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ADP- or pyrophosphate-dependent proton pumping of pea stem tonoplast-enriched vesicles

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Pea stem tonoplast-enriched vesicles have an ADPase and PP_iase activity capable of generating a transmembrane proton gradient (ΔpH) and an electric potential ($\Delta\psi$). Both proton translocating activities have a pH optimum around 6.5, are Mg^{2+}-dependent, require the presence of a monovalent cation (K^+, Rb^+ or Cs^+) and of a permeant anion, such as NO_3^-, Cl^- or Br^-. They are almost completely inhibited by 50 μM DIDS, DES and DCCD, 50% inhibited by 100 μM molybdate and unaffected by Na_3VO_4 or KNO_3. Hexokinase and ATP do not prevent H^+-ADPase and H^+-PP_iase activity, thus indicating that these functions are not caused by an ATP-dependent proton pumping and that they have catalytic sites different from those of H^+-ATPase, respectively. On the basis of these characteristics, ADP- and PP_i-dependent proton translocating activities seem carried out by a similar enzyme complex which appears different from the NO_3-inhibited, VO_4^{3-}-insensitive H^+-ATPase.

Acridine orange; Oxonol-IV; H^+-ADPase, H^+-PP_iase; (Tonoplast vesicle; *Pisum sativum*)

1. INTRODUCTION

It is now well established that the primary active transport across the plasmalemma, tonoplast and Golgi membranes of higher plant cells consists of an ATP-dependent electrogenic translocation of protons [1–4]. The electrochemical gradient thus generated, supplies the driving force for the secondary transport, i.e. fluxes of ions, amino acids, sugars and hormones by symport or antiport mechanisms [5,6].

Walker and Leigh [7] first demonstrated that beet vacuoles have a PP_iase and suggested that a proton pump is associated to this activity. Recently, the evidence for a PP_iase in the generation of an electrochemical proton gradient across tonoplast, microsomal and Golgi membranes has been found [8–12].

Pea stem microsomal fractions possess both ATPase and ADPase activity [13,14]. ATPase has a proton translocating activity [15,16] linked to the generation of an electrochemical potential [17] and to a H^+/Ca^{2+} antiport [18]. In addition, a H^+-ATPase has also been shown in pea root plasma membrane-enriched vesicles [19].

In this paper, we show that pea stem tonoplast-enriched vesicles have an ADP- or PP_i-dependent proton translocating activity, which appears

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Abbreviations: BSA, bovine serum albumin; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DTE, dithioerythritol; FCCP, carboxyl cyanide *p*-trifluoromethoxyphenylhydrazone; PP_i, inorganic pyrophosphate; H^+-ADPase, proton translocating ADPase; H^+-PP_iase, proton translocating inorganic pyrophosphatase; Oxonol-IV, bis(3-propyl-5-oxoisoxanol-4-yl)pentamethine oxonol

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distinct, on the basis of its characteristics, from the H^+-ATPase inhibited by nitrate and insensitive to vanadate.

2. MATERIALS AND METHODS

2.1. *Plant material*

Peas (*Pisum sativum* L., cv. Alaska) were grown for 7 days, in the dark, at 27°C and 75% relative humidity.

2.2. *Isolation of pea stem tonoplast-enriched vesicles*

50 g etiolated stems were ground in an ice-cold mortar with 200 ml of 20 mM Hepes-Tris (pH 7.6), 250 mM sucrose, 1 mM NaEDTA, 10 mM $MgCl_2$, 25 mM $K_2S_2O_5$. 0.5% (w/v) BSA and then filtered through 8 layers of gauze. The filtrate was centrifuged at 13000 × *g* for 15 min and the supernatant recentrifuged at 80000 × *g* for 30 min. The pellet (microsomal fraction) was resuspended in 2 ml of 10 mM Hepes-Tris (pH 7.0), 5 mM DTE, 125 mM sucrose and 0.5% BSA. The microsomes were layered onto a 3 step gradient consisting of 9.5 ml of 20, 35, 45% sucrose (w/v) in 10 mM Hepes-Tris (pH 7.0), 1 mM DTE, 0.5% BSA and centrifuged at 80000 × *g* for 90 min (Beckman SW 25.1 rotor). The 20–35% sucrose interface fraction was collected by a Pasteur pipette and stored at −20°C for two weeks without loss of activity. The activity of these vesicles was almost completely inhibited by 10 mM KNO_3 and only slightly inhibited (~10%) by 100 μM Na_3VO_4 ([20] and results to be published).

2.3. *Spectrophotometric assays*

Proton uptake in the vesicles was monitored by the decrease of absorbance at 495 nm of the ΔpH probe, acridine orange, with a double beam Perkin-Elmer, model 554, spectrophotometer. The electric potential of the vesicles (inside positive) was monitored as an increase in absorbance at 630 nm of Oxonol-VI.

