ATP synthesis catalyzed by a V-ATPase: an alternative pathway for energy conservation operating in plant vacuoles?

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ABSTRACT

The electrochemical H^+ gradient generated in tonoplast vesicles isolated from maize seeds was found to be able to drive the reversal of the catalytic cycle of both vacuolar H^+ -pumps (Façanha and de Meis, 1998). Here we describe the reversibility of the vacuolar V-type H^+ -ATPase (V-ATPase) even in the absence of the H^+ gradient in a water- Me_2SO co-solvent mixture, resulting in net synthesis of $[\gamma\text{-}^{32}P]ATP$ from $[^{32}P]P_i$ and ADP. The water- Me_2SO (5 to 20 %) media promoted inhibition of both PP_i hydrolysis and synthesis reactions whereas it slightly affected the ATP hydrolysis and clearly stimulated the ATP synthesis, which was unaffected by uncoupling agents (FCCP, Triton X-100 or NH_4^+). This effect of Me_2SO on the $ATP \rightleftharpoons ^{32}P$ exchange reaction seems to be related to a decrease of the apparent K_m of the V-ATPase for P_i . The results are in accordance to the concept that the energetics of ATP synthesis catalysis depends on the solvation energies interacting in the enzyme microenvironment. A possible physiological significance of this phenomenon for the metabolism of desiccation-tolerant plant cells is discussed. [Physiol. Mol. Biol. Plants 2008; 14(3) : 195-203] E-mail : arfacanha@yahoo.com.br

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Abbreviations : ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide *p*(trifluoromethoxy)-phenylhydrazone; Me_2SO , dimethyl sulfoxide; V-ATPase, V-type H^+ -ATPase; H^+ -PPase, membrane-bound H^+ pyrophosphatase.

It has been argued that the inorganic pyrophosphate (PP_i) of plant cells may function as an alternative source of energy to circumvent ATP-dependent processes during stress conditions or developmental stages when cellular adenylates are low (Stitt, 1998). In this context two enzymes have been extensively characterized, the pyrophosphate-dependent phosphofructokinase that represent a glycolytic bypass to the ATP-limited phosphofructokinase (Duff *et al.*, 1989) and the tonoplast H^+ -PPase which is considered as a backup system for a maintenance of the ATP-dependent H^+ gradient across the vacuolar membrane (Rea and Sanders, 1987). Nevertheless, there are many other key reactions where PP_i cannot replace ATP in the plant metabolism. In this regard, an alternative pathway has

been explored where ATP synthesis could be driven by the V-ATPase coupled to a H^+ gradient generated in the tonoplast by the H^+ -PPase (Dupaix *et al.*, 1989; Façanha and de Meis, 1998). Despite of this, a common sense prevails that under physiological conditions the reversal of vacuolar H^+ -ATPase would be unlikely due to differences in the ΔG values for ATP synthesis and PP_i hydrolysis (Schmidt and Briskin, 1993a and b). This assumption was based on thermodynamic calculations considering equilibrium constants determined in aqueous medium assumed to be representative of most physiological environments.

On the other hand, higher plants are able to produce structures such as seeds and pollen, which are naturally adapted to desiccation. In addition, the species termed resurrection plants possess desiccation-tolerant vegetative tissues (Vicré *et al.*, 2004). In fact, several abiotic stresses such as chilling, drought or salt stress

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cause a disruption of the plant cell water status. Thus, it is fairly hard to predict the water activity and solvation energies that influence the equilibrium of enzymatic reactions in the dynamic microenvironment of living cells. Furthermore, it is now well established that enzymes can function in the absence of bulk water, their activities can be modulated, their selectivities tailored, and their stabilities altered (Klibanov, 2001; Lee and Dordick, 2002; Gupta and Roy, 2004).

Although, in aqueous media the ATP hydrolysis and synthesis are accompanied by a large change of the free energy, under hydrophobic conditions these reactions are accompanied by only a small energy change (George *et al.*, 1970; Hayes *et al.*, 1978). It has been proposed that the large change in energy of hydrolysis/synthesis of several phosphate compounds is promoted by small changes in water structure in the enzyme microenvironment (Boyer *et al.*, 1977; de Meis *et al.*, 1985). Actually, it is now clear that the thermodynamic equilibrium of many catalytic reactions is more favorable in organic solvent than in water (Klibanov, 2001).

