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Proton pumping inorganic pyrophosphatase of pea stem submitochondrial particles

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Pea stem submitochondrial particles have an inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) which is able to couple PP_i hydrolysis with the generation of an inside positive proton gradient. The activity is Mg^{2+}-requiring, is stimulated by monovalent cations, inhibited by imidodiphosphate and, partially, by dicyclohexylcarbodiimide, diethylstilbestrol and oligomycin. This mitochondrial H^+-PP_iase is distinguishable from the tonoplast H^+-PP_iase and appears to be an additional coupling factor, which works in parallel with the well-known H^+-ATPase.

Introduction

It is known that inorganic pyrophosphate is released during several anabolic reactions, i.e., DNA and RNA synthesis, activation of fatty acids, $NADP^+$ and UDPG synthesis and amino acid acylation. In the light of a recent hypothesis, this substrate appears to be linked to two major energy-conserving reactions of plant cells: a cytoplasmic phosphofructokinase utilizing PP_i instead of ATP and a tonoplast proton pumping pyrophosphatase [1]. Inorganic pyrophosphate is also synthesized by the activity of H^+-PP_iases of *Rhodospirillum rubrum* chromatophores [2], mitochondria from beef heart [3] and rat liver [4]. The process has been shown to proceed concurrently with and independently of ATP synthesis and not to depend on ATPase activity.

The tonoplast proton pumping pyrophosphatase has been well characterized in several plant tissues [5], while there is little evidence of a similar enzyme in mitochondria [6]. Recently, it has been demonstrated that the tonoplast H^+-PP_iase works in vivo as a proton pump rather than as a pyrophosphate synthetase [7] and that the proton gradient generated can drive the synthesis of ATP [8].

In this work we show that pea stem mitochondria contain an H^+-PP_iase (pyrophosphate phosphohydrolase, EC 3.6.1.1) which may represent a coupling factor, in addition to the well-known oligomycin-sensitive H^+-ATPase.

Materials and Methods

Preparation of submitochondrial particles. Mitochondria were isolated from etiolated pea (*Pisum sativum* L., c.v. Alaska) stems as previously described [9]. The mitochondrial suspension (3 ml) was diluted (1:1) with 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/6 mM ATP/0.5% BSA (w/v) and then sonically irradiated four times (100 W) for 0.5 min with 1 min intervals in an ice-bath. The sonicate was centrifuged at $28000 \times g$ for 5 min to remove unbroken mitochondria. The supernatant was centrifuged at $100000 \times g$ for 30 min. The pellet, washed in 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/0.5% BSA, was recentrifuged. Finally, the pellet (submitochondrial particles) was resuspended in approx. 1 ml 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/0.3% BSA and stored at −40 °C.

Fluorescence assays. PP_i- or ATP-dependent proton pumping was followed as fluorescence quenching of acridine orange (AO) or quinacrine by a Perkin-Elmer fluorescence spectrometer model LS-3, at room tem-

Abbreviations: AO, acridine orange; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PP_i, inorganic pyrophosphate.

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perature. The excitation and emission wavelengths were 495 and 540 nm for AO and 430 and 500 nm for quinacrine, respectively. A slitwidth of 5 nm for both emission and excitation was used. The incubation medium was 10 mM Hepes-Tris (pH 7.5)/5 mM $MgSO_4$/50 mM KCl/1 mM EGTA/5 μM AO or quinacrine and 100 μl of submitochondrial particles (0.2 mg protein) in a final volume of 2 ml. The reactions were started by 50 μM PP_i or 0.5 mM ATP, respectively.

Enzyme assay. The PP_iase activity was evaluated as release of inorganic phosphate, measured as described in Ref. 10. The medium was as in AO experiments, except that the final volume was 1 ml and 2 μM nigericin and 50 μl of submitochondrial particles (0.05–0.1 mg protein) were added. The reaction was started by 100 μM PP_i and proceeded for 20 min at 37 °C. Tonoplast ATPase activity was determined as in Ref. 11, except that 25 μl of submitochondrial particles were added (approx. 0.04 mg protein).

