

ADENOSINE DIPHOSPHATASE AND ADENOSINE TRIPHOSPHATASE ACTIVITIES OF MAIZE TONOPLAST-ENRICHED VESICLES**An-Fei Hsu*, David Brauer, and Shu-I Tu**

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ABSTRACT: Microsomal fraction enriched with tonoplast vesicles was obtained from maize root (*Zea mays* L. FRB 73) by sucrose step gradient centrifugation. The vesicles contained an adenosine diphosphatase (ADPase) activity as determined by the hydrolysis of Mg-ADP. The ADPase and adenosine triphosphatase (ATPase) of the fraction have different sensitivities toward several modifiers. ADPase activity was not sensitive to nitrate, N,N'-dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES), and azide, but partially sensitive to vanadate and molybdate. In contrast, ATPase was very sensitive to nitrate, DCCD, and DES, but not to vanadate, azide, and molybdate. The presence of 2'-or (3')-trinitrophenol adenosine 5'-diphosphate (TNP-ADP) significantly reduced ADPase activity but not that of the ATPase activity. On the other hand, the addition of TNP-ATP abolished ATPase activity but not the ADPase activity. Since the influence of these modifiers to the activities are different, the binding and the hydrolysis of ADP and ATP in maize tonoplast vesicles may occur at different sites.

INTRODUCTION

It has been found that plant root cells contain a tonoplast ATPase which has an absolute specificity for Mg⁺⁺-ATP and is inhibited by nitrate, but not vanadate (1,2). The other ATPase, located in plasma membrane (3,4,5), is sensitive to

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vanadate but not to nitrate. The proton gradients and membrane potentials generated by these two types of H⁺-ATPases are believed to serve as the thermodynamic driving force for the movement of other ions and molecules in and out of the cytoplasm and vacuolar lumen (5,6). These H⁺-ATPase are energy transducers which convert chemical free energy available in the covalent bonds to electrochemical gradients across membranes. Two mechanisms have been proposed by which ATPase may convert the energy from ATP hydrolysis into an electrochemical gradient via direct or indirect coupling (7,8). When ATP hydrolysis is directly linked to proton transport, experimental treatments will affect ATP hydrolysis and proton transport to the same extent. Alternatively, the primary energy-releasing process, i.e. ATPase may be only indirectly linked to proton transport.

It was reported that pea stem microsomal fraction showed both ATPase and ADPase activities. These two enzymes are capable of translocating protons resulting in the generation of an electrochemical potential to drive a H⁺/Ca⁺⁺ antiport. The ATPase and the ADPase have a similar pH optimum, but different in physical and chemical properties (9). While a large volume of information on ATPases in tonoplast and plasma membrane are available, the role of ADPase in membrane vesicles is still poorly understood.

In this paper, we showed the maize tonoplast vesicles also possess both ATP and ADP hydrolyzing activities. We found that ATP hydrolysis, but not ADP hydrolysis induced a proton translocating activity. Different sensitivities of the ATPase and the ADPase toward various modifiers revealed that the binding of ADP and ATP in tonoplast may occur in different sites with little effects on each other.

MATERIALS AND METHODS

Material

ADP, (adenosine diphosphate) AO (acridine orange), ATP adenosine triphosphate), BTP (bis tris propane) DTI (dithiothreitol), EDTA (ethylenediaminetetraacetic acid), Hepes (4-2-hydroxyethyl-1-piperazinethane), EGTA, (ethyleneglycerol bis-3-aminoethyl ether), Mes (2-N-morpholinoethane sulfonic acid), sucrose, and malachite green were obtained from Sigma Co. DCCD (N, N1-dicyclohexylcarbodiimide) was purchased from Aldrich. TNP-ADP (2' or 3' trinitrophenol-ADP) and TNP-ATP were obtained from Molecular Probes, Inc. All other chemicals used were of analytical grade.

