the malachite green-molybdate assay as previously described (Tu et al., 1987). The ATP hydrolysis rate remained constant for about 15 to 20 min under the experimental conditions. Thus, the average rate over a time period of 10 min was used to represent the initial hydrolysis rate (R_{ATP}) of tonoplast H⁺-ATPase after subtracting the ATP hydrolysis in the same assay media with the addition of 10 nM bafilomycin A₁.

Proton Pumping and Membrane Leakage Measurement

ATP-driven H⁺-transport was followed by changes in absorbance of acridine orange at 492.5 nm. Typically, 200 μL of vesicles were diluted to 2.2 mL in the

same solutions used for ATP hydrolysis measurements. Assays were conducted with a Beckman DU-70 Spectrophotometer interfaced to a personal computer. The digitized data (sampling time = 1 s) were then analyzed according to a steady-state kinetic model developed in this laboratory (Tu et al., 1987). Briefly, the model assumes that the build-up of gradient induces a proton leakage and gradually decreases the net ATP-hydrolysis-induced proton transport. When the net transport rate approaches zero, i.e. when leakage equals initial pumping rate, a steady-state condition is reached. Mathematically, the ATP-supported proton pumping can be represented by the following equations:

$$\text{net proton pumping rate: } \quad d\partial/dt = R_o - k_1\partial \quad [1]$$

$$\text{steady-state approximation: } \quad R_o = k_1\partial_s \quad [2]$$

$$\text{time-course of pumping: } \quad \ln(1 - \partial/\partial_s) = k_1t \quad [3]$$

where: ∂ , ∂_s , R_o , and k_1 represent the extent of proton transport, the extent of transport at steady state, the initial proton pumping rate, and pumping inhibition constant (proton leakage with ATPase activity on), respectively. The initial ATP hydrolysis rate (R_{ATP}) and initial pumping rate (R_o) are related by:

$$R_{ATP} = m R_o \quad [4]$$

in which m is the stoichiometric ratio or the extent of coupling between proton pumping and ATP hydrolysis. A decrease in the value of “ m ” reflects a decrease in the coupling between ATP hydrolysis and proton pumping resulting in a decrease in net proton uptake by the tonoplast vesicles. We have previously suggested that this decrease reflects an altered and weakened conformational coupling between ATP hydrolytic domain and proton pumping domain in the enzyme structure (Tu et al., 1990).

A rapid quenching of ATP hydrolysis can discharge the proton gradient ∂_s by leakage through the de-energized membrane. Hexokinase-induced phosphorylation of glucose may be utilized to rapidly exhaust added ATP. The leakage of protons follows a simple first-order decay:

$$\ln(\partial/\partial_s) = -k_2t \quad [5]$$

Here, ∂ represents the residual gradient at time t after the quenching. It should be noted that k_2 represents the proton leakage constant of de-energized (ATP

hydrolysis silent) membrane. Because the structural features of the membrane need not be the same under different energetic conditions, it should not be surprising that the k_1 and k_2 may have different values.

RESULTS AND DISCUSSION

Poly(L-aspartate) Inhibition of the Proton Pumping in Tonoplast Vesicles

The proton pumping associated with the ATP hydrolysis in the tonoplast vesicles can normally be detected by the quenching of the absorbance of acridine orange. As shown in Figure 1, the inclusion of poly(L-aspartate) before the addition of ATP significantly decreased the initial rate and the extent (at steady-state) of proton transport. According to the steady-state model of proton pumping, there are three possible reasons for the inhibition described in Figure 1. First, the inhibition may result from a decrease in ATP hydrolysis rate (R_{ATP}) without any direct effect on the pumping process or the coupling mechanism. Second, the interaction between poly(L-aspartate) and the membrane may significantly increase the membrane proton leakage (k_1 and k_2) and thus decreased the net proton pumping rate. Third, the interaction between the membrane and the polyanion may affect the integrity of pumping mechanism to reduce its efficiency ("m") in utilizing the energy released from ATP hydrolysis to support proton pumping. Further experiments were performed to identify the origin of observed poly(L-aspartate) inhibition.