Dimethyl sulfoxide (Me_2SO) is a widely used dipolar organic solvent which can interact with water, salts, protein and lipids and penetrate cell membranes without irreversible damages. Water- Me_2SO mixtures have been described to increase the hydrophobicity of aqueous systems without affecting significantly the water dielectric constant (Otero and de Meis, 1982). Thus, this organic solvent has proved to be useful for studies on the influence of the water activity on the functional and conformational dynamics of several ion transport ATPases (Tribuzy *et al.*, 2002). Herein, Me_2SO was used as a tool to study the enzymatic behavior of the tonoplast H^+ pumps in plant cell environments with low water activity. The present study provides further evidences for the reversibility of the tonoplast V-ATPase and discusses the means by which this mechanism could take place *in vivo*.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L.) seeds were provided by the Laboratory for Plant Genetic Improvement of the State University of North Fluminense Darcy Ribeiro (UENF), Rio de Janeiro, Brazil. The seeds were surface sterilized in 2% sodium hypochlorite solution for 10 min, and soaked in water for 12 h before the cell fractionation procedures.

Tonoplast-Enriched Vesicles

Hydrating maize seeds were used for isolation of tonoplast vesicles by differential centrifugation as described by Façanha and de Meis (1998). Briefly, about 150 g of seeds were homogenized in 2 mL/g (fresh weight) of an ice-cold buffer containing 10 % (v/v) glycerol, 0.5 % (v/v) polyvinylpyrrolidone (PVP-40, KDa 40), 5 mM EDTA, 0.13 % (w/v) BSA and 0.1 M Tris/HCl buffer (pH 8.0). Just prior to use 150 mM KCl, 3.3 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at $8,000\times g$ for 10 min. The supernatant was centrifuged once more at $8,000\times g$ for 10 min and then at $100,000\times g$ for 40 min. The pellet containing microsomes was homogenized in a small volume of the storage ice-cold buffer: 10 mM Tris-HCl (pH 7.6), 10 % (v/v) glycerol, 1 mM dithiothreitol, 1 mM EDTA and 1 mM PMSF. In order to obtain the tonoplast vesicles the microsomal fraction was layered over a 10/30/46% (w/w) discontinuous sucrose gradient contained the respective sucrose concentrations diluted in the storage buffer. After centrifugation at $100,000\times g$ for 3 h in a swinging bucket rotor, the vesicles sedimented at the interface between 10 and 30% sucrose were collected, diluted with 3 volumes of ice-cold water and centrifuged at $100,000\times g$ for 40 min. Bafilomycin A_1 -inhibited H^+ -ATPase and K^+ -dependent H^+ -PPase activities were used as marker-enzymes for the vacuolar membrane (Sze, 1985). The pellet was homogenized in the storage medium and the vesicles were either used immediately or frozen under liquid nitrogen and stored at -70°C until use. Protein concentrations were determined by the method of Lowry *et al.* (1951).

ATPase and PPase activity

The ATP or PP_i hydrolysis activities were determined at 25°C by measuring the P_i release, either colorimetrically (Fiske and Subbarow, 1925) or using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as previously described (de Meis, 1988). Between 85 and 100% of the vesicle ATPase activity measured at pH 7.0 was inhibited by 50 mM KNO_3 or 50 nM Bafilomycin A_1 , two specific inhibitors of V-type H^+ -ATPases (Bowman *et al.*, 1988; White, 1994). In all experiments the ATPase activity was measured with and without Bafilomycin A_1 and the difference between these two activities was attributed to the maize seed V-ATPase. Pyrophosphatase and ATPase activities of tonoplast vesicles were unaffected by vanadate (0.1 mM), an inhibitor of plasma membrane ATPase, or oligomycin (10 nM), an inhibitor of mitochondrial ATPases.

