

006393

**THE EFFECT OF TRINITROPHENYL NUCLEOTIDE  
DERIVATIVES ON THE ADENOSINE TRIPHOSPHATASE  
ACTIVITIES IN MAIZE TONOPLAST VESICLES**

**ABSTRACT**

*The effects of trinitrophenyl (TNP) derivatives of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) on the coupled activities of the tonoplast ATPase in corn roots were investigated. The addition of TNP-ATP at micromolar concentrations (0.5–0.25 mM) significantly decreased both the ATP hydrolytic activity and the coupled proton pumping activity. The presence of TNP-ATP resulted in an increased  $K_m$  for ATP and a reduced maximum enzyme velocity. Unlike TNP-ATP that strongly inhibited proton transport at low concentration, TNP-ADP inhibited this activity only slightly. TNP-AMP stimulated proton transport at low concentrations. ADP and AMP inhibited the initial rate of proton transport in a different manner. The concentrations required to inhibit half of the initial rate of proton transport activity for ADP and AMP were 80  $\mu\text{M}$  and 2 mM, respectively. The results suggest that tonoplast vesicles may contain at least two kinds of nucleotide binding sites.*

**INTRODUCTION**

Enzymes that link the transport of ions to either hydrolysis or synthesis have been classified into three broad categories of transport adenosine triphosphatase

(ATPases), P-, V-, and F-type (Nelson and Taiz 1989). The involvement of multiple binding sites of nucleotides to *Escherichia coli* F1-ATPase is well documented (Weise *et al.* 1983). In plants, allosteric modulation (E<sub>1</sub>E<sub>2</sub>-type) of ion translocating ATPase by multiple binding of substrate has also been observed. For example, the regulatory effects of ADP on corn root plasma membranes has demonstrated that it may contain more than one binding site for ADP (Brauer and Tu 1994) and the presence of ADP may cause the root plasma membrane ATPase to be less sensitive to the wide range of inhibitors (Tu *et al.* 1987, 1990; Brauer *et al.* 1988) on tonoplast membranes, regulation of the activities of V-type ATPase by adenosine nucleotides is favored. Previous studies by Brauer and Tu (1994) demonstrated that several ATP analogs such as ADP (adenosine diphosphate), AMP-PNP (adenylylimidodiphosphate) or BzATP (3'-O-4benzoyl-benzoyl-adenosine-5' triphosphate) inhibited the kinetics of ATP utilization on the proton pumping of vacuolar H<sup>+</sup>-ATPase from maize roots. Their results favored at least two types of nucleotide binding sites on the V-type ATPase from maize root tonoplast membranes.

Information on the *in vitro* regulation of nucleotide binding with fluorescent trinitrophenyl (TNP) derivatives of AMP, ADP and ATP on maize tonoplast membrane is not yet available. However, these derivatives have been utilized to study the nucleotide binding sites of the isolated  $\beta$ -subunit of *Escherichia coli* F1-ATPase (Rao *et al.* 1988). Their results suggest the  $\beta$ -subunit of *Escherichia coli* F1-ATPase possesses a single nucleotide binding site. TNP-nucleotides were also used to assess the nucleotide binding sites on sarcoplasmic reticulum Ca-ATPase (Dupont *et al.* 1985) which revealed two distinct classes of binding sites in this enzyme. It also demonstrated that the hydrolytic activity of the high affinity ATP binding site was activated by ATP or TNP-ATP or TNP-AMP-PNP binding in the low affinity ATP binding site.

In this paper, we investigated the effects of different TNP-derivatives on the activities of tonoplast ATPase. By classical Michaelis-Menten kinetics, the results demonstrated that multiple nucleotide binding sites were present in the tonoplast vesicles of maize roots.

## MATERIALS AND METHODS

### Material

AMP, (adenosine monophosphate); ADP, (adenosine diphosphate); ATP, (adenosine triphosphate); AO, (acridine orange); BTP, (Bis-Tris-Propane); Hepes, N-(2-hydroxyl ethyl) piperazine-N'-(2-ethane sulfonic acid); Mes, 2-(N-Morpholino) sulfonic acid were obtained from Sigma Co. TNP-AMP, (2' or 3' trinitrophenol AMP); TNP-ADP and TNP-ATP were obtained from Molecular Probes, Inc. All other chemicals used were analytical grade.

