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Differentiation inhibition of tonoplast H⁺-ATPase activity by dansyl chloride

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ABSTRACT

Treatment of corn root (*Zea mays* L. FRB73) tonoplast vesicles with a primary amine specific reagent, dansyl chloride (DC), caused a modification of the membrane. Both aqueous and lipid fractions of tonoplast membrane were labeled with DC in a typical concentration dependent fashion. The labeling of membranes resulted in a differential inhibition to coupled activities of tonoplast ATP hydrolysis and proton transport. The transport activity was more sensitive to the treatment with dansyl chloride than the adenosine triphosphate (ATP) hydrolysis activity. The presence of micromolar DC (as low as 20 μ M) caused a steady decrease in ATP supported proton transport, and reduced the proton pumping rate by 60%. The build-up of the proton gradient was analyzed according to a steady-state kinetic model. It was found that proton membrane leakage was not affected by the dansyl chloride modification, while initial rate of proton transport decreased as the concentration of dansyl chloride increased. The results suggest that proton pumping is indirectly linked to ATP hydrolysis in tonoplast vesicles and the linkage between these processes is apparently weakened by dansyl chloride modification of the membrane vesicles.

INTRODUCTION

Tonoplast membranes of plant roots contains at least two enzymes, an adenosine triphosphatase (ATPase) and a pyrophosphatase (PPiase), which can convert the chemical energy from the hydrolysis of a high energy phosphate ester bonds into a trans-membrane proton electrochemical gradient (Churchill and Sze 1983, Benett and Spanswick 1984,

Chanson *et al.* 1985, Wang *et al.* 1986). The mechanism by which the hydrolysis of ATP converts the electrochemical gradient into movement of ions by the ATPase is not well understood. A general reaction scheme for p-type ATPases is consistent with a direct coupling mechanism between ATP hydrolysis and ion transport (Show 1985). However, for the V-type ATPases it appears that ATP hydrolysis and ion proton transport are indirectly linked since ATP hydrolysis and the transport activities could be differentially inhibited or activated by various treatments (Tu *et al.* 1987, 1990). Further evidence for indirect coupling was obtained by Hsu *et al.* (1992) who added N,N'-dicyclohexylcarbodiimide (DCCD) on plasma membranes. The results supported the indirect mechanism for coupled ATP hydrolysis and ion transport in vanadate-sensitive ATPases. According to the indirect mechanism, the ATPase may be composed of several functional domains, one leading to ATP hydrolysis and another one to ion transport. Neither of these two reactions share a common intermediate step but are linked by a third domain.

Previous studies by Tu *et al.* (1990) showed that treatment of tonoplast vesicles with fluorescamine a primary amine specific reagent, resulted in a differential inhibition of the coupled activities of proton transport and ATP hydrolysis. They suggested that the coupling step is linked to a molecular arrangement which is sensitive to the presence of acrylic fluorescamine derivatives in the membrane. The results also suggest that the presence of H⁺-bond forming group in the membrane, is essential for the inhibition.

To investigate further the mechanism by which proton transport is coupled to ATP hydrolysis, we have studied the effects of modifying tonoplast vesicles

with dansyl chloride, a primary amine modification reagent. The results support the concept of indirect coupling between ATP hydrolysis and proton pumping through a molecular arrangement which is sensitive to dansyl chloride.

MATERIAL AND METHODS

Preparation of corn root tonoplast vesicles:
Zea mays L. cv. FRB73, Illinois Foundation Seeds) were germinated and harvested as previously described (Hsu *et al.* 1991). Approximately, 40-60 g fresh weight of excised roots were ground with a mortar and pestle at 4°C in 3 volumes of buffer containing 0.3 M sucrose, 5 mM ethylenediamine, N,N,N',N'-tetraacetic acid (EDTA), 5 mM, 2-mercaptoethanol, 5 mM, dithiothreitol (DTT) and 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.6 (adjusted with Hepes free acid). Following the procedure of Tu *et al.* (1987), tonoplast vesicles were isolated from a crude microsomal pellet by differential centrifugation of the root homogenate at 6,000 g for 20 min. The supernatant was centrifuged at 80,000 g for 35 min. Microsomes were washed by the procedure as previously described (Tu *et al.* 1987). The washed microsome was resuspended in the homogenized buffer and overlaid on a 15-45% (w/w) linear sucrose gradient with 5 mM Hepes-Mes, pH 7.5 with 5 mM DTT and centrifuged at 4°C for 18 h at 84,000 g in a Beckman SW 28 rotor as previous described (Tu *et al.* 1987). The tonoplast vesicles were obtained between 19 and 24% sucrose density are were pooled and stored in -60°C freezer. These vesicles were used for the analysis.