Electrochemical Gradient of Protons

The accumulation of H⁺ by the membrane vesicles was determined at 25°C by measuring the fluorescence quenching of the probe 9-amino-6-chloro-2-methoxyacridine (ACMA), using a Hitachi (Tokyo, Japan) F-3010 fluorometer. The excitation wavelength was set at 415 nm and emission wavelength at 485 nm. The reaction medium contained 10 mM MOPS/Tris (pH 7.0), 2 mM ACMA, 3 mM MgCl₂, and 100 mM KCl. In different vesicle preparations tested, the pretreatment with 50 nM Bafilomycin A₁ or 500 μM imidodiphosphate in the assay medium almost complete inhibited the fluorescence quenching measured after the addition of ATP or PP_i, respectively (data not shown).

P_i ↔ PP_i and P_i ↔ ATP Exchange Measurements

Synthesis of PP_i and ATP by the tonoplast vesicles was assayed by measuring the amount of [³²P]PP_i and [γ-³²P]ATP formed during the cleavage of non-radioactive ATP or PP_i (de Meis, 1984; Behrens and de Meis, 1985; de Meis *et al.*, 1985). The assay medium contained 50 mM Mops-Tris buffer (pH 7.0), 3 mM MgCl₂, 5 mM [³²P]_i (20,000 cpm/nmol P_i), 100 mM KCl and tonoplast vesicles to a final concentration of 0.05 mg/mL protein. The reaction was started by addition of either 0.2 mM PP_i or 1 mM ATP and after 30 min at 25°C (or in different times for the figure 3) samples of the reaction medium were quenched with ice-cold trichloroacetic acid and used to determine the total amount of P_i esterified as either ATP or PP_i. The free ³²P_i was extracted as phosphomolybdate using isobutyl alcohol-benzene as previously described (de Meis, 1984; de Meis *et al.* 1985). The small amount of radioactivity found in controls (not incubated with the tonoplast vesicles) was subtracted from that found in the experiments with vesicles.

Identification of ATP and Pyrophosphate Labeled with ³²P_i

Identification of the products synthesized during the ³²P_i incorporation was achieved by the pretreatment with Bafilomycin A₁, a specific inhibitor of V-type ATPases, and by the accessibility of synthesized pyrophosphate to soluble inorganic pyrophosphatase purified from yeast (EC 3.61.1; Sigma I-4503 lyophilized powder). As previously reported, Bafilomycin A₁ was able to inhibit the ATP synthesis promoted by PP_i hydrolysis (Schmidt and Briskin, 1993b; Façanha and de Meis, 1998; Hirata *et al.*, 2000) as well as post incubation of medium containing the products synthesized with the yeast soluble pyrophosphatase promoted the

hydrolysis of the PP_i labeled by the ³²P_i incorporation (Tuena de Gómez-Puyou *et al.*, 1993; Façanha and de Meis, 1998). The radioactivity remaining in the aqueous phase after extraction of the free ³²P_i with isobutyl alcohol-benzene (representing total ³²P_i esterified) was subtracted from that of the sample previously treated with Bafilomycin A₁ and the difference was attributed to the ATP synthesized. In order to discriminate the fraction of ³²P_i incorporation representing PP_i synthesis, after the exchange reaction was completed, the reaction medium was filtered (0.22-μm filters, Millipore) to remove the tonoplast vesicles. Then, two equal aliquots of the mixture were withdrawn. To one of these samples 0.1 mM PP_i and 10 μg/mL yeast pyrophosphatase were added. The addition of 0.1 mM PP_i was required to optimize the hydrolysis activity (data not shown). Afterward, both samples were incubated for 30 min at 35°C. At this time, 0.5 mL of ammonium molybdate in H₂SO₄ was added to the two samples and all free ³²P_i was extracted as phosphomolybdate using isobutyl alcohol-benzene. The radioactivity present in the aqueous phase was determined. The difference in radioactivity between the samples that did or did not receive soluble yeast pyrophosphatase was considered to correspond to PP_i synthesized. The ATP synthesized was correlated with the Bafilomycin-sensitive ³²P_i incorporation.

Reagents

Bafilomycin A₁, FCCP, ADP and ATP were purchased from Sigma and Me₂SO from Merk. All other reagents used were also of analytical grade. A 500 mM P_i-Tris stock solution adjusted to pH 7.0 was prepared by mixing aqueous solutions of phosphoric acid and Tris base. One-mM stock solutions of FCCP or ACMA in ethanol were used. The final concentration of ethanol in the assay medium never exceeded 0.03%.