### **Preparation of Tonoplast Vesicles**

Corn (*Zea mays* L. cv. FRB73) seeds were germinated on filter paper moistened with 0.1 mM CaCl<sub>2</sub> for 3 days at 28C and harvested as described previously (Hsu *et al.* 1993, 1994). Highly purified tonoplast enriched vesicles were obtained by discontinuous sucrose gradient as described previously (Hsu *et al.* 1993). Briefly, an aliquot of 3 mL of the microsomal suspension was layered over a discontinuous density gradient containing 8 mL of 42, 30 and 15% (w/w) sucrose buffered with 5 mM Hepes, pH 7.5 after centrifugation at 100,000 x g for 150 min. The membrane collected between the 15 and 30% sucrose steps was removed and used as tonoplast vesicles. The tonoplast vesicles were stored in small aliquots at -60C until use.

Protein concentration was obtained by the procedure of modified Lowry method (Bensadoun and Weinstein 1976) by using bovine serum albumin as the standard.

### **Assays for Proton Pumping and Membrane ATPase Activities**

Proton pumping activity was measured by changes in the absorbance of acridine orange (AO) at 492 nm as described by De Michelis and Spanswick (DeMichelis and Spanswick 1986). Typically, 200  $\mu$ L of vesicles (containing 300-500  $\mu$ g protein) were diluted with 2 mL of 17.5 mM Mes-Bis-Tris-Propane (Mes-BTP), pH 6.45, 2.5 mM MgSO<sub>4</sub>, 1 mM ethylene glycol-bis ( $\beta$ -aminoethylether N, N'-tetraacetic acid (EGTA), 7.5  $\mu$ M AO, and 50 mM KCl. After equilibrium at room temperature for 10 min, the reaction was initiated by the addition of 20  $\mu$ L of 0.2M ATP (pH adjusted to 6.45 with BTP). The initial rate of proton transport was determined by analyzing the time course of the quenching of AO absorbance.

ATPase activity was determined by the release of inorganic phosphate by the Malachite green assay (Hsu *et al.* 1993).

### **Preparation of Inhibitors and Experimental Conditions**

TNP derivatives were prepared in desired stock concentrations in distilled water immediately before use. In measuring ATPase activities in the presence of TNP derivatives, background experiments were conducted by using the same concentration of TNP derivatives without the addition of tonoplast vesicles. In general, the addition of high concentrations of TNP derivatives resulted in an increase in background absorbance at 492 nm. This increase in absorbance interfered with the measurement of proton transport, since proton pumping activity was measured by the initial rate of decreased absorbance after the addition of ATP. In this study, the chosen concentrations of TNP-derivatives would not significantly alter the absorbance at 492 nm. The detailed conditions of each experiment are described in the figure legends.

## RESULTS

### Effects of TNP-ATP on ATP Hydrolysis and Proton Pumping

It was demonstrated (Hsu *et al.* 1994) that TNP-ATP is an inhibitor for the tonoplast ATPase. It is of interest to examine the potency of TNP derivatives (i.e., TNP-ATP, TNP-ADP and TNP-AMP) in the inhibition of the ATP hydrolysis. Tu *et al.* (1987, 1990, 1995) and Brauer and Tu (1994) showed that the sensitivity and characteristic inhibition of the transport activity and ATP hydrolysis from maize root tonoplast vesicles can be different. The presence of 0.25 mM of TNP-ATP in the normal assay containing 2 mM ATP inhibited 70% of the proton transport activity, while ATP hydrolysis was only reduced by 40%. At 0.5 mM of TNP-ATP, the activity of proton pumping was almost decreased less than 10%, while more than 40% of ATP hydrolysis activity remained (Fig. 1). The results indicated that the proton pumping activity of tonoplast vesicles

