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Plant Uncoupling Mitochondrial Protein Activity in Mitochondria Isolated from Tomatoes at Different Stages of Ripening[1]

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In the present study we have observed a higher state of coupling in respiring mitochondria isolated from green as compared to red tomatoes (*Lycopersicon esculentum*, Mill.). Green tomato mitochondria produced a membrane potential ($\Delta\Psi$) high enough to phosphorylate ADP, whereas in red tomato mitochondria, BSA and ATP were required to restore $\Delta\Psi$ to the level of that obtained with green tomato mitochondria. This supports the notion that such uncoupling in red tomato mitochondria is mediated by a plant uncoupling mitochondrial protein (PUMP; cf. Vercesi *et al.*, 1995). Nevertheless, mitochondria from both green and red tomatoes exhibited an ATP-sensitive linoleic acid (LA)-induced $\Delta\Psi$ decrease providing evidence that PUMP is also present in green tomatoes. Indeed, proteoliposomes containing reconstituted green or red tomato PUMP showed LA uniport and LA-induced H^+ transport. It is suggested that the higher concentration of free fatty acids (PUMP substrates) in red tomatoes could explain the lower coupling state in mitochondria isolated from these fruits.

KEY WORDS: Plant uncoupling mitochondrial protein (PUMP); fatty acids; ripening; *Lycopersicon esculentum*.

INTRODUCTION

It is generally accepted that the electrochemical proton gradient ($\Delta\mu H^+$) generated by respiratory chain redox proton pumps is used by the F_0F_1-ATP synthase to produce ATP. Moreover, $\Delta\mu H^+$ can also be used to drive other energy-requiring processes or be dissipated by the uncoupling protein (UCP) of mammalian brown adipose tissue mitochondria (BATM) for heat production (Nicholls and Locke, 1984).

[1] Abbreviations: AOx, alternative oxidase; BATM, brown adipose tissue mitochondria; FA, fatty acids; FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone; LA, linoleic acid; OctylPOE, octylpentaoxyethylene; PBFI, potassium-binding benzofuran isophthalate; PUMP, plant uncoupling mitochondrial protein; SHAM, salicylhydroxamic acid; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; UcP, uncoupling protein of brown adipose tissue mitochondria.

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In the presence of fatty acids (FA), UCP allows the protons extruded by the respiratory chain to reenter the matrix and bypass the ATP synthase, thus permitting dissipation of $\Delta\mu H^+$ as heat, in a process called nonshivering thermogenesis. UCP is now abbreviated as UCP1, to distinguish it from the two novel mammalian uncoupling proteins UCP2 and UCP3, discovered in 1997 (Boss *et al.*, 1997; Vidal-Puig *et al.*, 1997; Nègre-Salvayre *et al.*, 1997). Purine nucleotides such as GDP or ATP bind to a high-affinity site on the UCP1 (Heaton *et al.*, 1978) and inhibit its activity (Nicholls and Locke, 1984) by an allosteric mechanism (Ježek *et al.*, 1994). Recent results suggest that UCP1 mediates uncoupling via a fatty acid cycling mechanism (Skulachev, 1991; Garlid *et al.*, 1996; Ježek *et al.*, 1996a) in which UCP1 translocates fatty acid anions outward. Subsequently, fatty acids become protonated and enter mitochondria in their protonated form, leaving the proton in the matrix (Garlid *et al.*, 1996).

A distinct feature of plant mitochondria, in addition to their nonphosphorylating NAD(P)H dehydrogenases (Soole and Menz, 1995), is the presence of an alternative oxidase (AOx) (for detailed reviews, see Lambers, 1980; Day and Wiskich, 1995; Krab, 1995; Moore *et al.*, 1995). AOx is a cyanide-resistant quinol oxidase sensitive to hydroxamates such as SHAM and represents a nonprotonmotive terminal oxidase that can significantly decrease proton pumping. Together with succinate dehydrogenase (complex II) or the exogenous NAD(P)H dehydrogenases, AOx is capable of oxidizing substrates without any oxidative phosphorylation.

