An Essential Arginyl Residue in the Tonoplast Pyrophosphatase from Etiolated Mung Bean Seedlings

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ABSTRACT

Tonoplast membrane of etiolated mung bean (Vigna radiata, L.) seedlings contained H⁺-translocating pyrophosphatase (PPase). Modification of tonoplast vesicles and partially purified PPase from etiolated mung bean seedlings with arginine-specific reagents, phenylglyoxal (PGO) and 2,3-butanedione (BD), resulted in a marked decline in H⁺-translocating PPase activity. The half-maximal inhibition was brought about by 20 millimolar PGO and 50 millimolar BD for membrane bound and 1.5 millimolar PGO and 5.0 millimolar BD for soluble PPase, respectively. The substrate, Mg²⁺-pyrophosphate, provided partial protection against inactivation by these reagents. Loss of activity of partially purified PPase followed pseudo-first order kinetics. The double logarithm plots of pseudo-first order rate constant versus reagent concentrations gave slopes of 0.88 (PGO) and 0.90 (BD), respectively, suggesting that the inactivation may possibly result from reaction of at least one arginyl residue at the active site of H⁺-translocating PPase.

The H⁺-translocating enzymes of the tonoplast play an essential role in the maintenance and regulation of cell turgor and in the storage and transport of ions and metabolites (26). A line of evidence has revealed the presence of H⁺-translocating ATPase and PPase on the tonoplast membrane (12). Recent reports demonstrated that the tonoplast isolated PPase and ATPase activities are distinct on the membrane (12). The tonoplast ATPase from several sources has been purified and characterized by many workers (26, 29). However, information on the structure and function of tonoplast PPase is still limited.

Group-specific chemical probes, which react covalently and cause changes in enzymatic activity, have been widely used to provide more insight into structure and function at the active site (27). Many essential amino acid residues involved in the active site of yeast and Escherichia coli PPase were demonstrated (3, 11, 15, 23). However, the amino acid residues involved in the active of tonoplast PPase of higher plants still remains to be elucidated (8).

Several reports using guanidium-specific modifiers (either PGO or BD), have revealed the involvement of arginine residues in the binding of anionic substrate or cofactor at active site (22). In this manner, an essential arginyl residue was found for enzymes such as ATPases which hydrolyze the anionic substrate ATP (8, 14, 19). The physiological substrate of PPase, inorganic pyrophosphate, is also anionic in nature similar to ATP. In this paper we determine, using arginine modifier PGO and BD, whether the arginine residue(s) is involved in tonoplast PPase activity of higher plants. The results indicate that PPase in the vacuolar membrane of etiolated mung bean seedlings contains at least one arginyl residue essential for enzymatic activity and proton translocation.

MATERIALS AND METHODS

Plant Material

Seeds of Vigna radiata L. (mung bean), purchased from a local market, were soaked for 3 h in tapwater and then germinated at room temperature in the dark using a commercial seedling incubator. The hypocotyls of 4-d-old etiolated seedlings were excised, chilled on ice, and then used as starting materials.

Membrane Preparation

Tonoplast vesicles were prepared from etiolated seedlings as previously described (29). All procedures were carried out at 4°C. The tonoplast vesicles were isolated at the interface of a 0 to 4% (w/v) dextran (79,000) gradient following centrifugation of the microsomal fraction at 70,000g for 2 h.

Partial Purification of PPase

Soluble tonoplast PPase was prepared from ressealed tonoplast vesicles depleted of ATPase using detergent solubilization. The tonoplast vesicles were pretreated with low salt at 4°C for 30 min to deplete peripheral ATPase (16) in a medium containing 0.1 mM Mops-KOH (pH 7.9), 15% (w/v) glycerol and final membrane protein concentration 1 mg/mL. After incubation, the tonoplast vesicles were then centrifuged at 120,000g for 1 h and the pellet was resuspended in the buffer containing 0.1 mM Mops-KOH (pH 7.9), 30% (w/v) glycerol, and a protein concentration of 2 mg/mL. OG was added dropwise from a 200 mM stock solution to a final concentration of 20 mM. After 30 min incubation at 4°C, OG-treated tonoplast vesicles were centrifuged at 120,000g for 1 h. An aliquot (0.8 mL) of supernatant containing PPase activity was
then layered onto 4 mL 7 to 27% linear sucrose gradient containing 10 mM Tris-Cl (pH 7.3), 0.25 m sorbitol, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 15% (w/v) glycerol, and 7 to 27% sucrose. The gradient was centrifuged at 200,000g for 5 h. Aliquots (0.28 mL) were collected from the bottom of gradient and the fraction with highest PPase activity was used for this study.