RESULTS

The proton gradient of the tonoplast vesicles from maize seeds

Figure 1 shows that the tonoplast vesicles isolated from maize seeds were able to maintain an electrochemical H⁺ gradient generated by either ATP- or PP_i-dependent H⁺ translocation. The addition of the V-ATPase inhibitor Bafilomycin A₁ after the ATP-dependent H⁺ gradient has reached the steady-state induced a rapid recovery of the initial fluorescence, but H⁺ translocation was recuperated by the addition of PP_i 0.2 mM resulting in a striking H⁺ gradient. Addition of either 3 μM of the

protonophore FCCP or the detergent Triton X-100 (0.02%) abolished the H^+ gradient formed regardless the substrate used. No H^+ translocation could be detected in the presence of Me_2SO .

The initial velocities of ATP-dependent H^+ -transport as well as the ATP hydrolysis were several times slower than those of PP_i -dependent H^+ -transport (Fig. 1) and PP_i hydrolysis (Fig. 2a), respectively. This differential activation of the H^+ -PPase in relation of the H^+ -ATPase in corn seeds tonoplast is in accordance with the preponderance of the vacuolar H^+ -PPase previously reported for the early stages of plant development (Suzuki and Kasamo, 1993; Maeshima *et al.*, 1994; Swanson and Jones, 1996).

Effects of Me_2SO on H^+ -ATPase and H^+ -PPase activities in tonoplast vesicles

When PP_i was used as substrate for formation of the H^+ gradient, the forward (PP_i hydrolysis) and the reversal reactions (as measured by the $^{32}P_i$ incorporation) were both inhibited by increasing concentrations of Me_2SO (Fig. 2). On the other hand, using ATP as substrate, the ATP hydrolysis was not significantly affected by Me_2SO (Fig. 2a) whereas the $^{32}P_i$ incorporation was remarkably

Fig. 1. ATP and PP_i -dependent H^+ -gradient generated by the vacuolar H^+ -pumps of maize seeds. The H^+ transport was measured in a medium containing 10 mM MOPS/Tris (pH 7.0), 3 mM $MgCl_2$, 100 mM KCl and 2 μ M of the pH-sensitive fluorescent probe ACMA. The addition of 50 nM Bafilomycin A_1 dissipated specifically the ATP-dependent H^+ -gradient, whereas the addition of either 3 μ M FCCP or 0.02% Triton X-100 dissipated the H^+ -gradient generated by either ATP or PP_i . No H^+ -gradient was formed in the presence of Me_2SO (10–20%). Similar results were obtained in various assays using at least four different tonoplast vesicles preparations.

Fig. 2. ATP or PP_i hydrolysis (A) and $^{32}P_i$ incorporation (B) catalyzed by the vacuolar H^+ -pumps of maize seeds in the presence of different Me_2SO concentrations. The assay medium composition was 50 mM MOPS-Tris buffer (pH 7.0), 0.1 mM ADP, 100 mM KCl, 5 mM $^{32}P_i$, 5 mM $MgCl_2$ and 0.05 mg/mL tonoplast vesicles. The reaction was started by addition of (open circles) 0.2 mM PP_i or (open triangles) 1 mM ATP. Closed symbols in B represent the $^{32}P_i$ incorporation measured in the membrane vesicles permeabilized by the protonophore FCCP (3 μ M). The data represent means of four replicates from different tonoplast vesicles preparations.

enhanced (Fig. 2b). The lasting $^{32}P_i$ incorporation obtained in the presence of 20% Me_2SO reached similar values regardless the substrate used (Fig. 2b).

In the absence of Me_2SO , the addition of uncoupling agents such as Triton X-100 and FCCP blocked the [$^{32}P_i$] exchange reactions, however, in the presence of Me_2SO the [$^{32}P_i$] incorporation was detected even in permeabilized tonoplast vesicles (Figs. 2b and 3). When the reaction was triggered by ATP, in the presence of 2.5 μ M FCCP or 0.02% Triton X-100, 20% Me_2SO was able to promote equivalent levels of $^{32}P_i$ incorporation reached for the control without uncouplers (Figs. 2b and 3). The time course of the $^{32}P_i$ incorporation showed that the synthesis reaction seems to stop after 30 min and after 60 min some degradation of the products synthesized was verified in the presence of Me_2SO (Fig. 3).

