

Evidence for an indirect coupling mechanism for the nitrate-sensitive proton pump from corn root tonoplast membranes

David Brauer, Shu-I Tu, An-Fei Hsu and Deidre Patterson

Brauer, D., Tu, S.-I., Hsu, A.-F. and Patterson, D. 1993. Evidence for an indirect coupling mechanism for the nitrate-sensitive proton pump from corn root tonoplast membranes. – *Physiol. Plant.* 89: 588–591.

The nitrate-sensitive proton-translocating adenosine triphosphatase (H^+ -ATPase) of tonoplast membranes plays an important role in regulating the flow of nutrients and metabolic waste between the cytoplasm and vacuole in the cells of plant roots. Relatively little information is available regarding the coupling between ATP hydrolysis and proton pumping by the nitrate-sensitive, tonoplast H^+ -ATPase. The coupling may be achieved either directly, i.e. the two reaction pathways share at least one common molecular step, or indirectly, i.e. the two reaction pathways do not share an intermediate step. These coupling mechanisms may be differentiated by the responses of the two events to external perturbation. The effects of the presence of nitrate in the assay medium on the rates of ATP hydrolysis and proton transport catalyzed by the tonoplast H^+ -ATPase from maize (*Zea mays* L. cv. FRB 73) were investigated. The presence of nitrate inhibited proton transport activity of the tonoplast H^+ -ATPase to a much greater degree than ATP hydrolysis. This differential response of the two activities to nitrate is the basis for a proposed reaction model for the tonoplast H^+ -ATPase that features an indirect coupling mechanism between ATP hydrolysis and proton transport.

Key words – Coupling, H^+ -ATPase, hydrolysis, maize, transport, *Zea mays*.

D. Brauer (corresponding author) et al., Plant and Soil Biophysics Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Dept of Agriculture, Philadelphia, PA 19118, USA.

This paper is part of the contributions to the Proceedings of the 3rd International Symposium on Inorganic Nitrogen Assimilation, held in Tiberias, Israel, 6–11 September, 1992.

Introduction

Membranes isolated from plant roots contain at least two H^+ -ATPases (DeMichaelis et al. 1983, Sze 1984, 1985). One of these enzymes is localized on the tonoplast membrane and has sensitivities to nitrate and vanadate similar to other vacuolar-type H^+ -ATPases (Nelson and Taiz 1989, Sze 1984, 1985). This enzyme is probably involved in regulating the pH of the cytoplasm and mediating secondary transport processes across the tonoplast. The other enzyme is believed to be localized on the plasma membrane and shares the characteristics of the E1–E2 type H^+ -ATPase in forming an aspartyl phosphate intermediate, and being sensitive to vanadate (Nelson and

Taiz 1989, Sze 1984, 1985). The H^+ -ATPase of the plasma membrane may also be involved in regulating cytoplasmic pH, in addition to mediating transport into and out of the cell. 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 (Sze 1984, 1985).

By their actions, transport H^+ -ATPases are transducers of free energy, interconverting the free energy available in the chemical bonds of ATP and the free energy conserved in electrochemical gradients across membranes. The mechanism by which this free energy is transformed by these proteins is still poorly understood, despite a

large volume of research. Conceptually, there are two possible mechanisms by which ATPases may convert the energy from ATP hydrolysis into an electrochemical gradient: direct or indirect coupling. If the process leading to proton transport shares part of the molecular pathway responsible for ATP hydrolysis, then this is said to be direct coupling. An example of direct coupling is the redox-loop mechanism proposed for electron transfer-coupled proton movement in mitochondria (Mitchell 1975). When proton transport is directly coupled to ATP hydrolysis by an ATPase, experimental treatments will affect proton transport to the same extent as ATP hydrolysis. Alternatively, the primary energy-releasing process, i.e. ATP hydrolysis, may be only indirectly linked to proton transport, so that certain intermediate steps are required to couple ATP hydrolysis to the transport of protons. If proton transport is indirectly coupled to ATP hydrolysis, changes in proton transport may not be manifested by changes in ATP hydrolysis; i.e. the two reactions can maintain separate rates. Experimental conditions that can evoke such differential changes in the coupled activities of corn root tonoplast H⁺-ATPase have been sought by this laboratory. Membranes from maize roots of the cultivar FRB 73 were utilized because of the ability to isolate tonoplast vesicles to a very high degree of purity (Tu et al. 1987).

Assay to determine initial rates of proton influx

Proton transport by isolated membrane vesicles is typically followed *in vitro* by changes in the absorbance or fluorescence of pH sensitive dyes like acridine orange or quinacrine (Bennett and Spanswick 1983, Brauer et al. 1989). These assays measure net proton transport, not the unidirectional flux of protons. Therefore, the first experimental challenge was to devise a method for obtaining an estimate of the initial unidirectional rate of proton transport by the tonoplast H⁺-ATPase *in vitro*.

