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Tissue specificity of the regulation of ATP hydrolysis by isolated plant mitochondria

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Pea leaf mitochondria had a high ATP hydrolase activity following the collapse of the membrane potential by addition of valinomycin in state 4. In mitochondria isolated from potato tubers such ATP hydrolase activity was not observed. Pea leaf mitochondria also had a ΔpH, in contrast to what was previously found for potato tuber mitochondria. This ΔpH could, however, not explain the different results on ATP hydrolysis since this activity was also observed in the presence of nigericin. The results suggest a tissue-specific regulation of ATP hydrolysis in resting organs (potato tubers) as compared to active organs (leaves).

Plant mitochondria; F_0F_1 H^+-ATPase; Membrane potential; ΔpH; ATP-hydrolysis

1. INTRODUCTION

The mitochondrial F_0F_1 ATPases of several plant tissues have been extensively characterized [1]. With enzymes isolated from potato tubers, the maximal activity has been obtained by the use of non-ionic detergents [2]. The first observations in situ indicated a very low hydrolyzing activity of membrane-bound ATPase as compared to the synthesis of ATP, especially in potato tubers [3,4]. The ATP-hydrolyzing activity of inverted particles was increased by treatments known to remove the endogenous inhibitor protein [3,4]. However, in animal submitochondrial particles, it is not clear whether the removal of the inhibitor protein, favoured by the electrochemical proton gradient, triggers only ATP hydrolysis [5,6], or both synthesis and hydrolysis [7].

Moreover, a specific regulation of ATP hydrolysis activity in mitochondria from various tissues has been previously proposed [8], suggesting that a growing tissue may hydrolyze ATP, at variance with a resting organ as potato tubers [3].

In the present paper we have investigated the regulation of the ATP-hydrolyzing activity in situ in mitochondria extracted from potato tubers, a resting organ, and pea leaves, an active organ. The possible involvement of the pH gradient has especially been tested, after the demonstration of its presence in pea leaf mitochondria under our experimental conditions.

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Abbreviations: F_0 and F_1, membranous and extrinsic parts of the proton ATPase; $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\psi$, transmembrane potential difference; ΔpH, transmembrane pH difference; TPP^+, tetraphenylphosphonium ion.

2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria*

Extraction and purification of mitochondria from potato tubers (*Solanum tuberosum*, L.) were carried out at 4°C as previously described [9] except for the addition of 0.5% (w/v) polyvinylpyrrolidone in the grinding medium. Purified mitochondria were collected and stored in assay medium containing 0.4 M mannitol, 5 mM $MgCl_2$, 30 mM KCl, 0.1% (w/v) bovine serum albumin and 2 mM K-K_2-phosphate buffer (pH 6.9) at a protein concentration of 10–20 mg · ml^{-1}, determined using the Bradford method [10].

Pea leaf tissue (200 g) was chopped twice for 8 s, at high speed, in a Waring blendor containing 700 ml of medium composed of 0.3 M mannitol, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.4% (w/v) bovine serum albumin, 0.5% (w/v) polyvinylpyrrolidone, 8 mM cysteine, 50 mM 3-[*N*-morpholino]propane-sulfonic acid (MOPS) (pH 7.5). The homogenate was filtered through a nylon sieve (mesh diameter 25 μm), and centrifuged at 300 × *g* for 10 min. The supernatant was recentrifuged at 9,000 × *g* for 20 min and the mitochondrial pellet was resuspended in washing medium containing 0.4 M mannitol, 0.1% (w/v) bovine serum albumin, 10 mM MOPS (pH 7.2). After a centrifugation at 500 × *g* for 10 min, the supernatant was centrifuged at 9,000 × *g* for 20 min, and the purification was performed on a 28% self-generating Percoll gradient [9]. Pea mitochondria were resuspended in the same assay medium as potato mitochondria.

2.2. *Assays*

Measurements of oxidation rate, transmembrane potential and changes in pH of the medium were made simultaneously in a single assay at 25°C in a glass vessel containing 3 ml (final volume) of the assay medium. About 0.2 to 0.5 mg protein were added per assay.