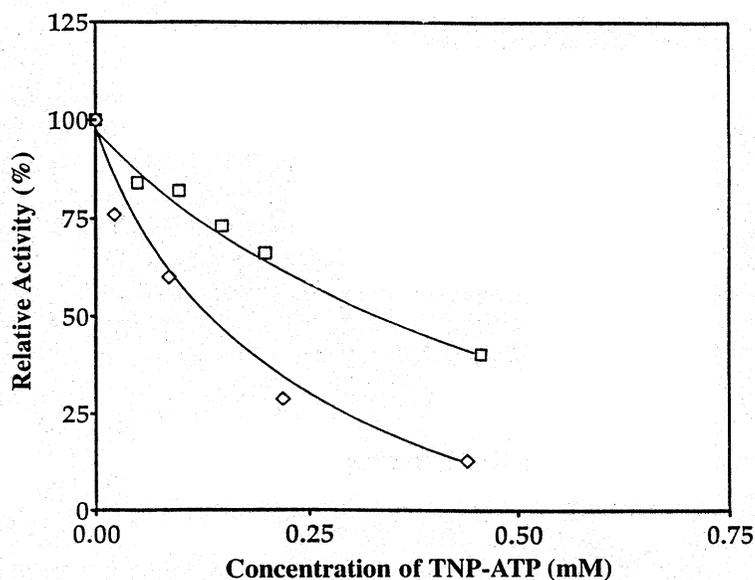


FIG. 1. DIFFERENTIATION EFFECT OF TNP-ATP ON TRANSPORT COUPLED ACTIVITIES OF MAIZE TONOPLAST VESICLES

Different concentrations of TNP-ATP were added at the same time when proton pumping was initiated by the addition of ATP. When proton pumping reached a steady state (no change in Absorbance), 100  $\mu$ L of incubation mixture was assayed for the release of phosphate. 100% activity represented the ATP hydrolysis (□-□) or proton pumping (◇-◇) without the addition of TNP-ATP. Data are the average of two experiments, each with three replicates and error range as  $\pm 5\%$ .

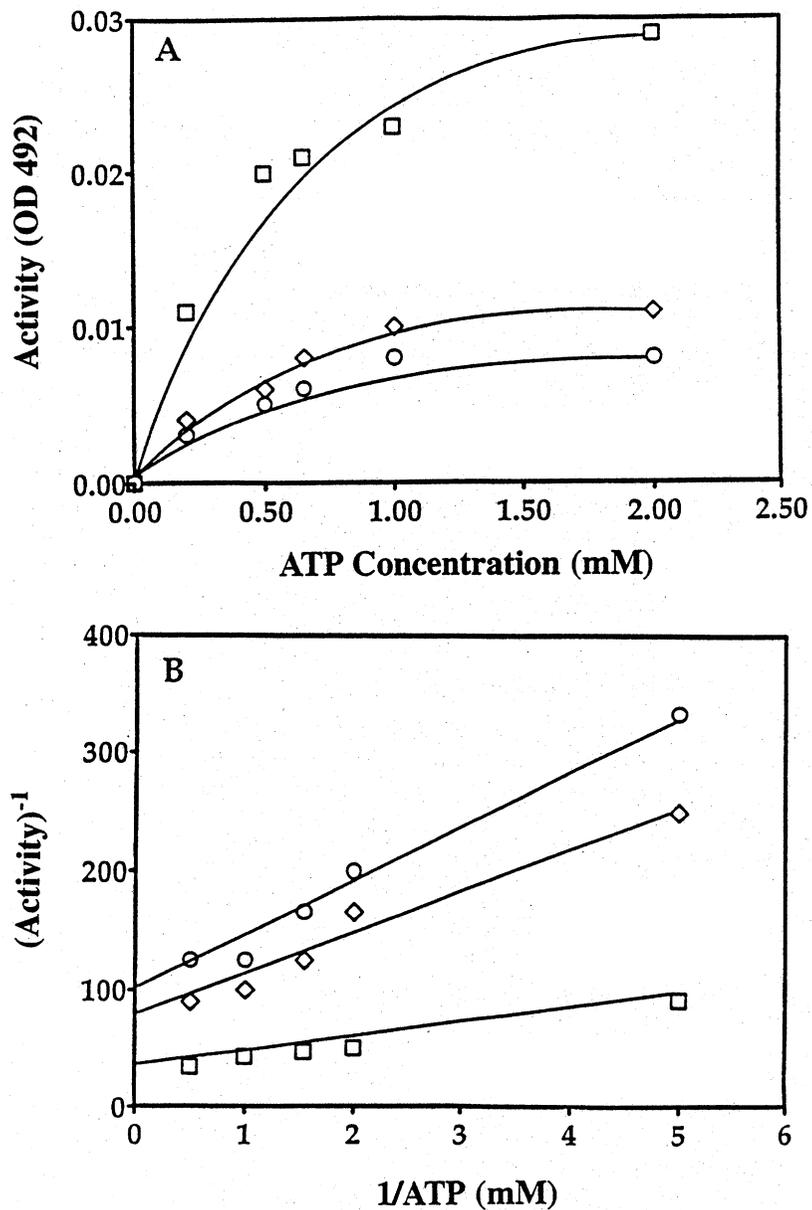


FIG. 2. EFFECTS OF TNP-ATP ON THE ATP DEPENDENCE OF THE INITIAL RATE OF PROTON TRANSPORT CATALYZED BY THE TONOPLAST  $H^+$ -ATPase FROM MAIZE ROOTS