Kinetic Origin of Poly(L-aspartate) Inhibition on Proton Pumping

We tested the responses of coupled activities of tonoplast H^+ -ATPase to the presence of poly(L-aspartate) at a concentration of 10 mg/mL. The results (Table 1) clearly indicated that poly(L-aspartate) did not have significant effect on the hydrolytic activity (R_{ATP}) of the ATPase in the medium with a relatively low ionic strength. The effects of different concentrations of poly(L-aspartate) to ATP hydrolysis were further tested in solutions of similar osmolarity but with different ionic strength (Figure 2). The data showed that the interactions between poly(L-aspartate) and the membrane only slightly affected the hydrolysis of ATP under a wide-range of experimental conditions. Analysis of the time courses of proton pumping under different conditions, including the rapid quenching by glucose and hexokinase, revealed that both k_1 and k_2 (Table 1) were not increased by poly(L-aspartate). Thus, the interaction did not change the proton leakage of the tonoplast

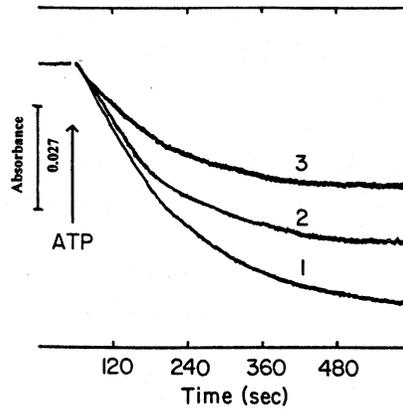


Figure 1. *Inhibition of Proton Pumping by Poly(L-aspartate).* Tonoplast vesicles (67 μg protein) in an aliquot of 1.0 ml containing 30 mM KCl, 240 mM sucrose and other components mentioned in Material and Methods were incubated with different concentrations of poly(L-aspartate) for 10 min at 22 $^{\circ}\text{C}$. Substrate ATP was then added to start the proton pumping which was monitored spectroscopically at 492.5 nm. The time courses 1, 2, and 3 were obtained with poly(L-aspartate) concentrations as 0, 10, and 20 $\mu\text{g ml}^{-1}$, respectively.

membranes. Since the initial proton pumping rate (R_0) decreased as the concentration of poly(L-aspartate) increased, the interaction is most likely due to a gradual decrease in the coupling ("m") between the hydrolytic activity and the proton pumping process.

Nature of the Interaction Between Poly(L-aspartate) and the Membrane

Biomembranes normally carry net negative charges at neutral pH. However, this does not preclude the possible existence of regions or domains rich in positive charges from membrane proteins and/or phospholipids. In a previous study (Tu et al., 1990), we demonstrated that the cytoplasmic surface of corn root tonoplast membrane contained primary amino groups which could react with fluorescamine, a primary amine specific fluorescent reagent. The covalent modification resulted in the insertion of derivatized fluorescamine functionality which interfered with the

Table I. Effects of Poly(L-aspartate) on Tonoplast H⁺-ATPase^{1,2}.

Poly(L-aspartate) $\mu\text{g ml}^{-1}$	R_{ATP} (%)	R_0 (%)	k_1 (%)	k_2 (%)	m (%)
0	100	100	100	100	100
10	94	67	98	95	71

1. The tonoplast vesicles were treated with poly(L-aspartate) as described in Figure 1 except the inclusion of 5 mM glucose in the assaying solution. After the attainment of steady-state proton pumping level, 20 units (1 unit = 1 μmol of glucose phosphorylated per min under experimental conditions) of hexokinase were added to rapidly deplete ATP and thus quenched proton pumping.

2. In the absence of poly(L-aspartate), the numerical values of R_{ATP} , R_0 , k_1 , k_2 , and m were 45.6 nmol P_i released $\text{min}^{-1} \text{mg}^{-1}$, 0.148 absorbance change $\text{min}^{-1} \text{mg}^{-1}$, 0.489 min^{-1} , and 0.973 min^{-1} , and 3.2×10^{-3} absorbance change (nmol P_i released) $^{-1}$ respectively. These values were assigned as 100%. The data shown represent averages of three independent experiments with relative errors no greater than $\pm 5\%$.

coupling between the hydrolytic and pumping activities of corn root tonoplast H⁺-ATPase. The primary amine groups on the surfaces of tonoplast membrane should be protonated at or near neutral pH. Presumably, the positive charges in a domain may exhibit net attractive interaction with poly(L-aspartate) if the repulsive interaction with the surrounding negative charges is compensated. The possible electrostatic nature of the interaction was tested by investigating the relationship between the inhibition efficacy of poly(L-aspartate) and the concentration of KCl in the assay solution.