Isolation of Tonoplast Enriched Fraction from Corn Root Microsome

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 described

previously (2). Crude microsomal pellets were obtained from differential centrifugation at 13,000 g for 15 min and 87,000 g for 45 min. The pellet was then suspended in homogenized buffer (5 mM EDTA, 10 mM DTI, and 0.1M Hepes titrated to pH 7.75) and layered over a discontinuous sucrose gradient (15/30/40% w/w). Further centrifugation at 38,000 g for two and half hours yielded the tonoplast enriched fraction at the interphase of 15 to 30%. The obtained tonoplast-enriched vesicles were frozen in small aliquot and stored at -20°C until use.

Assays for Proton Transport

The proton transport supported by ATP or ADP hydrolysis was assayed by the absorbance change of acridine orange (AO) at 492.5 nm as previously described (10). Typically, vesicles (100-200 μ L) were diluted with proton pumping assay medium containing 17.5 mM MES titrated to pH 6.45 with BTP, 0.2 mM EGTA, 2.5 mM $MgSO_4$, 7.5 μ M AO, and 50 mM KCl. After incubating at room temperature (24°C) for 5 min, proton transport was initiated by the addition of 20 μ L of 0.2 M ATP or 0.2 M ADP titrated to pH 6.45 with BTP. The collapse of the steady state pH gradient was induced by the addition of ADP.

ATP, ADP Hydrolysis and Protein Determination

The hydrolysis rate of ATP or ADP was determined by the addition of 5 μ L of 0.2 M of ADP to the incubation mixture containing tonoplast vesicles (20 μ g), 50 mM KCl, 1 mM EGTA, 5 mM $MgSO_4$, and 1 mM azide. The incubation was carried out at room temperature for 20 min. The release of inorganic phosphate was determined by Malachite-Green molybdate method (2). Protein concentration was measured by a modified Lowry method using bovine serum albumin as the standard (11).

ATP and ADP Hydrolysis in the Presence of TNP-ATP or TNP-ADP

Stock solutions of TNP-ATP and TNP-ADP were freshly prepared immediately before use in distilled water. Assay of ATP or ADP hydrolysis was conducted as described above except the addition of TNP-ATP or TNP-ADP in the incubation. The release of phosphate was determined as described above.

RESULTS

ADP Hydrolysis in Maize Tonoplast Enriched Vesicles

The presence of ADPase activity in tonoplast enriched fraction is shown in Fig. 1A. The hydrolysis of ADP exhibited a concentration dependent and saturable kinetics. Increasing the concentration of ADP from 0 to 2 mM increased the release rate of inorganic phosphate. However, in the absence of ADP, no detectable

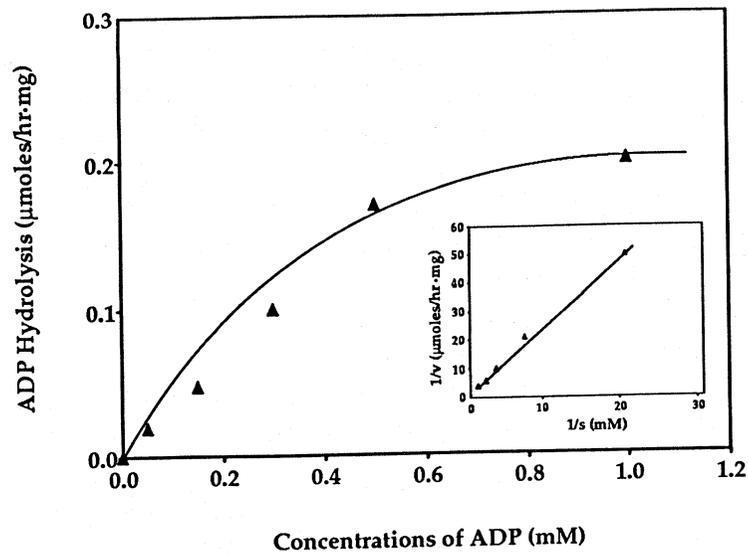


FIGURE 1. ADP hydrolysis of maize root tonoplast enriched fractions. ADP hydrolysis was determined as a function of ADP concentration. (1A). The release of inorganic phosphate was described in "Materials and Methods". Insert (Fig. 1b) represents the linear transformation by reciprocal plot of Figure 1A.