Protein determination. Protein was determined by the biuret method [12], after washing the submitochondrial particles with 5 mM $MgSO_4$ to remove BSA present in the resuspending medium.

Chemicals. MgATP, acridine orange, quinacrine, DCCD, DES, CCCP, nigericin, oligomycin, sodium vanadate and imidodiphosphate were purchased from Sigma Co., St. Louis, MO, U.S.A. Sodium pyrophosphate was obtained from Merck, Darmstadt, Germany. Bafilomycin A1 was a generous gift of Professor E. Komor, University of Bayreuth, Germany.

Data presentation. The results are representative of a typical experiment. In no case did standard deviation exceed 10%.

Results and Discussion

According to the chemiosmotic hypothesis, ATP synthesis in mitochondria is linked to the re-entry of protons ejected during oxidoreduction of respiratory substrates [13]. Submitochondrial particles are inside-out with respect to intact mitochondria and, therefore, may catalyse the hydrolysis of external ATP which is coupled to the vectorial translocation of protons across the membrane vesicles.

Fig. 1 shows that pea stem submitochondrial particles were able to build up a PP_i-dependent proton gradient followed as acridine orange (trace A) and quinacrine (trace B) quenching of fluorescence. Both gradients were immediately dissipated by the addition of nigericin or CCCP (not shown). Conversely, ADP did not induce the formation of ΔpH (trace C). In addition, trace D shows that these particles generated an ATP-dependent proton gradient which was completely inhibited by oligomycin (trace E). The proton pumping PP_iase activity exhibited a broad pH optimum

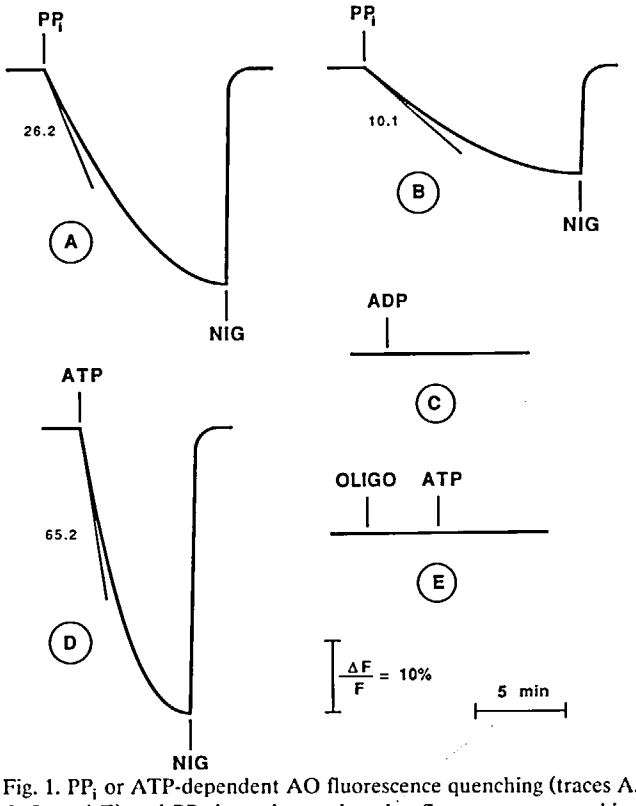

Fig. 1. PP_i or ATP-dependent AO fluorescence quenching (traces A, C, D and E) and PP_i-dependent quinacrine fluorescence quenching (trace B) in pea stem submitochondrial particles. Additions were: 50 μM PP_i, 0.5 mM ATP or ADP, 2 μM nigericin (NIG) and 4 μg/ml oligomycin (OLIGO). Figures next to each trace are expressed as (% $\Delta F/F$)/mg protein per min.