Oxygen uptake was measured polarographically with a Clark-type electrode. Membrane potential was measured with a TPP^+ sensitive electrode [9,11]. ATP synthesis and hydrolysis were estimated with a

glass electrode from the pH variation in the medium. Indeed the equilibrium:

$$(ADP + P_i)^{p-} + mH^+ \rightarrow ATP^{q-} + H_2O$$

involves protons. The coefficient $m = p - q$ depends on the protonation of ADP, ATP, and P_i, and then depends on the pH value [12]. Therefore, ATP synthesis consumes and ATP hydrolysis produces H^+ ions independently of the proton pumping activity of the enzyme. The total change in pH during ATP hydrolysis was maintained below 0.05 and the buffer capacity, estimated by HCl titration in each condition, was constant in this range. Kinetics of HCl titration also show that the response of the electrode was never rate-limiting.

3. RESULTS

Fig. 1 presents the primary observations concerning ATP hydrolysis by mitochondria from different tissues. In order to observe hydrolysis of ATP by mitochondria, electron transfer chain was inhibited by KCN to avoid the formation of a redox-driven electrochemical proton gradient. Under these conditions, $\Delta\psi$ generated in the presence of ATP can be attributed to the ATP hydrolase activity.

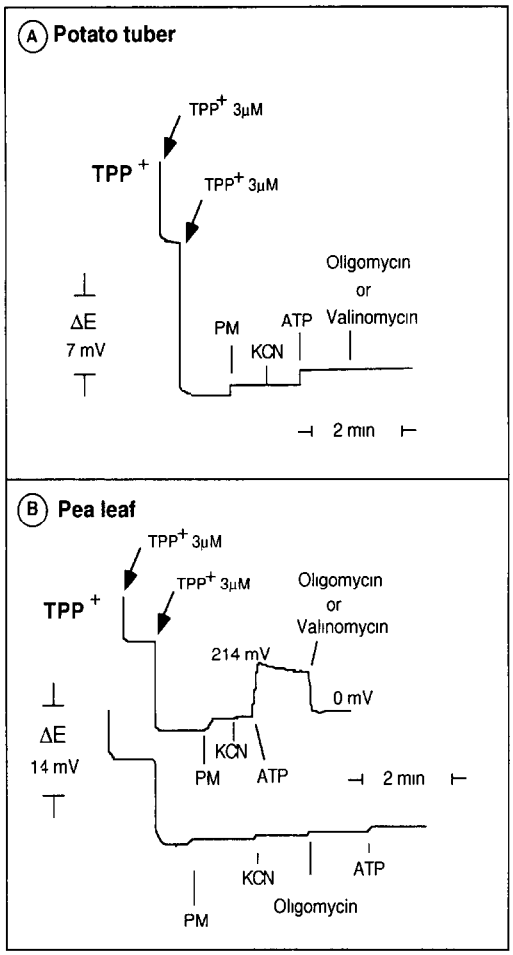

Fig. 1. ATP hydrolysis-driven membrane potential in plant mitochondria. Conditions as described in section 2. Purified mitochondria (PM), 130 µg protein · ml^{-1}; KCN, 100 µM; ATP, 1 mM; valinomycin, 40 ng · (mg protein)$^{-1}$; oligomycin, 0.2 mg · (mg protein)$^{-1}$. ΔE represents the TPP^+ electrode response in mV. A, potato tuber mitochondria; B, pea leaf mitochondria. The values plotted on the trace in B refer to membrane potential.

Fig. 1A shows that potato tubers mitochondria treated with KCN did not generate a membrane potential after addition of ATP. A slight TPP^+ uptake by mitochondria was observed, but it was not affected by addition of valinomycin (a K^+ ionophore), or oligomycin, a specific inhibitor of F_0F_1 ATPase. This TPP^+ signal can be attributed to a nonspecific binding of the cation, independent of $\Delta\psi$.

By contrast, in pea leaf mitochondria under the same experimental conditions (Fig. 1B), a transmembrane potential was generated after addition of ATP. This membrane potential was totally dissipated by valinomycin or oligomycin. Moreover, the presence of oligomycin before addition of ATP prevented the generation of the membrane potential.