(a) The initial rate of proton transport was determined in the absence ( $\square-\square$ ) and presence of  $80 \mu\text{M}$  ( $\diamond-\diamond$ ) or  $160 \mu\text{M}$  ( $\circ-\circ$ ) TNP-ATP. (b) Data were plotted as the linear transformation of reciprocal plot of Fig. 2A. Data are the average of two experiments, each with two replicates and error range as  $\pm 5\%$ .

was more sensitive to TNP-ATP inhibition than that of ATP hydrolysis. The different sensitivities of ATP hydrolysis and proton transport to TNP inhibition shown in Fig. 1 clearly demonstrated that TNP-ATP has a differential effect on the coupling of tonoplast ATPase activities.

The effect of TNP-ATP on the kinetics with respect to  $H^+$ -transport is shown in Fig. 2. The presence of TNP-ATP in the normal assay media containing 2 mM ATP inhibited proton transport. At 80  $\mu$ M TNP-ATP, the maximum initial rate of proton pumping was about 40% found in the absence of TNP-ATP (Fig. 2a). The effect of TNP-ATP on the kinetics of ATP supported proton transport indicated that ATP was not a simple competitive inhibitor of proton transport, since both  $K_m$  for ATP and  $V_{max}$  were altered (Fig. 2b). The addition of 80  $\mu$ M and 160  $\mu$ M of TNP-ATP increased  $K_m$  for ATP from 0.4 mM to 0.50 and 0.55 respectively.  $V_{max}$  was decreased by approximately 60% and 75%, respectively obtained in the absence of TNP-ATP. Therefore, the inhibition of proton transport of vacuolar  $H^+$ -ATPase by TNP-ATP was similar to that observed with ADP inhibition as reported by Brauer and Tu (1994).

#### **Effect of TNP Derivatives on ATP Hydrolysis**

Tonoplast vesicles were treated with increasing concentrations of TNP-ATP, TNP-ADP and TNP-AMP (from 0.05 to 0.20 mM) in the presence of 2 mM ATP. Figure 3 indicates that among these derivatives that TNP-ADP and TNP-AMP were not inhibitors for ATP hydrolysis, whereas TNP-ATP was a specific and sensitive inhibitor for ATP hydrolysis. At 0.2 mM of TNP-ATP, ATP hydrolysis activity decreased about 40%, while the same concentration of TNP-ADP and TNP-AMP did not cause any significant change of tonoplast ATP hydrolysis.

#### **Inhibition of Proton Uptake by ADP and AMP**

The tonoplast membrane vesicles were incubated in the proton pumping buffer with increasing concentrations of ADP or AMP (Fig. 4). Proton pumping was initiated by the addition of various concentrations of ATP while the total concentration of ATP+ADP, and ATP+AMP was kept constant. Therefore the molar ratio of X ( $ADP/(ADP+ATP)$  or  $AMP/(AMP+ATP)$ ) increased when the concentration of ADP or AMP decreased. The ADP inhibition data showed the presence of a lag approximately up to  $X = 0.75$ , then proton pumping was increased rapidly. The AMP inhibition on proton uptake increased initially very rapidly, with the X increased to greater than 0.50, no inhibition was observed. Figure 4 also indicated that a 50% inhibition on proton uptake was caused by either 80  $\mu$ M ADP or 2 mM for AMP. Apparently ADP was 25 times more potent than AMP in inhibiting proton transport initiated by ATP. Further analysis