**Treatment of PPase with BD and PGO**

The tonoplast vesicles or partially purified PPase were incubated with PGO in a medium containing 50 mM Mops-KOH (pH 7.9) at 37°C for the time periods indicated. Similarly, modification of tonoplast vesicles and soluble PPase with BD was conducted in 50 mM borate buffer (pH 7.9). MgCl2 and protection agents were added (when present) as described in figure legends. The final protein concentration of tonoplast vesicles was 0.8 to 1.0 mg/mL while that of partially purified PPase, 0.4 to 0.6 mg/mL.

**Enzyme Assay and Protein Determination**

PPase activities of the modified and control membrane preparations were determined by measuring the released Pi and PPI. Since 2 mol of Pi are released from hydrolysis of 1 mol PPI, we have expressed the activity as moles PPI consumed. Aliquots of resealed vesicles or soluble PPase were assayed in a 1.0 mL volume containing 25 mM Mops-KOH (pH 7.9), 3 mM MgSO4, 50 mM KCl, 3 mM K3PPI, 16 to 20 μg/mL membrane protein or 8 to 12 μg/mL partially purified PPase, with 0.5 mM NaN3, 0.1 mM Na-orthovanadate, 50 mM KNO3, and 0.1 mM ammonium molybdate to inhibit the ATPase of mitochondria, plasma membrane and tonoplast, and other possible acid phosphatase, respectively (26, 29).

Assays were carried out at 37°C for 15 to 30 min and terminated by adding a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) SDS and 0.02% (w/v) 1-aminoo-2-naphthol-4 sulfonic acid. The released Pi was determined spectrophotometrically (SLM-AMINCO U2000) as described elsewhere (29).

Protein concentration was measured according to the modified Lowry method (29) using BSA as the standard.

**Measurement of Proton Translocation**

Proton translocation was measured as fluorescence quenching of acridine orange (excitation wavelength 495 nm, emission wavelength 530 nm) with a Hitachi F-4000 fluorescence spectrophotometer. The reaction mixture contained 5 mM Mops-KOH (pH 7.9), 250 mM sorbitol, 3 mM MgSO4, 50 mM KCl, 3 mM PPI, 0.1 mM Na-orthovanadate, 50 mM KNO3, 0.1 mM ammonium molybdate, 0.5 mM NaN3, 5 μM acridine orange, and 10 to 20 μg/mL membrane protein. The fluorescence quenching was initiated by adding 3 mM MgSO4. The ionophore gramicidin (2 μg/mL) was added at the end of each assay.

**Kinetic Analysis**

The t1/2 values, time required for 50% inhibition of activity, at various concentration of modifiers were measured accord-

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![Graph](https://via.placeholder.com/150)