amount of phosphate was found. Thus, tonoplast enriched vesicles were capable of catalyzing the hydrolysis of ADP. Figure 1B indicated the double reciprocal plot of Figure 1A. Under the experimental conditions, K_m and V_{max} of ADPase are 0.5 mM and 2 $\mu\text{moles Pi/mg:hr}$ protein, respectively. The vesicles preparation also catalyzed the hydrolysis of ATP (data not shown) with K_m and V_{max} of 0.25 mM and 8 $\mu\text{moles Pi/mg: hr}$, respectively. Apparently, tonoplast enriched fraction contained both ATP and ADP hydrolysing activities.

Sensitivity of ATPase and ADPase to Nitrate

When the tonoplast enriched vesicles were incubated with ATP or ADP in the presence of nitrate, a substantial difference between sensitivity between ATP and ADP hydrolyses toward nitrate was observed (Fig. 2). It is apparent that ATP

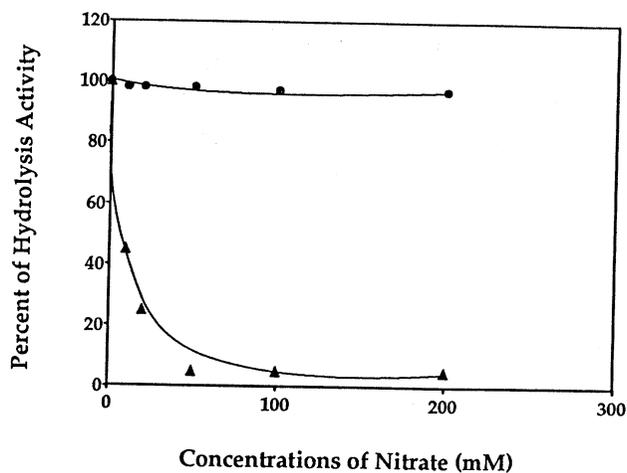


FIGURE 2. Nitrate inhibition on ATPase and ADP hydrolysis. The incubations of ATPase and ADPase were carried out as described in "Materials and Methods". Various concentrations of KNO_3 (0.25, 5.0, 100, and 200 mM) were added in the incubations containing ATP (▲-▲) or ADP (●-●) as a substrate. The percentage of activity was based on the control experiment (without the addition of KNO_3) which is assumed as 100%.

hydrolysis was extremely sensitive to nitrate. At 50 mM of KNO_3 , ATP hydrolysis activity almost decreased 90%, while ADP hydrolysis activity remained unchanged. This difference response was confirmed within a wide concentration range of nitrate (0-200 mM).

Response to Other Modifiers

In addition to nitrate, the ATPase and the ADPase in the tonoplast enriched vesicles were found to have different sensitivities toward other modifiers (Table 1). DCCD, DES, and vanadate were very effective inhibitors for the ATPase while the ADPase was not affected by these chemicals. ADPase was partially sensitive to vanadate and molybdate but the ATPase was completely insensitive to these anions. Both ATPase and ADPase were not affected by azide. The possibility that the

TABLE 1. The Effect of Various Modifiers on the ATPase and the ADPase Activity.

Modifiers	ATPase	ADPase
Control ^a	100%	100%
100 μ M Vanadate	100%	80%
5 mM Molybdate	100%	56%
5 mM azide	100%	100%
10 mM DCCD	5%	100%
10 mM DES	10%	100%
50 mM Nitrate	0%	100%

^aThe rate of ATP and ADP hydrolysis obtained without the addition of modifiers were assumed as 100%. All other hydrolysis activities measured in the presence of other modifiers were compared to the control value and are expressed as a relative percentage.