P_i -dependence of the $^{32}P_i$ incorporation in the presence of Me_2SO

The rate of $^{32}P_i$ exchange reaction varied with the P_i concentration of the medium (Fig. 4). The affinity of the tonoplast H^+ -pumps for P_i seems to be low, since the saturation was not reached in the absence of Me_2SO even at 12 mM P_i . Thus, it was not possible to measure the apparent K_m for P_i for the control without Me_2SO (Fig. 4). However, the addition of Me_2SO strikingly enhanced the enzyme affinity for $^{32}P_i$ incorporation

Fig. 3. Time course of $^{32}\text{P}_i$ incorporation in seed tonoplast vesicles in the presence or absence of Me₂SO. The assay medium composition was 50 mM MOPS-Tris buffer (pH 7.0), 0.1 mM ADP, 100 mM KCl, 5 mM $^{32}\text{P}_i$, 5 mM MgCl₂ and 0.05 mg/mL tonoplast vesicles. The reaction was started by the addition of 0.2 mM PP_i and arrested by the addition of trichloroacetic acid at determined times. The $^{32}\text{P}_i$ incorporation was measured in intact vesicles (open circles) and membrane vesicles permeabilized by the detergent Triton X-100 (0.02%) (open diamonds). Closed symbols represent data obtained in the presence of 20% Me₂SO. Essentially the same results were obtained in three experiments using different vesicle preparations.

Fig. 4. Phosphate-dependence of the $^{32}\text{P}_i$ incorporation in the presence or absence of Me₂SO. The reaction was performed at 25°C in a medium containing 50 mM MOPS-Tris buffer (pH 7.0), 0.1 mM ADP, 100 mM KCl, 5 mM MgCl₂ and 0.05 mg/mL tonoplast vesicles, using as substrate either 0.2 mM PP_i (A, open circles) or 1 mM ATP (B, open triangles). Closed symbols represent data obtained in the presence of 20% Me₂SO. The reaction was carried out in the presence of increasing P_i concentrations, maintaining the radioactive $^{32}\text{P}_i$: non-radioactive P_i ratio constant at 20,000 cpm/nmol of non-radioactive P_i. Essentially the same results were obtained in four independent experiments.

promoting a decrease of apparent K_m for P_i when ATP was the substrate (Fig. 4a) as well as when the reaction was started by PP_i (Fig. 4b).

Identification of the synthesized phosphate compounds

The identification of the ATP synthesized was estimated by the sensitivity of the $^{32}\text{P}_i$ incorporation to the V-ATPase inhibitor Bafilomycin A₁. The PP_i synthesized was accessed by its post-hydrolysis using a commercial soluble inorganic pyrophosphatase purified from yeast. Bafilomycin A₁ was able to inhibit the ATP synthesis promoted by PP_i hydrolysis as previously reported (Schmidt and Briskin, 1993b; Façanha and de Meis, 1998; Hirata *et al.*, 2000). In the presence of Me₂SO no significant amount of $^{32}\text{P}_i$ incorporation was measured after pre-incubation of vesicles with Bafilomycin A₁ and the total $^{32}\text{P}_i$ incorporation was minimally affected by treatment with yeast pyrophosphatase (Table I). These data indicate that most of $^{32}\text{P}_i$ incorporation represented ATP synthesized regardless of the substrate used suggesting that Me₂SO can inhibit both the forward and reversal reactions of the tonoplast H⁺-PPase.

Table 1. Identification of the products synthesized by using Bafilomycin A₁ and yeast soluble pyrophosphatase (YsPPase). The reactions were started by adding either 1 mM ATP or 0.2 mM PP_i. After 30 min the reactions were stopped by filtration to remove the membrane vesicles and the filtrates were divided in two aliquots. One of them was incubated with 0.01 mg/mL YsPPase in order to hydrolyze the PP_i synthesized. The reaction was stopped after 30 min by the addition of trichloroacetic acid (10% w/v). Then the medium was subjected to phosphomolybdate extraction, and the radioactivity remaining in the aqueous phase was counted. The $^{32}\text{P}_i$ incorporation sensitive to 50 nM Bafilomycin A₁ was related to the ATP synthesized by the V-ATPase. The data are expressed in nmol of $^{32}\text{P}_i$ incorporated per mg per 30 min. Values represent the mean ± SE of four independent experiments.