In spite of these energy-dissipating systems, our analysis of respiratory energy coupling in plant mitochondria suggested that these mitochondria may possess an UCP-like protein (Beavis and Vercesi, 1992). Indeed, a hydrophobic 32-kDa protein, which we named plant uncoupling mitochondrial protein (PUMP), was isolated from potato mitochondria and reconstituted into liposomes (Vercesi *et al.*, 1995). Its properties and functions were in all respects similar to UCP1 (Vercesi *et al.*, 1995; Ježek *et al.*, 1996b, 1997a), with the exception that PUMP does not translocate Cl^-. cDNAs encoding UCP homologous proteins were isolated from potato and *Arabidopsis* (Laloi *et al.*, 1997; Maia *et al.*, 1998). Transcripts of those UCP homologous genes were ubiquitously expressed, with high degree of expression in leaves (potato), flowers, and roots (*Arabidopsis*)(Laloi *et al.*, 1997; Maia *et al.*, 1998). In addition to potato, PUMP has also been isolated (Ježek *et al.*, 1997a) from tomatoes (*Lycopersicon esculentum*, Mill.), a climacteric fruit in which a respiratory burst and thermogenesis occur during ripening (Andrews, 1995). Considering that during tomato ripening the concentration of FA (the PUMP substrates) increases significantly (Güçlü *et al.*, 1989; Rouet-Mayer *et al.*, 1995; Galliard, 1980), the aim of this work was to analyze the activity of the PUMP-dissipating pathway in mitochondria isolated from tomatoes at different ripening stages.

MATERIALS AND METHODS

Biological Material and Chemicals

Tomato plants (*Lycopersicon esculentum*, Mill. cv. Santa Clara) were grown in a greenhouse at the Centro de Biologia Molecular e Engenharia Genética, UNICAMP. Fruits were harvested at three stages of development based on color and were designated green (nearly developed but completely green), intermediary (just starting to change color to yellow), and red (mature fruits). ATP, ADP, BSA, SHAM, *n*-propyl gallate, valinomycin, linoleic acid, β-NADH, succinate, FCCP, safranin O, and phospholipids were purchased from Sigma (USA). Fluorescent probes were from Calbiochem (SPQ) and Molecular Probes (PBFI). All other reagents were commercial products of the purest grade available.

Mitochondrial Isolation and Protein Determination

Usually ten tomatoes were sliced and, after removal of the seeds, the slices were homogenized in a domestic blender. The juice obtained was immediately mixed with 500 m*M* sucrose, 40 m*M* HEPES (pH 7.8), 0.2 m*M* EGTA, and 4 m*M* cysteine to final volume of 400 mL. The pH was kept between 7.2 and 7.6 by adding 1 *N* KOH. The homogenate was then filtered through a double layer of a polyester cloth and centrifuged at 500 *g* for 10 min at 4°C. The supernatant was centrifuged at 12000 *g* for 10 min and the pellet resuspended in a medium containing 250 m*M* sucrose, 10 m*M* HEPES buffer, pH 7.2, and 0.3 m*M* EGTA was centrifuged again at 12000 *g* for 10 min. The final pellet containing mitochondria was resuspended in the same medium at a protein concentration of 30 to 40 mg-mL^{-1}. Protein concentration was determined by

modifications of the biuret method (Gornall *et al*, 1949).

Membrane Potential ($\Delta\Psi$) Determinations

The mitochondrial membrane potential ($\Delta\Psi$) was estimated by following the fluorescence of safranin (5 μM), recorded on a F-4010 Hitachi fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with a slit width of 5 nm. The reaction medium contained 125 m*M* sucrose, 65 m*M* KCl, 10 m*M* HEPES (pH 7.2), 2.5 m*M* P_i, 1 m*M* $MgCl_2$, and 0.33 m*M* EGTA. Propranolol (333 μM), atractyloside (10 μM), and oligomycin (1 μg-mL^{-1}) were also routinely added to exclude the plant inner membrane anion channel (Beavis and Vercesi, 1992), ADP/ATP carrier and F_0F_1-ATPase activity, respectively. KCN was added to all membrane potential measurements in order to normalize fluorescence changes and to eliminate possible swelling artifacts upon fatty acid additions.