ASSAY OF PROTON TRANSPORT, ATPASE AND PROTEIN DETERMINATION

Proton transport was followed by changes in the absorbance of acridine orange (AO) at 492 nm as described by Brauer *et al.* (1988). Typically, 100 to 200 μ l of tonoplast vesicles were diluted with 2 mL of 17.5 mM Mes-bis Tri-Propane (BTP) (pH 6.45), 2.5 mM MgSO₄ 1 mM ethyleneglycerol bis-(β -aminoethylether)N,N'-tetraacetic acid (EGTA), 7.5 μ M AO and 50 mM KNO₃. After equilibration at 18°C for 5 min, the reaction was initiated by the addition of 20 μ l of ATP, (0.2 M). When the proton pumping reached a steady state (absorbance remains the same), aliquots then were removed and assayed for the phosphate release by malchite-green molybdate method (Tu *et al.* 1987).

Protein content was measured by a modified Lowry method (Bensadoun and Weinstein 1976) using bovine serum albumin as the standard.

MODIFICATION OF TONOPLAST VESICLES

Aliquots of two hundred μ l of purified tonoplast vesicles (20-40 μ g of protein) were warmed up to room temperature over 3 min, while vortexing, 2 μ l of acetone (containing 10 μ M dansyl chloride) were rapidly added and then mixed for 5-s. Immediately after vortexing, the vesicles were diluted to 2.0 ml with the proton pumping assay medium. After standing in room temperature (22°C) for 3 min, the proton pumping and ATP hydrolysis were assayed as described above. Samples prepared by the addition of acetone only were used as controls.

Membrane lipids (1 ml of tonoplast vesicles) were extracted with organic solvents as described by Moreau and Isett (1985). Briefly, the tonoplast vesicles were treated with 7 ml of Hexane-isobutanol (v/v 7:1). Organic solvents containing membrane lipids were then removed and dried at 60-70°C under N₂. The dried lipids were redissolved in 3 ml of acetone. The amount of the dansylated products in the aliquot of the acetone extract was quantitatively estimated from the change in fluorescence by the use of a Perkin-Elmer LS-5B Luminescence Spectrometer. The fluorescence was measured with 450 nm as the exciting wavelength and 520 nm as the emission wavelength.

KINETIC ANALYSIS OF PROTON TRANSPORT

The net rate of proton transport and the decrease in net transport due to a leaky pressure effect has been addressed previously by Brauer *et al.* (1989) and Tu *et al.* (1987). The model can be used to described the time course of the formation of a proton gradient driven by a H⁺-translocation ATPase. Net proton pumping at a given time during formation a gradient may be represented by:

$$(d\delta/dt) = R_H - k_1 \delta \quad (1)$$

δ , in which is the net amount of proton transported across the membrane at time t after the pump is activated by the addition ATP.

R_H , is the initial proton pumping rate k_1 , the rate constant of proton leakage through energized membrane (in the presence of ATP hydrolysis) At steady state, the net of proton pumping, $d\delta/dt$

approach as zero therefore

$$R_H = k_1 \delta_s \quad (2)$$

where the subscript s denotes steady-state and reduced the equation (1) to $d\delta/dt = k_1 (\delta - \delta_s)$ or $\ln(1 - \delta/\delta_s) = -k_1 t$

The initial rate of proton transport, R_H , may be related to the initial rate of ATP hydrolysis, R as follows

$$R_H = mR$$

in which m is a measure of the coupling between the two processes.

After a proton gradient is established, its collapse can be induced by the addition of protonophores or inhibitors of ATP hydrolysis. The return of transported proton δ , may be assumed to obey a first order kinetics:

in which k_2 , is the first-order rate constant for proton leakage of de-energized membrane, and t, is

$$\begin{aligned} d\delta/dt &= k_2 \delta \\ \text{or } \ln(d\delta/\delta_s) &= -k_2 t \end{aligned} \quad (3)$$

the time after proton pumping has been pertained.