ADPase might originate from the contamination of the plasma membrane ATPase was ruled out by its insensitivity to DES and DCCD (4,10) which are powerful inhibitor to the p-type of ATPase.

ATP and ADP Dependent Proton Transport

The time courses of proton pumping driven by ATP and ADP hydrolysis as measured by absorbance decrease due to Acridine Orange (AO) uptake in the tonoplast vesicles were shown in Figure 3. Upon the addition of ATP, the AO absorbance decreased and reached a steady state between 6-10 min (line 1). This ATP dependent proton transport was insensitive to vanadate (line 4) but sensitive to nitrate (line 3). Upon the addition of the ADP, the AO did not change (line 2). Apparently, the ADP hydrolysis did not result in a proton transport. When an ATP initiated proton transport reached a steady state (The absorbance of AO unchanged), the addition of various concentrations of ADP (2.5, 5, and 10 mM) resulted in an increase of the AO absorbance in a time dependent fashion (line 5, 6, and 7). The ADP could be acting as a buffer in the proton pumping solution, since the concentration of ADP added in the proton pumping buffer was much higher (10

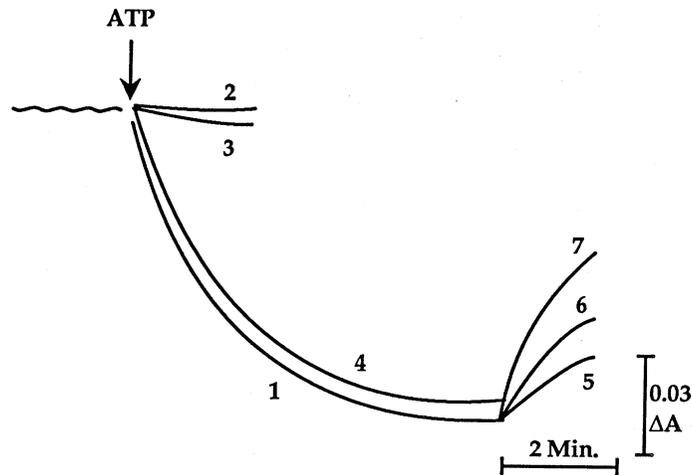
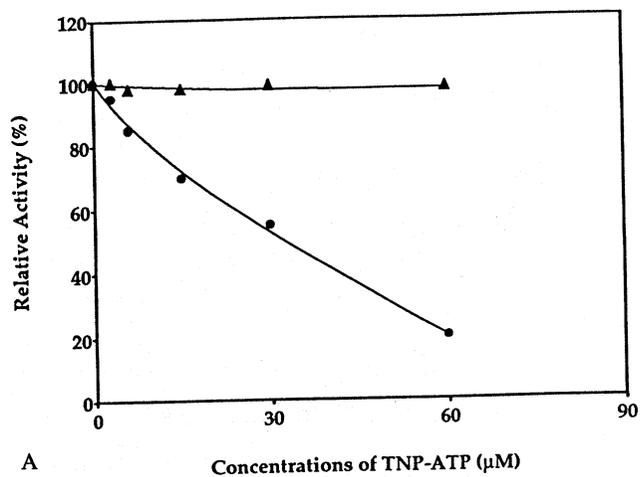


FIGURE 3. Changes in the absorbance of acridine orange at 492 nm as a function of different activities. Proton pumping of maize tonoplast enriched fractions (approximately 20 μg protein) in 2 mL of proton pumping buffer was initiated by the addition of 20 μL (0.2M) of ATP (line 1) or the addition of 20 μL of 0.2M ADP (line 2), or in the presence of 50 mM KNO_3 (line 3) or 200 μM of vanadate (line 4), or various concentrations of ADP (2.5, 5.0, and 10 mM) added after steady state of proton pumping (lines 4, 5, and 6).