It is known that chloride ions can activate the enzymatic activities of plant tonoplast H⁺-ATPase (Sze, 1984). We found that at pH 6.45 and with a total osmolarity as 300 mM (sucrose plus KCl), the requirement for chloride ions to

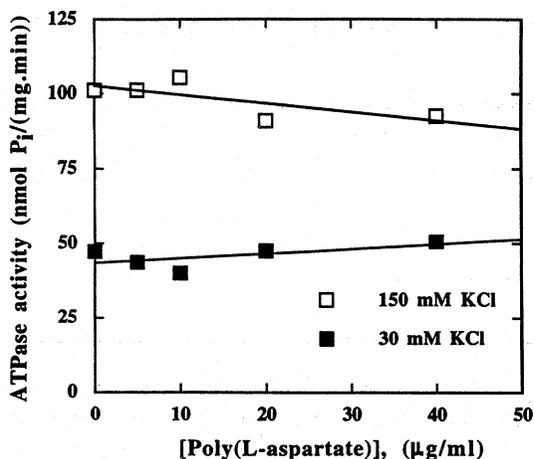


Figure 2. Effects of Poly(L-aspartate) on the Hydrolytic Activity of the Tonoplast Membrane Vesicles. Tonoplast vesicles (67 µg of protein) were incubated with indicated concentration of poly-(L-aspartate) in standard solutions for assaying proton pumping but with either 30 mM KCl and 240 mM sucrose (filled squares) or 150 mM KCl (open squares) for 10 min at 22 °C before the addition of ATP. The hydrolysis of ATP, measured as the release of inorganic phosphate, was determined as described in text.

stimulate ATP hydrolysis was quite different from that associated with proton pumping (Figure 3). As shown, ATP hydrolysis and proton pumping reached full stimulation levels with chloride ion concentrations close to 50 and 100 mM, respectively. Also, within the tested concentration range of KCl, the extent of stimulation of proton pumping was considerably higher than that associated with ATP hydrolysis. The results were consistent with the notion that proton pumping and ATP hydrolysis should have different molecular pathways which were coupled together indirectly through conformational interactions (Tin et al., 1990).

With tonoplast vesicles pre-treated with poly(L-aspartate), the chloride stimulation of ATP hydrolysis activity was only slightly decreased (less than 10%) over a wide range of KCl concentration (Figure 4). However, as shown, poly(L-aspartate) appeared to have a different effect over the proton pumping activity. As shown, the extent of inhibition to the proton pumping increased as the concentration of KCl

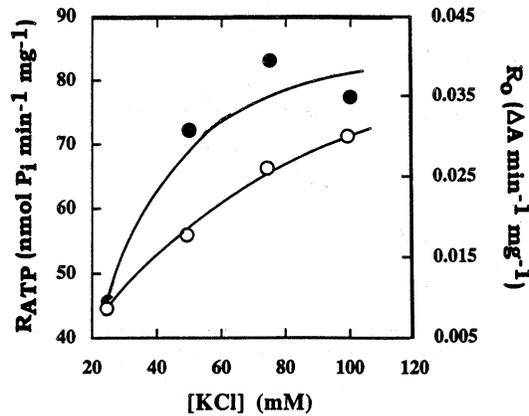


Figure 3. *Effects of Changing [KCl] on the Inhibition Potency of Poly(L-aspartate) to Coupled H⁺-ATPase Activities..* Tonoplast vesicles (67 μg) were incubated in 1 ml of media containing 20 mM MES-BTP pH 6.45, 1 mM EGTA, 2.5 mM MgSO₄, 10 μM acridine orange, 0 to 100 mM KCl, and 300 to 100 mM sucrose for 10 min at 22 °C before the addition of 2 mM ATP. The total osmolality (sucrose and KCl) was kept as 300 mM. The ATP hydrolysis rate, R_{ATP} (solid circles) and proton pumping rate, R_o (open circles), were then determined.

decreased. The results indicated that the observed inhibition of proton pumping might originate from at least two different sources. First, the [KCl]-independent decrease in ATP hydrolysis caused by poly(L-aspartate), should produce an inhibition, about 10%, over proton pumping. The second process affecting only proton pumping should involve an electrostatic interaction between poly(L-aspartate) and the tonoplast membranes because the inhibition decreased as [KCl] increased. A possible mode of interaction would involve salt bridge formation between -NH³⁺ groups of tonoplast membranes and -COO⁻ groups of poly(L-aspartate).