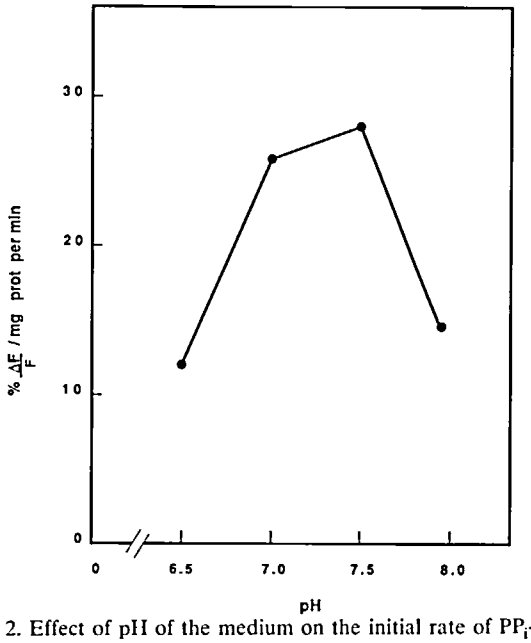

Fig. 2. Effect of pH of the medium on the initial rate of PP_i-dependent AO fluorescence quenching in pea stem submitochondrial particles.

TABLE I

Effect of monovalent cations on the initial rate of PP_i-dependent AO fluorescence quenching and PP_iase activity of pea stem submitochondrial particles

Addition (50 mM)	$\% \frac{\Delta F}{F}$/mg protein per min	μmol P_i/mg protein per h
None	0	2.6
KCl	25.6	30.0
LiCl	0	6.8
RbCl	20.3	32.0
CsCl	7.5	13.6
NaCl	5.1	10.4

between 7.0 and 7.5 (Fig. 2), a K_m and a V_{max} value, determined by the double reciprocal plot, of approx. 7 μM and 28.8 (%ΔF/F)/mg protein per min, respectively (result not shown). This H^+-PP_iase, evaluated as fluorescence quenching of AO or release of P_i, required Mg^{2+} and was strongly stimulated by some cations in the following order of magnitude: $K^+ = Rb^+ > Cs^+ > Na^+$. Lithium had no effect (Table I). Therefore, the activity of this enzyme is dependent by some monovalent cations, as for the vacuolar H^+-PP_iase [5] which is activated by K^+ only at the cytosolic face [14]. The H^+-PP_iase activity was inhibited by the competitive inhibitor imidodiphosphate [15] and, partially, by DCCD, DES and oligomycin. Vanadate and molybdate were ineffective (Table II).

These results show that pea stem submitochondrial particles possess a PP_iase which is able to couple PP_i hydrolysis with a vectorial transport of protons, similarly to that found in beef heart and rat liver mitochondria [3,4]. This activity is clearly distinguishable from the oligomycin-sensitive H^+-ATPase (F-ATPase) on the basis of its lesser sensitivity to oligomycin, inhibition by imidodiphosphate and the different substrate utilized, although they have the same pH optimum. On the other hand, the submitochondrial H^+-PP_iase is also distinct from that of pea stem tonoplast,

TABLE II

Effect of inhibitors on the initial rate of PP_i-dependent AO fluorescence quenching and PP_iase activity in pea stem submitochondrial particles

Inhibitor	$\% \frac{\Delta F}{F}$/mg protein per min	μmol P_i/mg protein per h
None	26.2	29.7
DCCD, 50 μM	13.1	19.6
DES, 50 μM	15.0	22.9
IDP, 50 μM	0	8.2
VO_4^{3-}, 100 μM	28.7	29.1
MoO^{2-} 100 μM	28.1	29.4
Oligomycin, 4 μg/ml	14.4	20.7

the latter being inhibited by molybdate, insensitive to oligomycin, having a pH optimum near to 6.5 and, in addition, showing an ADP-dependent proton pumping [16]. In agreement, submitochondrial particles were devoid of tonoplast contamination, because the ATPase activity, evaluated at pH 6.5, was insensitive to bafilomycin A1 (control: 28.0 μmol P_i/mg prot per h; 1 μM bafilomycin-treated: 28.9 μmol P_i/mg prot per h), a specific inhibitor of the tonoplast H^+-ATPase activity [17]. Therefore, the mitochondrial H^+-PP_iase appears to be an additional coupling factor which works in parallel with the well-known H^+-ATPase [18].