RESULTS AND DISCUSSION

ISOLATION AND MODIFICATION OF TONOPLAST VESICLES

Relatively pure tonoplast vesicles were obtained by following a previous established procedure (Tu *et al.* 1987) and were free of other membrane contaminants. When DC was added to the assay medium containing a simple amine (e.g. phenylalanine) a rapid increase in fluorescence was observed (data not shown). The reaction essentially reached completion within 2 min. When DC was added to tonoplast vesicles in the assay medium the fluorescence increased in both the total fraction and the lipid fraction following a similar kinetic pattern (Fig. 1). Unlike fluorescamine (FL), DC was relatively stable, no detectable hydrolysis of DC occurred during the labeling process. Therefore, tonoplast vesicles were allowed to react with DC for 3 min before testing functional effects by the modification of primary amine in tonoplast ATPase.

Fig. 1 showed a time course of DC modification by following the absorbance increase at 450 nm. The relative labeling efficiency was estimated by titrating a fixed amount of benzylamine with PC at different pH

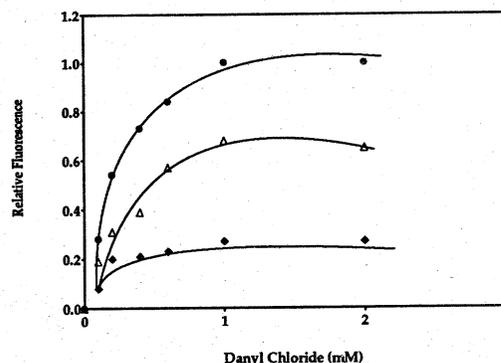


Fig. 1: Time dependence of dansyl chloride labeling on tonoplast vesicles: 10 μM of dansyl chloride (DC) was added in the tonoplast vesicles as described under the "Material and Methods". After separating the total labeling (●—●), lipids (Δ—Δ) and aqueous phase (◆—◆). The fluorescence measurement was determined at the excitation wavelength of 450 nm.

values. It was found that at pH 7.5 the labeling efficiency was maximum (data not shown).

DC reacted with the primary amines of tonoplast vesicles to yield fluorescent products. The labeling of the vesicles showed a concentration dependence. Fig. 1 indicated that the labeling of the total and lipid fractions reached a maxima between 1 to 2 min at room temperature. To identify the labeled lipid(s), extracted materials were separated by high performance thin layer chromatography according to the procedure of Moreau *et al.* (1985). However, the obtained results were not conclusive enough to identify the labeled lipids (data not shown).

THE EFFECT OF DC ON COUPLED OF PROTON PUMPING AND ATP HYDROLYSIS

As shown in Fig. 2, DC labeling of the tonoplast vesicles inhibited ATP hydrolysis significantly less than proton pumping. At the concentration of 20 μM of DC, ATP hydrolysis retained more than 90% of the control activity (without the treatment of DC), while H^+ -pumping activity decreased more than 40%. At 40 μM of DC, the ATP hydrolysis activity decreased less than 10%, while H^+ -pumping activity was completely eliminated. The observed preferential inhibition of proton pumping was not the result of interaction between the fluorescent label and the dye, acridine orange, used for monitoring proton

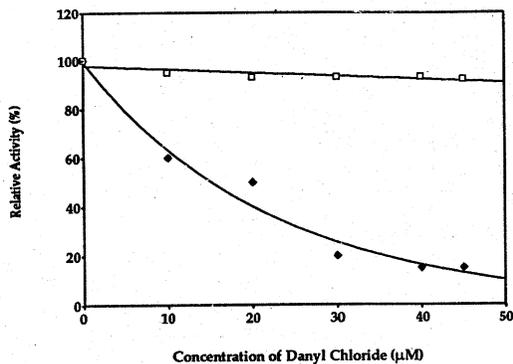


Fig. 2: Differentiation inhibition of coupled activity of ATP hydrolysis supported proton transport activity of tonoplast vesicles. Different concentrations of dansyl chloride (DC) were preincubated with tonoplast vesicles for 3 min before proton transport activity was initiated, as described in Materials and Methods. When proton pumping reached a steady state (no change in absorbance), 100 μ l of incubation mixture was assayed for released phosphate by malchite green molybdate method as described in Materials and Methods. One hundred percentage represented the ATP hydrolysis or proton transport rate without the addition of dansyl chloride.

movement. This was confirmed by following the procedure described by Tu *et al.* (1989). Neither the spectral properties (i.e. extinction coefficient or absorption maximum), nor the response of AC to pH changes was affected by DC modification (data not shown). Similarly the sensitivity and accuracy of the machite green assay method for phosphate determination were not affected by the presence of DC (data not shown).