PUMP Isolation and Reconstitution

PUMP was isolated and incorporated into liposomes as described by Ježek *et al.* (1997a). The mitochondrial pellet was solubilized with detergent (OctylPOE) and incubated in a hydroxylapatite spin column. The flowthrough contained diluted PUMP and subsequent stepwise elution (Ježek and Freisleben, 1994) yielded four fractions, each of which contained PUMP (approx. 0.1 mg protein-mL^{-1}). The isolated PUMP present in these fractions was added to a lipid/OctylPOE micellar solution containing a fluorescent indicator, either 2 m*M* SPQ (Orosz and Garlid, 1993) or 300 μM PBFI (Ježek *et al.* 1997a, b). The detergent was removed using Bio-Beads SM2 (Bio-Rad, USA), thus allowing the formation of sealed vesicles containing PUMP in their membrane. The external probe was washed out using Sephadex G25-300. Ion transport in proteoliposomes was detected on a F-4010 Hitachi fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). The detection of H^+ fluxes was based on the quenching of SPQ probe by the Tes anion (the internal buffer), but not by neutral Tes (Orosz and Garlid, 1993). Anion (fatty acid) uniport monitoring was performed indirectly by following the concomitant K^+ fluxes using PBFI, which indicates the net charge uptake resulting from the anion influx. Excitation and emission wavelenghts used were 340 and 440 nm for SPQ and 340 and 490 nm for PBFI, with slit widths of 10 and 20 nm, respectively. Both methods have been described in detail elsewhere (Ježek *et al.* 1997a, b).

RESULTS

Membrane Potential in Green and Red Tomato Mitochondria

Figure 1 shows that the transmembrane electrical potential ($\Delta\Psi$) of red tomato mitochondria was much smaller than that of green and intermediately ripe tomato mitochondria, when isolated without BSA. Green tomato mitochondria were able to phosphorylate ADP when oligomycin and atractyloside were omitted, as indicated by the transient decrease in $\Delta\Psi$ (line g, dotted). In these mitochondria, ATP addition had no effect, while BSA caused a small increase in the $\Delta\Psi$

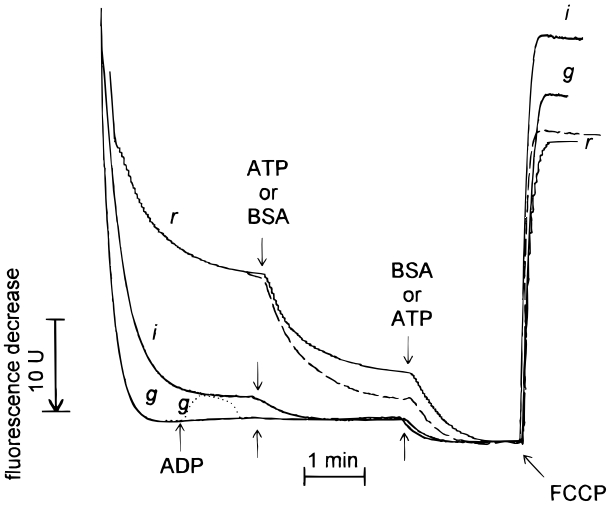

Fig. 1. Membrane potential ($\Delta\Psi$) of mitochondria isolated from the three stages of tomato development. Mitochondria (0.33 mg-mL^{-1}) from green (lines g), intermediate (line i), and red (lines r) tomatoes were assayed when respiring with 5 m*M* succinate (K^+ salt), in a medium containing 125 m*M* sucrose, 65 m*M* KCl, 10 m*M* HEPES (pH 7.2), 2.5 m*M* P_{i1} 1 m*M* $MgCl_2$, 0.33 m*M* EGTA, 333 μM propranolol, 10 μM atractyloside, and 1 μ-mL^{-1} oligomycin. ADP (K^+ salt, 0.2 m*M*), 0.5 m*M* Tris-ATP, 0.1% w/v BSA, and 1μM FCCP were added as indicated. Oligomycin and atractyloside were omitted for line g (dotted). In solid lines g, i, and r, the arrows indicate additions of ATP and BSA, respectively. In contrast, the dashed line r shows the addition of BSA followed by the addition of ATP. $\Delta\Psi$ was monitored by safranin fluorescence as decribed in section on Materials and Methods.