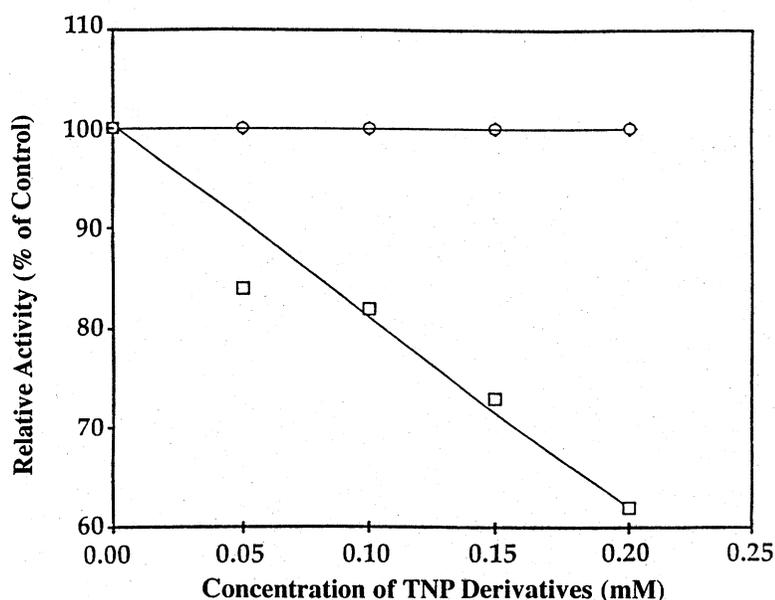


FIG. 3. EFFECT OF TNP-ATP, TNP-ADP AND TNP-AMP ON THE ATP HYDROLYSIS. Tonoplast vesicles were incubated with increasing concentrations of TNP-ATP (□—□), TNP-ADP or TNP-AMP (◇—◇) at room temperature for 30 min as described under "Materials and Methods" to determine the amount of phosphate released. Data are plotted relative to the activity of 100% in the absence of TNP derivative. All other activities were compared to the activity of 100%. Data were the average of two experiments. Each with three replications. Data are the average of two experiments, each with two replicates and error range as  $\pm 5\%$ .

on the inhibition of proton pumping indicated that the addition of ADP resulted in an increase in  $K_m$  and a decrease in  $V_{max}$ .

#### Effect of TNP Derivatives on the Initial Rate of Proton Transport Catalyzed by the Tonoplast ATPase

Figure 5 indicates that the tonoplast  $H^+$ -ATPase supported proton transport was inhibited both by TNP-ADP and TNP-ATP, while the proton transport activity reached maximum stimulation in the presence of  $0.4 \mu M$  of TNP-AMP. Proton transport activity was extremely sensitive to TNP-ATP inhibition and less sensitive to TNP-ADP inhibition. At low levels of TNP-ATP (concentration less than  $0.1 \mu M$ ) caused more than 70% inhibition in proton transport, but TNP-ADP at the same concentration inhibited less than 30% of proton pumping activity. These results suggest that there are two, rather than one type of function-link binding process for TNP-ATP, TNP-ADP and TNP-AMP.