The results described in Fig. 2 provided further evidence to support our previous indirect coupling hypothesis (Tu *et al.* 1987, Brauer *et al.* 1989) for corn root tonoplast vesicles. Fig. 2 showed a more pronounced sensitivity of proton pumping to DC treatment than did ATP hydrolysis. In our previously proposed indirect coupling model which emphasized the conformational interactions between different membrane components and predicts a differential influence on coupled activities by certain treatments. The applicability of this model is supported by the effects of nitrate ions (Tu *et al.* 1987), temperature change (Tu *et al.* 1988), and mercuric ions (Tu *et al.* 1991).

EFFECT DC MODIFICATION ON THE KINETIC PROPERTIES OF PROTON TRANSPORT

In previous reports (Tu *et al.* 1987, Brauer *et al.* 1989), a steady-state kinetic model was used to describe the time course of the formation of a proton gradient driven by a H^+ -translocating ATPase. The effect of DC modification on initial rate of proton pumping was shown in Fig. 3, which indicated that the proton leakage of energized membrane (k_1) decreased as the concentration of DC increased k_1 was calculated from the slope of the logarithm of $(1-\delta/\delta_s)$ vs time. The addition of 0, 10, 20, and 40 μ M of resulted a k_1 value of 1.36, 1.45, 1.56 and 1.67 min^{-1} respectively. In contrast, Fig. 4 indicated that the leakage rate of de-energized membrane (k_2), calculated from the logarithm of δ/δ_s vs time, was not affected by the addition of various concentrations of DC (0, 10 20 and 40 μ M). The induced leak rate constants were 0, 0.36, 0.37 and 0.39 min^{-1} respectively.

In this study, the effects of DC on proton pumping were reflected by a decrease in the quantity "m", a measure of the response of protonogenic domain to events occurring at the catalytic domain. We noticed that the tonoplast membrane proton leakage (k_2) was not affected by of DC treatment. This result would suggest that the de-energized membrane structure was not significantly affected by the labeling with DC. Thus, the mechanism for proton conduction

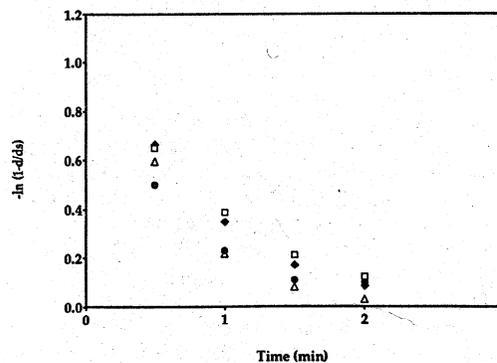


Fig. 3: The effect of dansyl chloride modification on the initial rate of tonoplast proton transport changes in acridine orange absorbance during the buildup of proton gradient was described in "Materials and Methods". Control (\square — \square), 10 μ M DC (\blacklozenge — \blacklozenge), 20 μ M DC (\bullet — \bullet), 40 μ M DC (Δ — Δ).

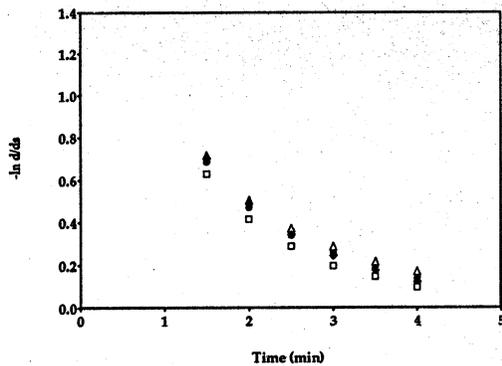


Fig. 4: The effect of dansyl chloride modification on the leakage rate of proton transport data from the collapse of the gradient was transformed and plotted according to equation (3) in "Material and Methods". Leakage rate was calculated from the log arithan of δ/δ_0 vs time. Control (□—□), 10 μM DC (◆—◆), 20 μM DC (●—●), 40 μM DC (△—△).

in deenergized membranes is not affected by the treatment. This observation would be consistent with the suggestion that metabolic variation could result in a reorganization of the energy transducing regions of the membrane as observed in the mitochondrial inner membrane (Lam *et al.* 1980). The results presented here are consistent with the previous observation that DC modification of tonoplast vesicles inhibits proton transport and ATP hydrolysis in a differentiation fashion.

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