In higher plant cells there are considerable levels of PP_i [19]. The latter is localized mainly in the cytoplasm, where there is also little or no alkaline pyrophosphatase activity; conversely, chloroplasts have negligible amounts of PP_i and a high activity of alkaline pyrophosphatase [20,21].

Although pyrophosphate does not permeate through the mitochondrial inner membrane, it may undergo an exchange with ADP [22]. The PP_i exported from mitochondria to the cytoplasm may therefore represent for plant cells an additional source of energy for the major energy-conserving reactions catalysed by PP_i-phosphofructokinase, UDPG pyrophosphorylase and the tonoplast proton pumping pyrophosphatase [20]. This contention is supported by a recent finding that stresses the importance of PP_i as an autonomous energy donor in the cytosol of higher plants [23].

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References

1 Taiz, L. (1986) J. Theor. Biol. 123, 231–238.
2 Strid, Å., Karlsson, I.-M. and Baltscheffsky, M. (1987) FEBS Lett. 224, 348–352.
3 Mansurova, S.E., Shakhov, Y.A. and Kulaev, I.S. (1977) FEBS Lett. 74, 31–34.
4 Volk, S.E. and Baykov, A.A. (1984) Biochim. Biophys. Acta 791, 198–204.
5 Rea, P. and Sanders, D. (1987) Physiol. Plant. 71, 131–141.
6 Maslowski, P., Kowalczyk, S. and Kazubska, E. (1978) Acta Biochim. Pol. 25, 175–179.
7 Johannes, E. and Felle, H. (1990) Plant Physiol. 93, 412–417.
8 Dupaix, A., Johannin, G. and Arrio, B. (1989) FEBS Lett. 249, 13–16.
9 Macrì, F., Vianello, A., Braidot, E. and Zancani, M. (1991) Biochim. Biophys. Acta, 1058, 249–255.
10 Cross, J.M., Briggs, R., Dohrmann, V.C. and Rayle, P.M. (1978) Plant Physiol. 61, 581–584.
11 Vianello, A., Macrì, F. and Dell'Antone, P. (1987) Physiol. Plant. 71, 44–78.
12 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
13 Mitchell, P. (1980) Ann. N.Y. Acad. Sci. 341, 564–584.

14 Davies, J.M., Rea, P.A. and Sanders, D. (1991) FEBS Lett. 278, 66–68.
15 Verstappen, R. and Rausch, T. (1988) FEBS Lett. 236, 420–424.
16 Macrì, F. and Vianello, A. (1987) FEBS Lett. 215, 47–52.
17 Bowman, E.J., Siebers, A. and Altendorf, K. (1988) Proc. Natl. Acad. Sci. USA, 85, 7972–7976.
18 Douce, R. (1985) Mitochondria in Higher Plants, pp. 113–125, Academic Press, Orlando.
19 Dancer, J. and ap Rees, T. (1989) Planta 177, 261–264.
20 Wiener, H., Stitt, M. and Heldt, H.W. (1987) Biochim. Biophys. Acta 893, 13–21.
21 Gross, P. and ap Rees, T. (1986) Planta 167, 140–145.
22 Kowalczyk, S. and Maslowski, P. (1981) Phytochemistry 20, 2611–2615.
23 Dancer, J., Veith, R., Feil, R., Komor, E. and Stitt, M. (1990) Plant Sci. 66, 59–63.