Me ₂ SO (%)	ATP	ATP + YsPPase	PP _i	PP _i + Bafilomycin
0	5.0 ± 1.6	2.1 ± 1.4	27 ± 4.8	12.5 ± 3.3
5	7.5 ± 2.4	5.5 ± 2.5	24 ± 7.0	7.3 ± 2.6
10	10 ± 2.6	9.2 ± 2.8	20 ± 4.5	3.6 ± 1.5
15	13 ± 3.1	12 ± 2.5	18 ± 3.6	3.3 ± 1.7
20	14 ± 2.7	13 ± 3.7	15 ± 4.0	3.4 ± 1.5

DISCUSSION

It is generally assumed that the energy derived from an ion gradient would represent an absolute energetic requirement for the net ATP synthesis catalyzed by ion pumps. However, it was previously reported that using water-Me₂SO co-solvent media the catalytic cycle of some of these enzymes could be reversed even in the absence of an ion gradient. For instance, in the presence of water-Me₂SO, F-type ATPases of mitochondria (Sakamoto and Tonomura, 1983; Yoshida, 1983; Cross *et al.*, 1984) and chloroplasts (Labahn and Graber, 1992) carried out spontaneous synthesis of ATP that remained tightly bound at the catalytic site of the enzymes. Moreover, it was also demonstrated that P-type Ca²⁺-ATPases can catalyze net ATP synthesis in the absence of a transmembrane Ca²⁺ gradient (de Meis and Carvalho 1974; de Meis and Vianna, 1979). In the present work, it is described for the first time the reversal reaction of a V-ATPase in absence of a H⁺ gradient by using aqueous-Me₂SO media.

Façanha and de Meis (1998) have previously reported that the H⁺ gradient generated by hydrolysis of either PP_i or ATP could be used to promote reversal of the catalytic cycle of both the vacuolar H⁺-PPase and V-ATPase. The present data show that the addition of Me₂SO to the reaction medium promotes a clear stimulation of the ATP synthesis by the V-ATPase whereas markedly inhibits the forward and reversal reaction of the tonoplast H⁺-PPase (Figs. 2). Interestingly, Grazinoli-Garrido and Sola-Penna (2004) have showed that the soluble yeast inorganic pyrophosphatase is also inactivated by several organic solvents (methanol, ethanol, propanol and acetone) depending on their hydrophobicity. This indicates that both the soluble and membrane bound pyrophosphatases seem to be highly sensitive to organic solvents.

The differential effect of Me₂SO on the two tonoplast H⁺ pumps could be due to their highly distinct structures. The tonoplast H⁺-PPase is an extremely hydrophobic protein of about 80 kDa (Zancani *et al.*, 2007) that form homodimers in the native membranes (Yang *et al.*, 1998). It is likely that the Me₂SO molecules disturb the subunits stability and/or cause conformational disturbances in the catalytic site of this enzyme that is localized in a short cytoplasmic loop very close to the membrane spans. Conversely, the V-ATPase is an oligomeric enzyme with molecular mass of about 700 kDa, which poses a large extramembranous catalytic portion composed by six subunits. In general,

such extramembranous parts of membrane proteins may behave much like soluble proteins (Haltia and Freire, 1995). Although, structure parameters of membrane- and water-soluble proteins are surprisingly similar, given the different dielectric environments, there are differences in the sensibility to hydrophobic and electrostatic forces (Cramer *et al.*, 1992).

In the early stages of seed hydration, the mitochondria are functionally deficient and the adenylate energy of the cell is quite low (Ivanov and Khavkin, 1976; Bewley and Black, 1985). Contrary to ATP, the cellular PP_i content was found to be five-fold higher in non-dormant than in dormant oat seeds and this level persisted during the hydration (Larondelle *et al.*, 1987). The prevalence of H⁺-PPase activity in hydrating seeds (Fig. 1) supports the idea that the physiological significance of this enzyme would be to maintain the H⁺ gradient under conditions of limited ATP supply, such as that occur during the seed germination and early stages of plant development (Façanha and de Meis, 1998).