The standard deviation of these determinations did not exceed 5%.

2.4. *Protein determination*

Protein was estimated by the biuret method [21], using BSA as a standard, after washing the samples with 5 mM $MgCl_2$.

2.5. *ADPase and PP*$_i$ase activity

The reaction medium was 10 mM Hepes-Tris (pH 6.5), 5 mM $MgSO_4$, 125 mM sucrose, 1 mM EGTA, 30 mM KCl and 20 μl (ADPase) or 100 μl (PP_iase) vesicle suspension in a final volume of 1 ml. The reactions were started by adding 0.5 mM ADP or PP_i and proceeded for 15 min at 37°C. Phosphate released was determined as described by Cross et al. [22].

2.6. *Chemicals*

ATP, ADP, FCCP, DCCD, oligomycin, DES, DIDS, and acridine orange were purchased from Sigma (St. Louis, USA). The purity of ADP was verified by TLC. Oxonol-VI was obtained from Molecular Probes (Junction City, OR, USA). Lyophilized hexokinase (EC 2.7.1.17) was purchased from Boehringer, Mannheim.

3. RESULTS

Fig.1 shows the ADP- and PP_i-dependent acridine orange or Oxonol-VI shift of absorbance in pea stem tonoplast-enriched vesicles. Trace A shows that the addition of 0.5 mM ADP causes a decrease of absorbance of the acridine orange, indicating the building up of a proton gradient (ΔpH) across the vesicles (inside acid), which completely collapses with 6 μM FCCP. The addition of 10 μM PP_i (trace B) also determines the decrease of absorbance and, hence, creates a proton gradient. Both ADP and PP_i, in the absence of a permeant anion, induce a shift of absorbance of Oxonol-VI that indicates the generation of an electric potential ($\Delta\psi$) which is dissipated by FCCP (traces C and D). Trace E shows that the addition of ADP (d) or PP_i (e) to the vesicles, after the building up of a proton gradient by 0.5 mM ATP, causes an increase of ΔpH, but to an extent which is lower than that generated by ADP or PP_i alone.

Fig.2 shows the effect of pH of the medium on the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease. Both rates show a strict dependence on the pH, with an optimum at 6.5 for the PP_i-dependent and 6.5–7.0 for the ADP-dependent activity. The rates drop sharply at pH values other than optimal.

The initial rate of ADP- and PP_i-dependent absorbance decrease due to acridine orange uptake in pea tonoplast-enriched vesicles is dependent on the

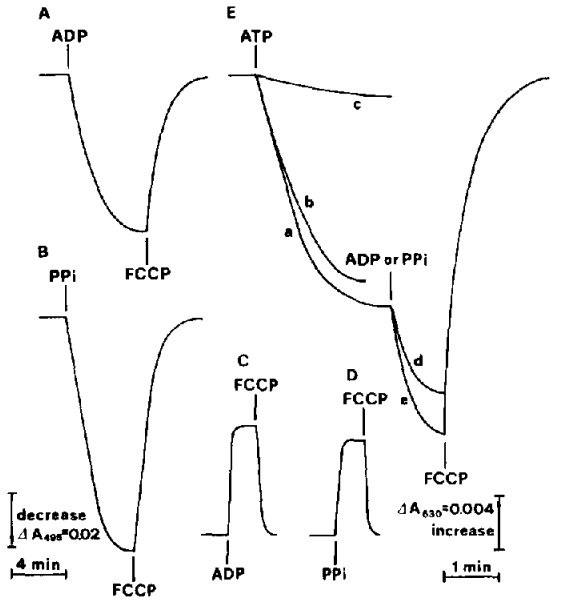

Fig.1. ADP- and PP_i-dependent acridine orange absorbance decrease or Oxonol-VI absorbance increase in pea stem tonoplast-enriched vesicles. The incubation medium was: 10 mM Hepes-Tris (pH 6.5), 5 mM $MgSO_4$, 125 mM sucrose, 1 mM EGTA, 30 mM KCl, 10 μM acridine orange or 5 μM Oxonol-VI and 100 μl of vesicles suspension (~150 μg/ml protein) in a final volume of 2 ml. Additions were: 0.5 mM ADP and ATP adjusted at pH ~6.5 with Tris, 10 μM PP_i adjusted at pH ~6.5 with Hepes and 6 μM FCCP. a, control; b, 100 μM Na_3VO_4; c, 10 mM KNO_3; d, ADP addition; e, PP_i addition.

presence of some monovalent and divalent cations. Potassium, Rb^+, and Cs^+ are required for the activity to occur. Sodium, Li^+ or choline are instead without effect (table 1). The presence of Mg^{2+} is also required while other divalent cations (Ca^{2+}, Co^{2+}, Mn^{2+}) are not necessary (not shown). In addition, table 1 shows that the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease is stimulated by permeant anions such as Cl^-, Br^-, NO_3^- and is very slightly affected by F^- and SO_4^{2-}.