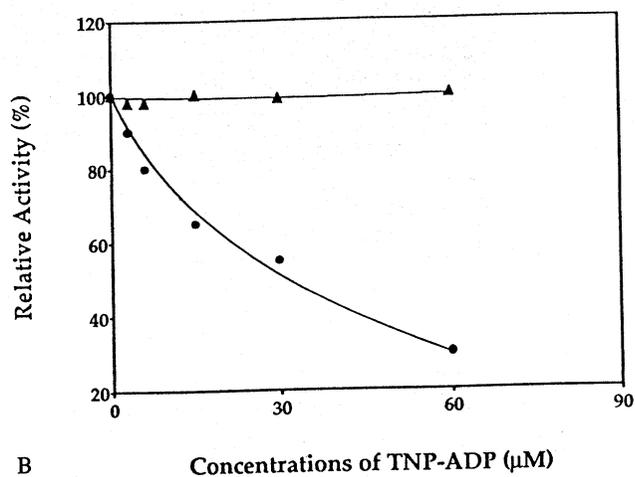
times) than that of ATP. However, the addition of equal or lower concentrations of ADP did not change the AO absorbance (data not shown).

Effect of TNP-ATP and TNP-ADP on ATP or ADP Hydrolysis Activity

The interaction of TNP-ATP and TNP-ADP with tonoplast ATPase and ADPase were shown in Figure 4A and 4B. Increasing the concentrations of TNP-ATP from 0 to 20 μM rapidly decreased ATP hydrolysis activity but ADP hydrolysis was not affected. In contrast, when the concentrations of TNP-ADP increased, ATP hydrolysis activity was not affected while ADP hydrolysis was significantly inhibited.



A



B

FIGURE 4. Effect of TNP-ATP and TNP-ADP on ATPase and ADPase. The determinations of ATPase hydrolysis and ADP hydrolysis are described under "Materials and Methods". Figure 4A shows the ATP hydrolysis with (• - •) or without (▲ - ▲) the addition of TNP-ATP (0, 2.5, 5.0, 10, and 20 mM). Figure 4B. shows the ADP hydrolysis with (• - •) or without (▲ - ▲) the addition of TNP-ADP (0, 2.5, 5.0, 10, and 20 mM). Data are plotted relative to the activity of ATP hydrolysis and ADP hydrolysis in the absence of TNP-ATP or TNP-ADP.

TABLE 2. Effects of Monovalent Ions on the ATPase and the ADPase of Tonoplast Enriched Vesicles.

Additions	% of Control	
	ATPase	ADPase
KCl	100%	100%
NaCl	95%	8%
RbCl	99%	98%
CsCl	97%	96%
LiCl	95%	4%
KBr	98%	92%
KF	94%	2%
KNO ₃	2%	96%

Conditions for ATPase and ADPase measurement were described in "Materials and Methods". The control values were obtained in a medium containing 50 mM KCl, other experiments were performed by replacing KCl to 50 mM of various monovalent ions as indicated above. The percent of activity was expressed by using KCl experiment as 100%.

Effect of Cations and Anions on ATP and ADP Hydrolysis Activity in Maize Tonoplast Enriched Vesicles

The normal (control) incubation medium for assaying ATP or ADP hydrolysis contained 50 mM KCl. Replacing KCl by other chlorides such as RbCl, or LiCl did not affect ATP hydrolysis activity. Yet ADP hydrolysis activity was significantly reduced when KCl was replaced by NaCl or LiCl. The anion effect on ATP or ADP hydrolysis was also shown in Table 2 which indicated that replacing Cl⁻ by F⁻ did not alter ATPase activity while nitrate diminished ATP hydrolysis. However, replacing Cl⁻ by F⁻ abolished ADP hydrolysis activity. Thus, unlike the ATPase, ADP hydrolysis activity showed a cation dependence of K⁺ > Na⁺ > Li⁺.