**Figure 1.** Inactivation of H+-PPase activity as a function of concentration of PGO and BD. Membrane protein (1.0 mg/mL) and partially purified PPase (0.6 mg/mL) were incubated at 37°C in Mops-KOH (pH 7.9) (PGO) or borate-KOH, pH 7.9 (BD) with various concentrations of modifiers as indicated. The incubation periods for tonoplast vesicles were 30 min (BD) and 15 min (PGO); for partially purified PPase, 15 min (BD) and 5 min (PGO), respectively. After treatments, aliquots (20 μL) were removed and assayed for pyrophosphatase activities and H+-translocation, expression as a percentage of residual activities with modifiers. The assay medium for pyrophosphatase hydrolysis contained: 25 mM Mops-KOH (pH 7.9), 3 mM MgSO4, 50 mM KCl, 3 mM potassium pyrophosphate, 0.1 mM Na orthovanadate, 50 mM KNO3, 0.1 mM ammonium molybdate, 0.5 mM NaN3 and 16 to 20 μg/mL membrane protein or 8 to 12 μg/mL partially purified PPase. The control activities were 7.48 and 30.2 μmol PPI consumed/mg protein/h for membrane bound and soluble PPase, respectively. The reaction conditions for H+-translocation were described as shown in Figure 2. (A) PGO; (B) BD; (C―C), H+-translocation; (■―■), pyrophosphate hydrolysis activity of membrane bound PPase; (△―△), pyrophosphate hydrolysis activity of partially purified PPase.
ing to semi-logarithmic plots of percent residual activity versus time as described by Levy et al. (17). The reaction order \( (n) \) with respect to modifier was determined from double-log plots of \( 1000/t_{1/2} \) versus concentration of modifiers (17). The \( K_i \), dissociation constant for the modifier and \( T_{1/2} \), \( t_{1/2} \) at excess concentration of modifiers, were calculated from the plots of \( t_{1/2} \) as a function of the reciprocal of modifier concentration according to Carlson (4).

**Chemicals**

PGO and BD were purchased from Sigma. ATP was obtained from Merck. All other chemicals were of analytic grade and used without further purification.

**RESULTS AND DISCUSSION**

**Inactivation of PPase by PGO and BD**

The effects of PGO and BD on the enzymatic activity of partially purified and membrane bound tonoplast \( \text{H}^+\)-PPase were examined. Incubation of the vesicles and partially purified protein with various concentrations of PGO and BD resulted in a loss of enzymatic activity of the \( \text{H}^+\)-PPase (Fig. 1 [○—○; △—△]). The degree of inactivation of \( \text{H}^+\)-PPase activity was at least twofold higher by PGO than by BD as judged from their concentration dependence. For instance, 20 mM PGO inhibited approximately 50% of membrane bound \( \text{H}^+\)-PPase activity. However, more than 50 mM of BD was required to cause similar loss of enzymatic activity. The greater hydrophobicity of PGO might offer the advantage for the modifier to access the active site. Furthermore, the \( f_{50} \) values are approximately 10-fold higher for the membrane bound than partially purified tonoplast PPase. Recently, Bai-kov et al. (1) demonstrated the differential reactivities of sulphydryl reagents to free and membrane bound inorganic PPase from submitochondria particles of rat liver. They speculated that the active site of PPase was deeply embedded in the membrane. The same conclusion could be drawn from the sensitivities to BD and PGO that inorganic PPase of plant vacuoles might be also submerged in the membrane. Thus, PGO was used preferentially as the selective modifier of arginyl residues in this report.

In parallel experiments, PGO and BD inhibited the PPase-supported \( \text{H}^+\)-translocation across the tonoplast vesicle as determined by fluorescence quenching of \( \Delta \text{pH} \) probe, acridine orange (Fig. 2). The concentration dependence of inactivation of \( \text{H}^+\)-translocation of PGO and BD coincides with that of enzymatic activity (Fig. 1 [Ο——Ο]), implying the possible involvement of arginyl residues in PPase activity as well as its associated \( \text{H}^+\)-translocation.

**Kinetic Analysis**

Inactivation kinetic studies of partially purified PPase activity as a function of time revealed to be pseudo-first-order according to semilogarithmic plots of percent residual activity versus time (Fig. 3). The reaction order with respect to PGO and BD was determined from double-log plots of \( 1000/t_{1/2} \) as function of modifier concentrations. Accordingly, straight lines were obtained with slopes equal to the number of molecules of modifier labeled at each active site of PPase. Slopes of 0.90 and 0.88 were determined for BD and PGO as modifier, respectively (insets of Fig. 3). It is likely that the inactivation of \( \text{H}^+\)-translocating PPase was primarily due to the modification of at least one arginyl residue at active site of enzyme by PGO and BD. It might be possible that more than one arginyl group exists at the active site. However, this possibility seems unlikely since the semi-logarithm of percent residual activity shows a simple exponential function of incubation time.