(line g). The $\Delta\Psi$ of intermediate (line i) and red (line r, solid and dashed) tomato mitochondria increased cumulatively following the additions of ATP (0.5 m*M*) and BSA 0.1% (w/v), suggesting that this uncoupling may be mediated by the plant uncoupling mitochondrial protein (PUMP). Inclusion of BSA prior to ATP to red tomato mitochondria (line r, dashed) did not present an additional increment in $\Delta\Psi$. The coupling effects of BSA and ATP were not affected by omitting oligomycin or atractyloside (inhibitors of F_0F_1-ATPase and the ADP/ATP carrier, respectively) from the assay medium.

FA-Induced PUMP Activity in Green Tomato Mitochondria

The results of Fig. 1 suggest that PUMP may not be present in green tomatoes. In order to evaluate this possibility, we investigated the effect of linoleic acid (LA) on green tomato mitochondria. Figure 2A (line a) shows, that similarly to what was observed in potato (Ježek *et al.*, 1996b) and red tomato mitochondria (not shown), the addition of 20 μM LA decreased $\Delta\Psi$ in green tomato mitochondria respiring with 5 m*M* succinate (K^+ salt). This LA-induced $\Delta\Psi$ decline, mediated by PUMP, was reduced and was only transient when 1 m*M* ATP (Tris salt, line b) was present from the beginning of the experiment. Total PUMP inhibition requires very high ATP concentrations (Ježek *et al.* 1997a). Figure 2B (line a) shows the addition of an equal amount of LA to rat liver mitochondria (where PUMP is not present) resulted in a significant ATP-insensitive decrease in $\Delta\Psi$ (line b), most probably caused by a fast diffusion of the protonated fatty acid by a flip-flop mechanism (Kamp and Hamilton, 1993; Ježek *et al.* 1997c). This mechanism should also be responsible for part of the initial $\Delta\Psi$ decline observed in line b (panel A and B).

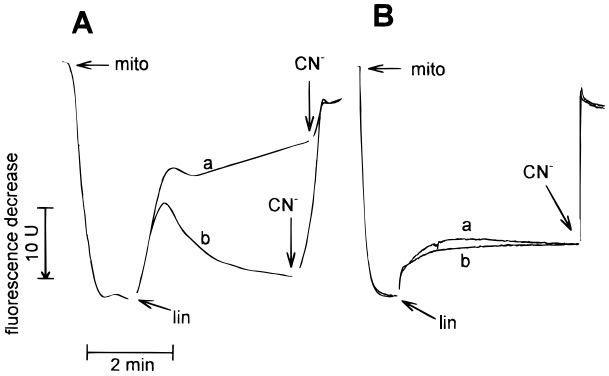

Fig. 2. Linoleic acid-induced membrane potential decrease in green tomato mitochondria. The addition of 20 μM linoleic acid (lin) induced a substantial decrease in $\Delta\Psi$ in mitochondria (0.33 mg-mL^{-1}) isolated from green tomatoes (panel A, line a), respiring with 5 m*M* succinate (K^+ salt) in the medium described in Fig. 1 legend. (A) Line b shows the inhibition of this decline by 1 m*M* ATP (Tris salt, present from the beginning). (B) Line a demonstrates the effect of linoleic acid on rat liver mitochondria (0.5 mg-mL^{-1}), and line b shows the effect of 1 m*M* ATP (Tris salt, present from the beginning). Linoleic acid and CN^- (1.5 m*M*) were added as indicated. $\Delta\Psi$ was monitored by safranin fluorescence as described in section on Materials and Methods.