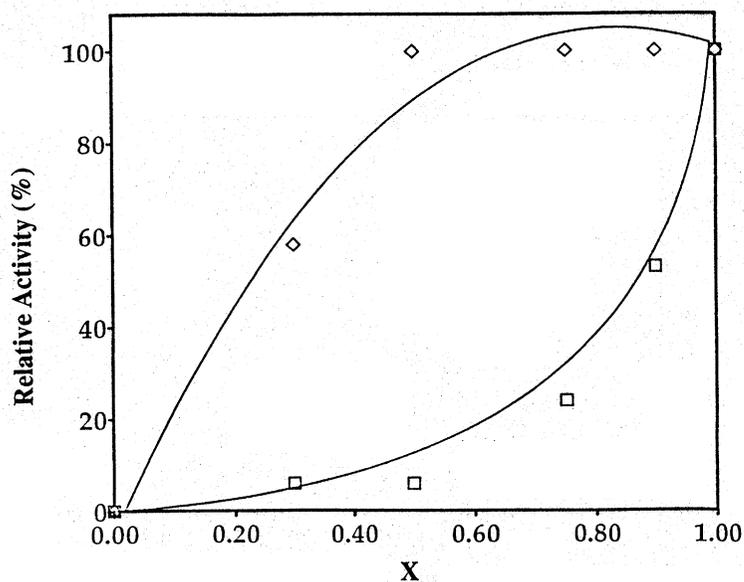


FIG. 4. EFFECT OF ADP AND AMP ON THE ATP DEPENDENCE OF THE INITIAL RATE OF PROTON TRANSPORT CATALYZED BY THE TONOPLAST ATPase. The tonoplast membrane vesicles were incubated in the proton pumping buffer at room temperature for 10 min, then various concentrations of ATP+ADP or ATP+AMP were added in the incubation mixture to initiate the proton pumping. The final concentrations of ATP+ADP or ATP+AMP were kept constant. The initial rate of proton uptake measured by the absorbance of acridine orange was assigned as 100%. The molar fraction of  $X_{ADP}$  or  $X_{AMP}$  is defined as  $ADP/ADP+ATP$  ( $\square-\square$ ) or  $AMP/AMP+ATP$  ( $\diamond-\diamond$ ). Data are the average of two experiments, each with two replicates and error range as  $\pm 5\%$ .

## DISCUSSION

The results present here indicate that ATP derivatives TNP-ATP, TNP-ADP and TNP-AMP have different effects on the hydrolysis and proton transport activity of V-type ATPase from maize tonoplast vesicles. The data support the possibility of the presence of more than one binding site in ATPase for the different nucleotides. At least one site is for the catalytic binding of ATP, where TNP-ATP might directly compete with ATP and inhibit hydrolysis and its supported proton pumping. ADP and TNP-ADP bind to a distinct site which may influence the conformational changes at the catalytic site, and therefore, inhibiting the proton pumping in a manner dissimilar to that of the competitive inhibitor,

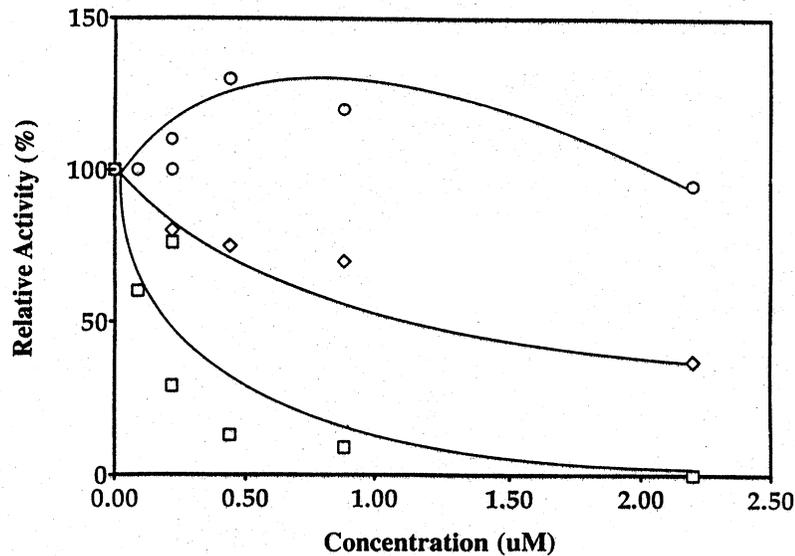


FIG. 5. EFFECT OF TNP DERIVATIVES ON THE INITIAL RATE OF PROTON TRANSPORT CATALYZED BY THE TONOPLAST ATPase

Proton pumping activity was measured as described under "Materials and Methods" with the addition of various concentrations of indicated TNP derivatives. The proton transport activity obtained in the absence of added TNP derivatives was assigned as 100%. The line represent the best curve fit by the analysis of best curve fit. TNP-ATP ( $\square$ - $\square$ ), TNP-ADP ( $\diamond$ - $\diamond$ ), TNP-AMP ( $\circ$ - $\circ$ ). Data are the average of two experiments, each with three replicates and error range as  $\pm 5\%$ .

such as TNP-ATP. It is not known from these studies whether these inhibitors bind to several distinct sites. The interaction between them needs to be examined more thoroughly before definite conclusions can be reached.

The mechanism of energy driven proton movement across biological membranes has been suggested as either direct (Mitchell 1975) or indirect (Tu *et al.* 1987, 1990, 1995), Hsu *et al.* (1993, 1994) and Brauer and Tu (1994) in nature. The differences in sensitivity and inhibition of the transport activity and ATP hydrolysis observed in maize root tonoplast vesicles support the indirect mechanism. This suggests that proton pumping and ATP hydrolysis may be indirectly coupled; in the sense that at least some intermediate steps, whether chemical or conformational are required for the coupling between these two events. The indirect mechanism can be further supported by inhibition of TNP-ATP on coupled activity of ATP hydrolysis and proton transport as present in this report. Figure 1 clearly demonstrated a differential sensitivity of the ATP hydrolysis and its coupled proton transport activity for TNP-ATP. The results suggested that the

coupling between ATP hydrolysis and proton pumping is indirect. Thus the primary effect of TNP-ATP on the ATPase reaction may be only linked to proton translocation through conformational changes.