Furthermore, a plot of \( tT_{1/2} \) versus reciprocal of modifier concentration gives \( K_i \) and minimum \( t_{1/2} \) (4, 6). The minimum \( t_{1/2} \) values \((T)\) of 3.0 min and 4.0 min at excess concent-
The effects of $V_{\text{max}}$ and $K_m$ by PGO were also investigated (Fig. 4) after inactivating PPase with or without 5.0 mM PGO for 6 min at 37°C. In the control, the $K_m$ for PPase was 2.5 mM and $V_{\text{max}}$ 57.1 μmol PPI consumed/mg protein/h. In the presence of PGO the $V_{\text{max}}$ was not changed, while the $K_m$ increased three-fold to 7.7 mM. The mode of inhibition of partially purified PPase by PGO is competitive as determined by its effect on $K_m$ but not $V_{\text{max}}$. Thus, it is confirmed kinetically that the modified arginine residue locates at active site.

Protection against PGO Inactivation

Protection against modifier-induced inactivation was studied. The PPase was preincubated 5 min with protectors in the presence and absence of 5.0 mM MgCl$_2$. After addition of PGO to a final concentration of 5.0 mM for 12 min at 37°C, the enzymatic activity of PPase was determined as mentioned above. Table I summarizes the protection effects of its substrate (pyrophosphate), inorganic phosphate, and substrate analogs, such as $p$-nitrophenyl phosphate, phosphoserine, phosphothreonine, and imidodiphosphate. Substantial protection was provided by the physiological substrate of the enzyme, pyrophosphate, with 5 mM MgCl$_2$. The presence of Mg$^{2+}$ is absolutely crucial for the partial protection against modifiers. In its absence, pyrophosphate exerted almost no protection effect. It is believed that native substrate is Mg$^{2+}$-pyrophosphate. However, we cannot exclude the possibility that Mg$^{2+}$ induces the conformational change of PPase resulting in the less accessibility of $\alpha$-dicarbonyl reagents. In addition, inorganic phosphate has a fairly reasonable partial protection (42.9%). Inorganic phosphate is the product of PPase. It can also bind to the active site and exert partial protection effect. Substrate analogs such as $p$-nitrophenyl phosphate and imidodiphosphate provided 51.8% and 60.6% protection, respectively. However, phospho-derivatives of amino acid residues like phosphoserine and phosphothreonine had negligible protection effect. The stereohindrance of amino acid moieties might make them less accessible to the active site of PPase.

ATP, ADP, and AMP contain a moiety similar to pyrophosphate at one end of the molecule. It is interesting to investigate their possible protection against PGO at native site of PPase. The hydrolytic activities of ATP, ADP, and AMP were low compared with pyrophosphate. ATP offered the protection against PGO as well as pyrophosphate. However, ADP and AMP had hardly any protection effects. Since ATPase and PPase use the anionic substrates, the possible similarity at active site of ATPase and PPase deserves special attention. Several studies revealed that at least one arginyl residue locates at the catalytic site of ATPase from various sources (6, 19, 24). For ATPase, the degree of protection of arginyl residue against $\alpha$-dicarbonyl reagents highly depended on the number of phosphate, as well as on the species of nucleotide moieties (6). It is believed (8) the arginyl residues in question bridged the $\alpha$- and $\beta$-phosphate of ATP at the ATP binding site of these ATPases. In our study, ATP, which contains three phosphate moieties, had a reasonable protec-
tion effect for PPase. We speculate that active pocket of PPase has room enough only for the binding of $\beta$- and $\gamma$-phosphate of ATP. The adenine and ribose moiety of ATP were left out of the active cleft of enzyme. Thus, ATP still offered protection while nucleotides such as ADP and AMP, with relatively higher stereohindrance at active cleft, had very little protection effect.

From the protection studies above, it is confirmed that the essential arginyl residues are in the active site of PPase. However, there might be other alternatives to explain the protection effect against modifiers. For instance, protectors could directly block the modification reaction with essential arginine. No spectrophotometrical evidence indicates the possibility of complex formation between protectors and modifiers (data not shown). On the other hand, we cannot exclude the possibility that the presence of protectors could cause a conformational change which would make the arginine less susceptible to attack. The later possibility requires further elucidation.