In another experiment, induction of ATP hydrolysis by addition of valinomycin to isolated plant mitochondria in state 4 [13] and in the presence of saturating ATP has been directly investigated (Fig. 2). In order to avoid problems due to the 'conditioning' of mitochondria [14], study of ATP hydrolysis was carried out after two consecutive state-3/state-4 cycles as defined in [13]. Uncoupling of the membrane by valinomycin was achieved only during the subsequent state 4, in order to have a maximal electrochemical proton gradient for the possible activation of the membrane-bound ATPase [5–7,15–18]. To reach the maximal membrane potential in state 4, before ATP hydrolysis, we used glycine as a respiratory substrate for pea leaf mitochondria [19–21], and succinate for potato mitochondria, as no glycine oxidation activity is present in mitochondria from non-green tissue.

Fig. 2 shows how ATP hydrolysis has been checked. In A, the experiment was carried out with potato tuber mitochondria. Addition of ADP induced a transient increase in oxidation rate, a release of TPP^+ from mitochondria (decrease of the transmembrane potential) and an increase in the external pH. Once net phosphorylation stopped (state 4), and after addition of ATP, a saturating concentration of valinomycin was injected, immediately collapsing the membrane potential. Under these conditions, hydrolysis would be detected as an acidification of the medium [12]. However, with potato tuber mitochondria, no change of the external pH was observed, i.e. no hydrolysis occurred, whereas a high activity of ATP synthesis was observed (1,660 ± 60 nmol ATP min^{-1} · (mg protein)$^{-1}$ for 9 different assays). In B, the same experiment was carried out with pea leaf mitochondria using glycine as respiratory substrate [19–21]. A significant acidification of the external medium was observed upon valinomycin addition. This phenomenon disappeared when oligomycin was injected before the uncoupling by valinomycin, proving that it