DISCUSSION

It is well known that maize tonoplast vesicles contain a H⁺-ATPase and the coupling mechanism of the enzyme has been extensively studied (4,5). However,

the presence of an ADPase and its functional roles in tonoplast vesicles is not well documented. In this study, we isolated tonoplast enriched vesicles and demonstrated that the vesicles possess both the ADPase and ATPase activities. This ADPase showed distinctive physical and chemical properties which are different from that of the ATPase in tonoplast vesicles. Previous study (8) showed that pea stem tonoplast enriched vesicles contains an ADPase activity capable of generating transmembrane proton gradient (Δ pH) and an electric potential (Δ). However, we are unable to demonstrate proton transport initiated by the ADP hydrolysis in the corn system. Results presented here showed that the ATPase is very similar to that of pea stem of ATPase which can be inhibited by nitrate, DCCD and DES, but not by vanadate. The ADPase discussed in this work displayed a different sensitivity from that of pea stem toward modifiers. Both ADPases were not inhibited by nitrate, but unlike the pea ADPase, the corn tonoplast ADPase was partially inhibited by vanadate. The possibility that the ADPase originated from the plasma membrane contamination was ruled out by its insensitivity toward DCCD or DES. Both the pea and corn ADPases exhibited a similar pH optima (pH 7.0, data not shown).

The results that ATP derivative (TNP-ATP) did not affect the ADPase activity and TNP-ADP only affect the activity of ADPase suggest that the binding of ADP and ATP should occur at different sites in the membrane. Further studies are needed to define the functional roles of the ADPase in maize tonoplast vesicles.

REFERENCES:

1. de Michaelis, M.I. and R.M. Spanswick. 1986. H⁺-pumping driven by the vanadate sensitive ATPase in membrane vesicles from corn roots. *Plant Physiol.* 81:717-724.
2. Tu, S.-I., G. Nagahashi, and J.N. Brouillette. 1987. Proton pumping kinetics and origin of nitrate inhibition of tonoplast-type H⁺-ATPase. *Arch. Biochem. Biophysics.* 256:625-637.
3. de Michaelis, M.I., M.C. Pugliarello, and F. Rasi-Caldgno. 1983. Two distinct proton translocation ATPase are present in membrane vesicles from radish seedling. *FEBS Lett.* 162:85-90.
4. Sze, H. 1984. H⁺-translocation ATPase of the plasma membrane and tonoplast of plant cells. *Physiol. Plant.* 61:683-691.

5. Sze, H. 1985. H⁺-translocation ATPase: Advances using membrane vesicles. *Annu. Rev. Plant Physiol.* 36:175-208.
6. de Michaelis, M.I., M.C. Pugliarello, and F. Rasi-Caldgno. 1983. Two distinct proton translocating ATPases are present in membrane vesicles from radish seedling. *FEBS Lett.* 162:85-90.
7. Mitchell, P. 1975. Vectorial chemistry and the molecular mechanism of chemiosmotic coupling-power transmission by proticity. *FEBS Lett.* 59:137-139.
8. Brauer, D., S.-I Tu, A.-F. Hsu, and D. Paterson. 1993. Evidence for an indirect mechanism for the nitrate sensitivity proton pump from corn root tonoplast membranes. *Physiol. Plant.* 89:588-591.
9. Gabathuler, R. and R.E. Cleland. 1985. Auxin regulation of a proton translocating ATPase in Pea Root Plasma membrane vesicles. *Plant Physiol.* 79:1080-1085.
10. Hsu, A.-F., S. Rodenbach, and S.-I. Tu. 1992. Effects of dicyclohexylcarbodiimide (DCCD) treatment of coupled activity sensitive ATPase from plasma membrane of maize root. *Physiol. Plant.* 85:17-22.
11. Bensadoun, A. and D. Weinstrein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241-250.