Our approach to solving this problem was to determine a means of extracting the information from our current assays for following net proton transport by changes in the absorbance and fluorescence of acridine orange (Brauer et al. 1989, Tu et al. 1987). A priori, the rate of net proton transport to the interior of membrane vesicles at a given time is the difference between influx catalyzed by the proton pump and the sum of inhibitory pathways, which lead to the efflux of protons from the lumen of the membrane vesicle. Mathematically, this relationship can be expressed as:

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

in which δ is the net amount of protons transported across the membrane at time t after the addition of ATP, and R_H is the initial rate of ATP-supported proton pump. The rate constant, k_i represents the processes that lead to the efflux of protons from vesicles. The quantity δ can be directly related to changes in absorbance of acridine orange

(Brauer et al. 1989, Perlin et al. 1986). The initial rate of proton transport can be related to the rate of ATP hydrolysis by:

$$R_H = m \times R_{ATP} \quad (2)$$

where R_{ATP} is the initial rate of ATP hydrolysis and m is a measure of the coupling between ATP hydrolysis and proton transport. The average rate of ATP hydrolysis over the time period of proton gradient build-up is approximately equal to R_{ATP} (Brauer et al. 1989, Tu et al. 1987). Alternatively, R_{ATP} may be determined by measuring the formation of ADP by a coupled enzyme assay (Brauer et al. 1989, Tu et al. 1987).

At steady state, the net rate of proton transport, $d\delta/dt$, approaches zero and therefore,

$$R_H = k_i \times \delta_s \quad (3)$$

where the subscript s denotes the steady state level. Application of the steady state approximation yields

$$d\delta/dt = k_i (\delta - \delta_s) \quad (4)$$

Upon integration of Eq. (4), the following is obtained

$$\ln(1 - \delta/\delta_s) = -k_i \times t \quad (5)$$

Therefore, Eq. (5) provides a convenient means of estimating k_i the rate constant that represents the loss of protons from the vesicles during proton pumping. Thus, the initial rate of proton influx into the vesicles (R_H) is determined from Eq. (3).

This mathematical treatment of the proton transport time course enables the researcher to quantify both the initial rate of proton movement into the vesicles and rate of proton loss from vesicles. Such treatment of the proton transport data allows the investigator to determine if changes in proton transport arise from direct treatment effects on the proton transport processes of the H⁺-ATPase or from changes in the rate of efflux from the vesicles.

Application of assay to nitrate-sensitive proton transport

When highly purified corn root tonoplast vesicles were incubated in the presence of acridine orange, there was an ATP-dependent decrease in absorbance at 492.5 nm. This decrease in absorbance was consistent with the movement of protons into the lumen of the vesicle (Fig. 1A). It was apparent from the time course that the net rate of proton transport into the vesicles decreased as the magnitude of the gradient increased. Eventually, the net rate of proton transport became zero. It was also apparent from Fig. 1A that nitrate had a negative effect on net proton transport.

When the data in Fig. 1A were fitted to the mathe-

higher inhibitor concentrations, the link between the two domains is completely blocked. Consequently, the turnover of the enzyme cannot be completed, and the result is an inhibition of ATP hydrolysis.

The possible implications of nitrate inhibition of proton transport by the H⁺-ATPase on the physiology of plant cells in situ has not been resolved. Based on the analysis of Granstedt and Huffaker (1982), the cytoplasm of plant cells may contain millimolar levels of nitrate. Such levels of nitrate could yield sufficiently high molar fractions of nitrate in the cytoplasm to inhibit the nitrate-sensitive proton pump of the tonoplast membrane, provided the chloride concentration is less than 100 mM. Therefore, nitrate-induced inhibition of the tonoplast H⁺-ATPase may occur in situ.

References

- Bennett, A. B. & Spanswick, R. M. 1983. Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles: kinetic analysis of Cl⁻ effects on a proton-translocating ATPase. – *J. Membr. Biol.* 71: 95–107.
- Brauer, D., Tu, S.-I., Hsu, A.-F. & Thomas, C. E. 1989. Kinetic analysis of proton transport by maize root microsomes. – *Plant Physiol.* 89: 464–471.
- DeMichaelis, M. I., Pugliarello, M. C. & Rasi-Calogno, F. 1983. Two distinct proton translocating ATPases are present in membrane vesicles from radish seedlings. – *FEBS Lett.* 162: 85–90.
- Granstedt, R. C. & Huffaker, R. C. 1982. Identification of the leaf vacuole as a major nitrate storage pool. – *Plant Physiol.* 70: 410–413.
- Mitchell, P. 1975. Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: power transmission by proticity. – *FEBS Lett.* 59: 137–139.
- Nelson, N. & Taiz, L. 1989. The evolution of H⁺-ATPase. – *Trends Biochem. Sci.* 14: 113–116.
- Perlin, D. S., San Francisco, M. J. D., Slayman, C. W. & Rosen, B. P. 1986. H⁺/ATP stoichiometry of proton pumps from *Neurospora crassa* and *Escherichia coli*. – *Arch. Biochem. Biophys.* 248: 53–61.
- Sze, H. 1984. H⁺-translocating ATPase of the plasma membrane and tonoplast of plant cells. – *Physiol. Plant.* 61: 683–691.
- 1985. H⁺-translocating ATPases: Advances using membrane vesicles. – *Annu. Rev. Plant Physiol.* 36: 175–208.
- Tu, S.-I., Nagahashi, G. & Brouillette, J. N. 1987. Proton pumping kinetics and origin of nitrate inhibition of the tonoplast-type of H⁺-ATPase. – *Arch. Biochem. Biophys.* 266: 289–297.