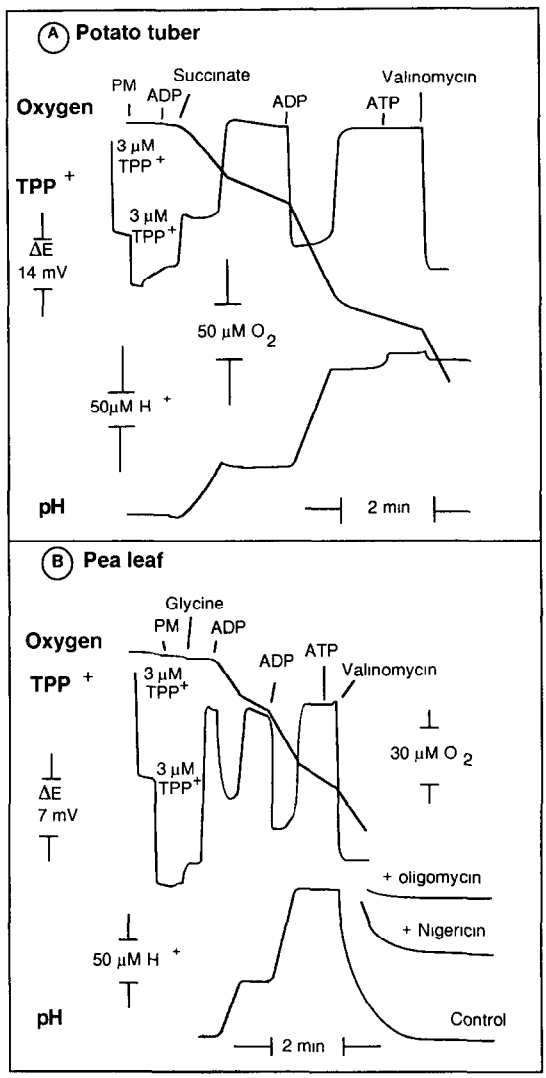

Fig. 2. Synthesis and hydrolysis of ATP by (A) potato tuber mitochondria and (B) pea leaf mitochondria. Conditions as described in section 2. A, potato tuber mitochondria. Purified mitochondria (PM), 170 μg protein · ml^{-1}; ADP additions, 100 μM and 200 μM; ATP, 1 mM; valinomycin, 40 ng · (mg protein)$^{-1}$. Rates of oxygen uptake in nmol O_2 min^{-1} · (mg protein)$^{-1}$: in state 3, 527; in state 4, 85. Membrane potential in mV: in state 3, 168; in state 4, 235. B, pea leaf mitochondria. PM, 170 μg protein · ml^{-1}; NAD^+, 250 μM; glycine, 8 mM; ADP additions, 50 μM and 100 μM; ATP, 1 mM; valinomycin, 40 ng · (mg protein)$^{-1}$. Oligomycin, 0.2 mg · (mg protein)$^{-1}$ or nigericin, 1 μg · (mg protein)$^{-1}$, is injected, if mentioned, between ATP and valinomycin. In B, the time course of ATP hydrolysis with nigericin or oligomycin was shifted to the right to be more visible. Rates of oxygen uptake in nmol O_2 min^{-1} · (mg protein)$^{-1}$: in state 3, 162; in state 4, 45. Membrane potential values in mV: in state 3, 173; in state 4, 229.

reflects ATP hydrolysis. The activity started to decay immediately after dissipation of the membrane potential, suggesting that the electrochemical proton gradient plays a key role in the enzyme activation, as in chloroplasts [15–17]. This rapid deactivation of the enzymes makes difficult a precise estimation of the initial rate of ATP hydrolysis. An additional problem is raised by transmembrane proton movements in the first seconds following valinomycin addition (see below). For this reason, we have estimated the instantaneous rate of ATP hydrolysis 15 s after membrane deenergization. In this condition, the activity had already started to decay, but the pH variations were now only due to the production of protons by the ATP hydrolysis. The reaction rate so measured varied from 900 to 1,800 nmol ATP min^{-1} · (mg protein)$^{-1}$, while the steady state rate of ATP synthesis during state 3 varied from 600 to 1,200 nmol ATP min^{-1} · (mg protein)$^{-1}$, depending on the preparation.

In contrast to potato mitochondria [9], nigericin increased $\Delta\psi$ under state 4 conditions in pea leaf mitochondria, indicating the existence of a ΔpH (not shown). In Fig. 2B, valinomycin-triggered ATP hydrolysis was reinvestigated in pea leaf mitochondria, but in the presence of nigericin to obtain a similar situation as in potato tuber organelles. An important ATP hydrolysis was still detected in this case. However, it was only a fraction of the control and the deactivation was slightly accelerated. These effects might well be the consequence of the dissipation of ΔpH, but the enzyme is still active, in contrast with potato tuber mitochondria. Thus, one may exclude that the lack of ΔpH in potato tuber mitochondria is responsible for the total absence of ATP hydrolysis.

Since the presence of a ΔpH means a higher pH in the matrix than in the medium, it would be possible that the ability to hydrolyze ATP is a question of matrix pH and not of ΔpH. Indeed, the catalytic part F_1 is exposed to the internal compartment. To check this hypothesis, we have first compared the pH-dependency of ATP-hydrolysis with and without nigericin, in pea leaf mitochondria, which are thought to maintain a significant ΔpH in addition to $\Delta\psi$. Fig. 3 shows the instantaneous rate of ATP hydrolysis as a function of pH, 5 s (Fig. 3A) or 15 s (Fig. 3B) after uncoupling by valinomycin alone (control) or by valinomycin plus nigericin. At t = 5 s the shape of the curve is quite different with and without nigericin (Fig. 3A). This is probably due to a fast proton extrusion from the matrix upon $\Delta\psi$ dissipation, induced by the acceleration of the redox chain, and probably also by the triggering of ATP hydrolysis. This phenomenon occurs only during a transmembrane ΔpH variation (in the absence of nigericin) and interferes with the consumption of protons used to monitor ATP hydrolysis. This effect becomes predominant at acidic pH, where the ratio m between H^+ produced and ATP hydrolysed is low [12]. When the rate of ATP hydrolysis was determined 15 s instead of 5 s after valinomycin addition (Fig. 3B), the shape of the curve obtained with nigericin was not changed, although the maximal activity was decreased. Without nigericin, the situation was different: one then obtained a bell-shaped curve. After a longer time of deactivation, the maximal activities, identical with and without nigericin, continue to de-