It has been reported that the interaction between poly(L-lysine), a positively charged polypeptide, and red blood cell membrane can induce a lateral clustering of negatively charged membrane proteins (e.g. band 3 protein) and phospholipids (Clague and Cherry, 1989) resulting in an aggregation of red blood cells. A similar

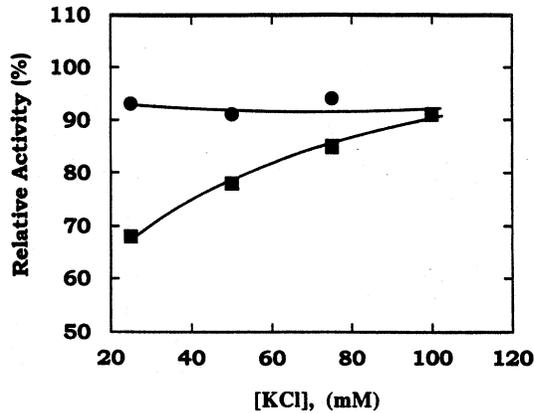


Figure 4. *Effects of Changing [KCl] on the Inhibition Potency of Poly(L-aspartate).* Tonoplast vesicles were treated as described in Fig. 3 except 10 μ g poly(L-aspartate) was added during incubation. The obtained R_{ATP} (solid circles) and R_o (solid squares) were plotted relative to control values described in Fig. 3. The data shown represent averages of two independent experiments with errors no greater than $\pm 5\%$.

aggregation of plant tonoplast vesicles caused by poly(L-lysine) was recently reported by our laboratory (Tu et al., 1995). Those studies indicated that the binding of polypeptides with multiple positive charges to the membrane could induce lateral movement of negatively charged membrane components. Using turbidity measurement, we did not find any evidence for poly(L-aspartate) induced aggregation of tonoplast vesicles. Presumably, the overall negative charges of the membrane surfaces prevented close contact of vesicles. However, the possibility of inducing lateral clustering of surface $-NH_3^+$ groups within limited regions can not be ignored. Thus, observed inhibition to the proton pumping process could arise from a lateral movement of certain $-NH_3^+$ groups from their normal locations in the membrane. The slight inhibition to ATP hydrolysis might also be expected if poly(L-aspartate) could interact with some of the $-NH_3^+$ groups involved in the binding of negatively charged ATP.

The involvement of -NH_3^+ and -COO^- groups of membrane components in the architecture of the proton conduction pathway has been suggested for bacteriorhodopsin, a transmembrane proton pump (Henderson et al., 1990; Chou, 1993). The arrangement of charged groups may also influence the proton movement along the membrane surface (Tocanne and Teissie, 1990). Thus, if some of these -NH_3^+ groups either are directly involved in the proton pumping or indirectly affecting the integrity of the specific pumping pathway, then the interaction with poly(L-aspartate) would be expected to perturb the proton pumping supported by ATP hydrolysis.

In a separate experiment, we tested the effects of changing the order of addition for ATP and poly(L-aspartate) to the kinetics of the proton pumping. Surprisingly, we found that once the proton pumping process was activated by ATP, further addition of poly(L-aspartate) exerted no detectable effects on proton pumping, i.e., no changes in the time course of proton pumping (data not shown). This result suggested that the functionally effective -NH_3^+ groups became inaccessible to poly(L-aspartate) once proton pumping was activated by ATP. Like those found in other eukaryotic systems (Forgac, 1989), the multimeric plant tonoplast H^+ -ATPase is composed of a peripheral (cytoplasmic) sector containing the ATP hydrolytic site and a membrane integral sector that forms the proton transport pathways (Sze et al., 1992). Thus, changes in the accessibility of membrane groups to poly(L-aspartate) induced by different membrane energetic status could reflect the dynamic conformation/configuration relationship between the two sectors as previously proposed in an indirect coupling mechanism (Tu et al., 1992).

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