The data in Fig. 5 indicates that TNP-ATP, TNP-ADP and TNP-AMP did not have the same effect on the proton transport activity. At the concentration lower than 0.1 mM, the initial rate of proton transport was strongly inhibited by TNP-ATP, while TNP-ADP only slightly reduced the proton transport activity. TNP-AMP appeared to have a completely different effect on proton transport, which significantly stimulated the proton transport activity. The data indicate that tonoplast H<sup>+</sup>-ATPase may contain more than one binding site.

### REFERENCES

- BENSADOUN, A. and WEINSTEIN, D. 1976. Assay of protein in the presence of interfering materials. *Anal. Biochem.* **70**, 241-250.
- BRAUER, D. and TU, S-I. 1994. Effect of ATP analogs on the protein pumping by the vacuolar H<sup>+</sup>-ATPase from maize roots. *Plant Physiol.* **91**, 442-448.
- BRAUER, D., TU, S-I. and THOMAS, A-F. 1988. Kinetic analysis of proton transport by the vanadate sensitive ATPase from maize microsomes. *Plant Physiol.* **89**, 464-471.
- DEMICHELIS, M.I. and SPANSWICK, R. 1986. H<sup>+</sup>-pumping driven by the vanadate sensitive ATPase in membrane vesicles from corn roots. *Plant Physiol.* **81**, 524-547.
- DUPONT, Y., POUCEOIS, R., RONJAT, M. and VERJOVSKV-ALMIDA, S. 1985. Two distinct classes of nucleotide binding sites in Sarcoplasmic reticulum Ca-ATPase revealed by 2', 3'-O-(2,4,6 Trinitrocyclohexadienylidene)-ATP. *J. of Biol. Chem.* **260**(12), 7241-7249.
- HSU, A-F., LEE, D. and TU, S-I. 1994. Differentiation inhibition of tonoplast H<sup>+</sup>-ATPase activity by danyl chloride. *Plant Physiol. (Life Sci. Adv.)* **13**, 3-87.
- HSU, A-F., RODENBACH, S. and TU, S-I. 1993. Separation of tonoplast vesicles enriched in ATPase and pyrophosphatase activity from maize roots. *J. of Plant Nutrition* **16**(7), 1179-1192.
- MITCHELL, P. 1975. The correlation of chemical and osmotic force in biochemistry. *J. Biochem.* **97**, 1-8.
- NELSON, N. and TAIZ, L. 1989. The evaluation of H<sup>+</sup>-ATPase Trends. *BioChem. Sci.* **14**, 113-116.
- RAO, R., AL-SHAWI, M.K. and SENIOR, A.E. 1988. Trinitrophenyl-ATP and -ADP bind to a single nucleotide site on isolated  $\beta$ -subunit of Escherichia coli F1-ATPase. *J. Biological Chem.* **263**(12), 5569-5573.

- TU, S-I., BRAUER, D. and NUNGESSE, E. 1990. Differentiation inhibition of tonoplast H<sup>+</sup>-ATPase activities by fluoescamine and derivatives. *Plant Physiol.* *93*, 1102-1109.
- TU, S-I., NAGHAHASKE, G. and BROUILLETTE, J.N. 1987. Proton transport and origin of nitrate inhibition of tonoplast-type H<sup>+</sup>-ATPase. *Arch. Biochem. Biophys.* *266*, 289-297.
- TU, S-I., PATTERSON, D., BRAUER, D. and HSU, A-F. 1995. Inhibition of corn root membrane ATPase activities by oryzalin. *Plant Physiol. Biochem.* *33*(2), 141-148.
- WEISE, J.G., DUNCAN, T.M., LATCHNEY, L.R., COX, D.N. and SENIOR, A.E. 1983. Properties of F1-ATPase from unc D412 mutant of *Escherichia coli* *Biochem. J.* *215*, 343-350.