Table 2 shows that the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease is insensitive to 50 μM Na_3VO_4, 10 mM KNO_3 and 2 μg/ml oligomycin, almost completely inhibited by 50 μM DIDS, DES, DCCD and ~50% inhibited by 100 μM Na-molybdate.

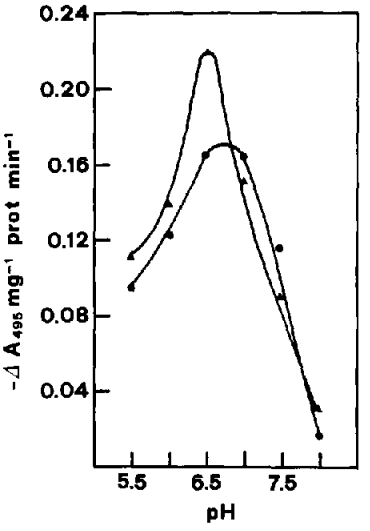

Fig.2. Effect of pH of the medium on the initial rate of ADP- (●) and PP_i- (▲) dependent acridine orange absorbance decrease in pea stem tonoplast-enriched vesicles. Conditions as in fig.1.

The ADPase and PP_iase activities, evaluated as release of P_i, are 37.6 ± 4.6 and 5.9 ± 0.7 nmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$, respectively, and are unaffected by 50 μM Na_3VO_4 and 20 mM KNO_3.

Table 1

Effect of monovalent cations and anions on the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease in pea stem tonoplast-enriched fractions

Additions	$-\Delta A_{495} \cdot mg\ protein^{-1} \cdot min^{-1}$		% control	
	ADPase	PP_iase	ADPase	PP_iase
KCl (control)	0.161	0.250	100	100
NaCl	0.015	0.020	9	8
RbCl	0.168	0.245	104	98
CsCl	0.165	0.240	102	96
Choline Cl	0.000	0.010	0	1
LiCl	0.000	0.010	0	4
K_2SO_4	0.029	0.050	18	20
KBr	0.170	0.230	106	92
KF	0.016	0.005	10	2
KNO_3	0.139	0.240	86	96

The conditions were as in fig.1. Percent values were calculated by considering control value as 100

Table 2

Effect of several inhibitors on the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease in pea stem tonoplast-enriched fractions

Additions	$-\Delta A_{495}$ · mg protein^{-1} · min^{-1}		% control	
	ADPase	PP_iase	ADPase	PP_iase
Control	0.171	0.245	100	100
50 μM Na_3VO_4	0.168	0.224	98	91
10 mM KNO_3	0.178	0.264	103	107
2 μg/ml oligomycin	0.155	0.230	97	94
50 μM DIDS	0.016	0.036	9	15
50 μM DES	0.006	0.000	4	0
50 μM DCCD	0.012	0.030	8	12
100 μM Na-molyb.	0.079	0.112	46	46

The conditions were as in fig.1. Percent values were calculated by considering control value as 100

The substrate concentration dependence of the initial rate of ADP- and PP_i-dependent acridine orange absorbance decrease is shown in fig.3. Panel A shows that ADP-dependent activity approximates to a saturable kinetic. The double reciprocal plot of the data (inset) yields a straight

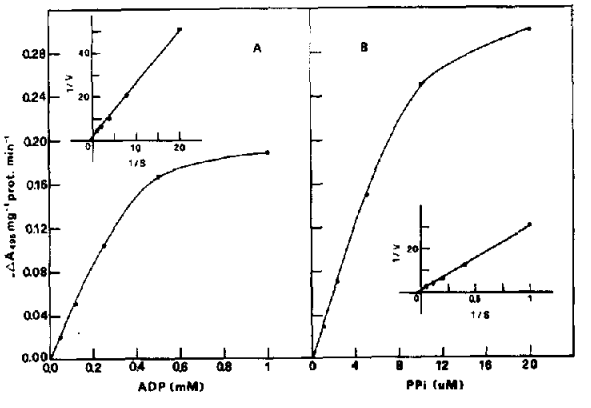

Fig.3. Initial rate of ADP-dependent (panel A) and PP_i-dependent (panel B) acridine orange absorbance decrease as a function of substrate concentration in pea stem tonoplast-enriched vesicles. Insets represent the linear transformation, by the double reciprocal plot, of the curves. Experimental conditions as in fig.1.