PPases from various sources were purified and molecular masses of their subunits were measured ranging from 20 to 70 kD (3, 11, 18). However, little is known on amino acid composition, sequence, and possible homology in active domain. Even under such conditions, many reports demonstrated by using chemical modifiers that PPases from these sources contain essential lysyl (15, 25), histyl (23), tyrosyl (2, 10, 20, 23), sulfhydryl (1, 3, 21, 28), arginyl (5, 9), methionine (30), and carboxylates (9) residues. The lysine residue was supposed to be involved in the protonation in the process of enzymatic activity and in the stabilization of transition state (15). The histyl and tyrosyl residues probably resided in the active site and participated in the mechanism of stability against heat inactivation (23). Circular dichroism studies showed yeast inorganic pyrophosphatase contained 5 to 10 residues of tyrosine and 1.4 to 2.5 residues of tryptophan exposed to the solvent (20). The modification of several residues of tyrosine and tryptophan resulted in total loss of activity indicating their important roles on the enzyme (10, 20). More than one carboxyl group was demonstrated to be essential in the mechanism of pyrophosphatase, probably by direct interaction with the substrates (9). The involvement of methionine and cysteine in pyrophosphatase activity were also studied and the roles of these residues in enzymatic process were also proposed (2, 21, 30).

A conserved essential arginine residue was suggested in the enzymes such as ATPase using anionic substrates (6, 7, 13). It is not surprising that PPase, as another example of enzymes using anionic substrates, contains an essential arginine residue (9). The arginine residue might participate in the binding of pyrophosphate in the active cleft. The absolute requirement of Mg$^{2+}$ for protection suggests its role in the anchoring of substrate in active site, probably through the carboxylates of the enzyme (9, 27).

This paper is first to report that H$^+$-translocating pyrophosphate of tonoplast vesicles from mung bean etiolated seedlings

![Figure 4. Double reciprocal plot of partially purified PPase in the presence and absence of PGO. Partially purified PPase (0.4 mg/ml) was modified by 5.0 mm PGO in 240 $\mu$L of 50 mm Mops-KOH buffer (pH 7.9) for 6 min at 37°C. The pyrophosphate hydrolysis activities of aliquots (20 $\mu$L) of control (•) or modified (○) soluble PPase were measured in the assay medium as described in Figure 1.](image-url)

### Table I. Protection of PPase Activity against Inactivation by PGO

<table>
<thead>
<tr>
<th>Protectors (5 mm)</th>
<th>Activity as Substrate</th>
<th>Protection against PGO (5 mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol PPI consumed</td>
<td>mg protein $^{-1}$ h$^{-1}$ (%)</td>
</tr>
<tr>
<td>K$_3$PPi</td>
<td>32.12 (100.0)</td>
<td>113.0</td>
</tr>
<tr>
<td>$\beta$-Nitrophenyl phosphate</td>
<td>2.55 (8.0)</td>
<td>51.8</td>
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<tr>
<td>Inorganic phosphate</td>
<td></td>
<td>42.8</td>
</tr>
<tr>
<td>Imidodiphosphate</td>
<td>4.19 (13.1)</td>
<td>60.6</td>
</tr>
<tr>
<td>Phosphothreonine</td>
<td>3.06 (9.5)</td>
<td>$&lt;1.0$</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>3.02 (9.4)</td>
<td>$&lt;1.0$</td>
</tr>
<tr>
<td>ATP</td>
<td>6.57 (20.5)</td>
<td>95.4</td>
</tr>
<tr>
<td>ADP</td>
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<td>25.0</td>
</tr>
<tr>
<td>AMP</td>
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<td>28.6</td>
</tr>
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</table>
was inhibited by incubation with two α-dicarbonyl compounds, PGO and BD. The inactivation by PGO and BD is possibly due to the specific modification of positively charged guanidium group of arginine residue at active site of pyrophosphatase. The kinetic analysis reveals that one essential arginyl residue is involved at each site of enzyme. The partial protection of enzymatic activity against PGO inhibition in the presence of Mg²⁺ indicates the binding of Mg²⁺-pyrophosphate at an active site. We conclude from the evidence above that H⁺-PPase of mung bean tonoplast contains at least one arginyl residue essential to its enzymatic activity.

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LITERATURE CITED