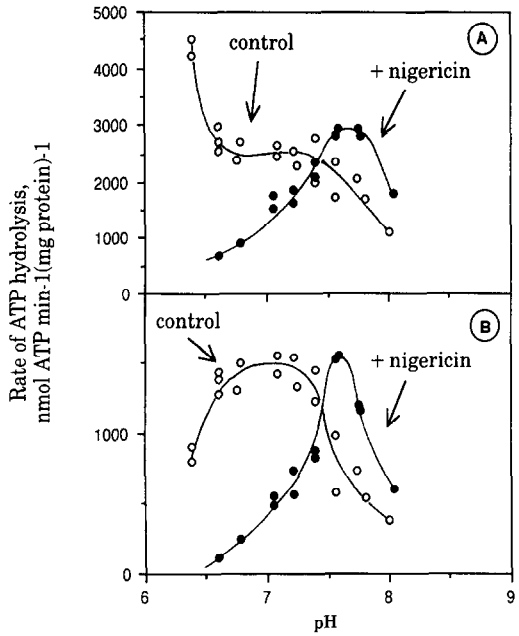

Fig. 3. pH-dependency of instantaneous ATP hydrolysis with (●) and without nigericin (○), 5 s (A) or 15 s (B) after valinomycin addition in pea leaf mitochondria. Purified mitochondria, 67 μg protein · ml^{-1} per assay; NAD^+, 250 μM; glycine, 8 mM; ADP additions, twice 50 μM; ATP, 1 mM; nigericin, 1 μg · (mg protein)$^{-1}$; valinomycin, 40 ng · (mg protein)$^{-1}$.

crease, but the shapes of the two curves no longer change (not shown). This shows that after 15 s (Fig. 3B) the contribution of the transmembrane proton movement to the measured activity was already negligible, because a pseudo-stationary ΔpH was probably reached. One can thus compare with confidence the data obtained with and without nigericin in Fig. 3B. These data show that the activity curve without nigericin is grossly shifted towards the low pH values, as compared to the curve obtained with nigericin. This is consistent with the existence of a ΔpH, varying between 0.5 and 1.0 pH unit, and with the control of ATP hydrolysis by matrix pH in the absence of a membrane potential.

A similar experiment was carried out on potato tuber mitochondria, increasing the external pH from 7 to 8. In this range, ATP hydrolysis was never observed, even in the presence of nigericin. Thus in potato tuber mitochondria, increasing the pH in the internal compartment does not trigger ATP hydrolysis activity.

4. DISCUSSION

In contrast to potato tuber mitochondria, organelles isolated from pea leaves present a high ATP hydrolase activity after addition of valinomycin ± nigericin in state 4. This activity, probably underestimated because of the rapid deactivation of the enzymes, is however consistently higher than ATP synthesis. This was unexpected, as it has been shown, in the case of membrane-bound chloroplast ATPase, that the maximum rate of catalytic turnover was 4 times higher for ATP synthesis than for ATP hydrolysis [22]. However, one should recall that in plant mitochondria, ATP synthesis is controlled mainly by the respiratory chain, and not by the ATPase [23], in contrast to what has been described for animal mitochondria [24]. The maximal turnover rate is therefore far from being reached during ATP synthesis in plant mitochondria.

The effect of nigericin on $\Delta\psi$ and on ATP hydrolysis demonstrated that pea leaf mitochondria maintain a significant ΔpH in addition to $\Delta\psi$, in contrast with potato mitochondria [9]. However, neither transmembrane ΔpH nor matrix pH are responsible for the different modes of regulation of ATPase encountered in pea leaf and potato tuber mitochondria.

The physiological process responsible for this switch between latent and active forms of the enzyme remains to be elucidated. A possible role of the ATP/ADP exchanger should also be precised. The result of this specific regulation could be the decrease of a wasteful ATP consumption in resting organs such as potato tubers. Finally, it would also be interesting to determine whether the loss of ATP hydrolase activity in potato tuber mitochondria is due to a rapid inactivation of the ATPase after dissipation of the proton motive force, as in chloroplasts [17], or whether it reflects an asymmetrical functioning of the enzyme, as it was proposed for the ATPase of animal mitochondria [5,6].

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