The effects of Me₂SO on the reversal of the catalytic cycle of V-ATPase observed *in vitro* could reproduce, to some extent, the low water activity condition of a living seed cell. Under physiological conditions, lacking the chemical side effects of the Me₂SO on the H⁺-PPase and membrane ΔpH (Fig. 1), it is possible that the energy derived from the PP_i-dependent H⁺ gradient could act synergically with the hydrophobic forces for the ATP synthesis by V-ATPases. For the reversibility of most ATPases so far studied, the electrochemical gradient seems to improve the ratio between the rates of synthesis and hydrolysis of ATP mainly by increasing the affinity of the enzymes for P_i (de Meis, 1993; Boyer, 1997; Weber and Senior, 1997). A proposed mechanism was that immediately after the products of the hydrolysis are released from the enzyme surface and before returning to the conformation that allows the beginning of a new catalytic cycle, some ATPases can re-bind the products, reverse its catalytic cycle and synthesize a new substrate molecule (Sakamoto and Tonomura, 1983; de Meis *et al.*, 1986; de Meis, 1981 and 1989; Tuena de Gómez-Puyou *et al.*, 1993). Given the capacity of Me₂SO to circumvent the H⁺ gradient requirement, it is perhaps not surprising that the affinity of the V-ATPase for P_i was strongly enhanced by this organic solvent (Fig. 4), suggesting that it induces stabilization of a conformation in which the enzyme could bind easier the P_i (and probably also ADP) for the reversal reaction.

CONCLUSIONS

The present study provides further evidences for the reversibility of the catalytic cycle of the plant vacuolar V-type H⁺-ATPase. The Me₂SO effects suggest that given appropriate thermodynamic conditions the tonoplast H⁺-ATPase may operate as a system for energy conservation, playing a role in the cytosolic ATP homeostasis in plant cells. The data could be interpreted in terms of a specific enzyme conformation of higher affinity for ADP and P_i induced by Me₂SO interactions through a rearrangement of water structure in the catalytic microenvironment of the V-ATPase and/or in its membrane vicinities. It is tempting to speculate that similar conformational changes could also occur in dehydrating environments of seed cells and other plant tissues adapted to desiccation, resulting in a physiologically relevant net synthesis of ATP.

FUTURE PERSPECTIVES

The mechanism by which the energy derived from ion gradients formed across biomembranes is transduced for the synthesis of ATP still remains a central theme in bioenergetics even after the elucidation of the F-type ATP-synthase mechanism (Boyer, 1997). In this regard, taking advantage of the similarities found between the mechanisms of F-type and V-type ATPases (Yokoyama *et al.*, 1998, Hirata *et al.*, 2003), it is possible to compare the conformational changes induced by the H⁺ gradient membrane energization and that derived from the Me₂SO hydrophobic forces. It will be interesting to investigate if electrochemical gradients could also provoke a rearrangement of water molecules surrounding the enzyme that could affect its dielectric environment and other effects, such as small distortions around the active site or rearrangements of counter ions.

At this moment, the inhibition of the tonoplast H⁺-PPase promoted by Me₂SO precludes any speculation on the effects of hydrophobicity on thermodynamic of reactions catalyzed by this enzyme. Further researches are required to determine how Me₂SO behaves as a structure perturbing solvent using biochemical and biophysical approaches on the H⁺-PPase purified and/or incorporated in specific liposomes. Actually, catalysis in low water containing organic solvents is a hot point in enzyme technology where considerable progress has been achieved on gaining an improved mechanistic understanding of enzyme function, deactivation and activation in dehydrated environments (Klibanov, 2001; Lee and Dordick, 2002; Gupta and Roy, 2004).

On the other hand, vegetative cells often present a conspicuous vacuole that occupies most of their inner space; therefore, it is likely that in specific regions of the tonoplast some of its H⁺ pumps could be in conditions favoring their reversibility. For instance, it will be very interesting to verify if ATP synthesis occurs in particular sectors around the vacuoles where the tonoplast make contact with the plasmalemma or organelles membranes, from which the cytosol was withdrawn creating microenvironments of low water activities. The use of the new fluorescent probes for confocal scanning of living cells (Kim *et al.*, 2006) will be a powerful tool for studying this phenomenon especially for the vitrifying cytoplasm of vegetative cells of resurrection plants.

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