Table 3

Effect of hexokinase and ATP on the initial rate of ADP- or PP_i-dependent acridine orange absorbance decrease in pea stem tonoplast-enriched fractions

Additions	$-\Delta A_{495}$ · mg protein^{-1} · min^{-1}	
	ADPase	PP_iase
Control	0.155	0.236
15 IU hexokinase + 5 mM glucose	0.161	0.231
30 IU hexokinase + 5 mM glucose	0.149	0.240
40 mM KNO_3	0.145	0.234
40 mM KNO_3 + 0.5 mM ATP	0.160	0.230
40 mM KNO_3 + 1 mM ATP	0.149	0.237

Conditions were as in fig.1

line from which a K_m of approx. 2 mM is calculated. A similar kinetic model is exhibited by the PP_i-dependent activity (panel B). The calculated K_m (inset) is approx. 20 μM.

Table 3 shows that the initial rate of ADP- or PP_i-dependent acridine orange absorbance decrease is unaffected by the presence of hexokinase plus glucose, used as an ATP trap, or ATP, at a concentration saturating the catalytic sites, plus KNO_3; the K_m for ATP-dependent proton uptake is ~0.4 mM (results to be published). Nitrate was used to prevent ATP-dependent acridine orange uptake.

4. DISCUSSION

Pea stem tonoplast-enriched vesicles possess an ADPase and PP_iase activity capable of generating a transmembrane proton gradient (ΔpH) and an electric potential ($\Delta\psi$). These proton translocating activities are dependent on the presence of Mg^{2+}, K^+, Rb^+, Cs^+, require a permeant anion such as NO_3^-, Cl^- or Br^- and are not inhibited by VO_4^{3-} and NO_3^-. The proton pumping ADPase and PP_iase are almost completely inhibited by 50 μM DIDS, DES, DCCD and 50% inhibited by 100 μM molybdate. Their insensitivity to oligomycin indicates that our tonoplast-enriched fraction is scarcely or not contaminated by mitochondrial fragments. The dependence on the pH of the medium also appears very close for both H^+-pumping activities. The main difference be-

tween H^+-ADPase and H^+-PP_iase is associated with the sensitivity to substrate; H^+-PP_iase has a 100-fold greater affinity than H^+-ADPase. ATP, in the presence of NO_3^- at a concentration capable of inhibiting ATP-dependent proton pumping, does not prevent H^+-ADPase and H^+-PP_iase activity. This indicates that ATP does not compete with ADP or PP_i for the active sites of the enzymes.

On the basis of their sensitivity to inhibitors, monovalent and divalent cations and anions, H^+-ADPase and PP_iase are very similar and, therefore, seem to be carried out by a similar enzyme complex. This H^+-ADP/PP_iase, on the other hand, appears to be different, in its characteristics, from the nitrate-inhibited, chloride-stimulated and vanadate-insensitive H^+-ATPase of pea stem microsomes [15,16] and tonoplast-enriched fractions (results to be published), and from the vanadate-inhibited, nitrate-insensitive H^+-ATPase of pea root plasmalemma-enriched fraction [19]. The ATP- and ADP- or PP_i-dependent proton pumping activity seems to be located on the same type of vesicles, since the ATP-generated ΔpH appears to counteract the ADP- or PP_i-dependent proton pumping activity (fig.1E,F). Pea stem tonoplast-enriched vesicles, hence, have two separate proton pumps, utilizing ATP and ADP or PP_i as substrates, to generate a transmembrane electrochemical potential.

The H^+-pumping ADPase might reflect the activity of H^+-ATPase utilizing ATP generated by conversion of 2 mol of ADP in a reaction catalyzed by various enzymes, such as adenylate kinase. However, H^+-ADPase was also recovered in the presence of hexokinase plus glucose, used as a trap for ATP eventually synthesized. On the other hand, the specific insensitivity to NO_3^- and the stimulation by some monovalent cations support the view that the electrochemical gradient generated by ADP is not determined by the activity of the H^+-translocating ATPase, which is NO_3^--inhibited, stimulated by permeant anions and insensitive to cations (fig.1E, traces a and b and results to be published).

The primacy of ATP in the energization of plasmalemma, tonoplast and Golgi plant cell membranes is well established [1–4]. However, emerging evidence supports the involvement of PP_i, utilized by a H^+-pumping PP_iase physically separable from the principal H^+-ATPase [12,23], in the generation of an electrochemical potential in tonoplast [10–12] and Golgi vesicles [11]. The characteristics of this H^+-PP_iase are very similar to those found in pea stem tonoplast-enriched vesicles. In particular, the proton pumping PP_iase activity of oat root tonoplast vesicles is also stimulated by K^+, Rb^+ and Cs^+, requires a permeant anion, is not inhibited by VO_4^{3-} and NO_3^-, is inhibited by DCCD and is unaffected by Li^+ and Na^+ [12]. However, pea stem tonoplast H^+-PP_iase appears strictly associated with a H^+-ADPase activity which so far has not been found in other plant vesicles.

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