H^+ Efflux and Fatty Acid Uniport in Proteoliposomes Containing Tomato PUMP

The partial purification of PUMP is based upon the finding that most of the tomato fruit proteins are retained on hydroxylapatite, except the hydrophobic PUMP (Ježek *et al.*, 1997a). Thus, PUMP from both red and green tomatoes, contained in the hydroxylapatite-passthrough, was reconstituted into the lecithin/cardiolipin liposomes and LA-induced H^+ transport or the uniport of LA anion were measured using the fluorescent ion indicators SPQ and PBFI, respectively. Figure 3 shows that valinomycin, added after LA to proteoliposomes with the reconstituted PUMP, induced H^+ efflux (botton line), which was partially inhibited by external ATP (line +ATP) and completely inhibited by undecanesulfonate (Table I, cf. Ježek *et al.*, 1997a). In the same experiment, BSA (0.1% w/v) completely inhibited the H^+ efflux (line +BSA) leading to the interpretation that it is mediated by PUMP. This is further supported by experiments with liposomes lacking PUMP (not shown) in which no H^+ efflux was induced by valinomycin (Ježek *et al.*, 1997a, b). The rates of LA-induced H^+ efflux in proteoliposomes containing PUMP from green and red tomatoes are presented in Table I.

Figure 4 shows that reconstituted red tomato PUMP was able to translocate the anionic forms of LA in an ATP-sensitive manner. BSA again completely abolished this process. In addition, a fatty acid which is unable to flip-flop, phenylvaleric acid (Ježek *et al.*, 1997b, c), was not translocated by PUMP. Undecanesulfonate and hexanesulfonate, but not CI^- were also translocated (not shown). The low degree of ATP or GDP inhibition seen in these reconstitution experiments reflects the two possible orientations outward or inward of the nucleotide-binding site, in which only

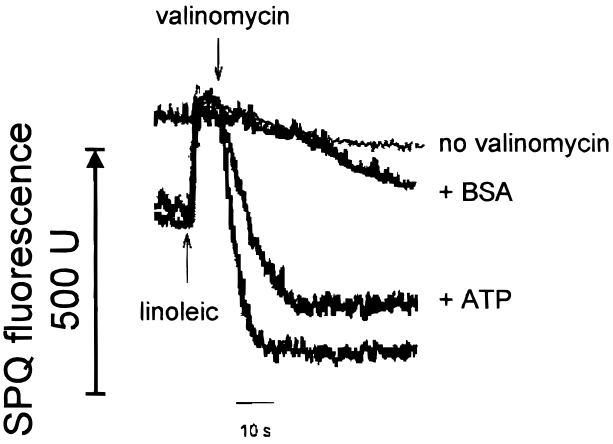

Fig. 3. Fatty acid cycling in proteoliposomes containing PUMP isolated from red tomato mitochondria. Bottom line, addition of 66.7 μM linoleic acid caused interior acidification of the vesicles, reflecting flip-flop of the neutral fatty acid into the inner lipid leaflet and its subsequent dissociation. PUMP function was manifested as the efflux of H^+ (internal alkalinization, indicated by the decrease in SPQ fluorescence) initiated by 1.3 μM valinomycin, added after LA. Line +ATP, Inhibition (43%) of PUMP-mediated LA cycling by 2.5 mM ATP (Tris salt). Line +BSA, absence of FA cycling in the presence of BSA (0.1% w/v); Line no valinomycin, represents an experiment performed as in bottom line, but in the absence of valinomycin. Vesicles (25 μL per assay) contained 84.4 mM tetraethylammonium sulfate, 28.85 mM tetraethylammonium-Tes, pH 7.2, (tetraethylammonium was 9.2 mM), and 0.6 mM Tris-EGTA. In the external medium, 84.4 mM K_2SO_4 replaced the tetraethylammonium sulfate.

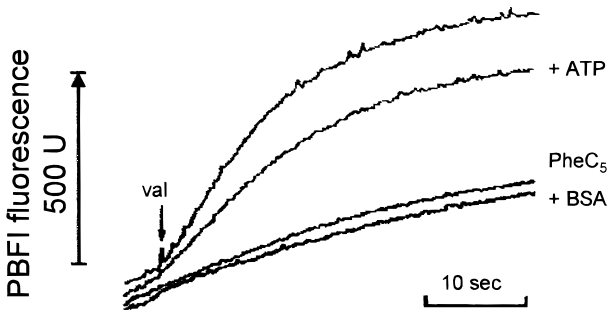

Fig. 4. Fatty acid uniport in proteoliposomes containing PUMP isolated from red tomato mitochondria. The two upper lines were measured in the presence of 40 μM linoleic acid, in the absence and presence (line +ATP) of 4 mM ATP (Tris salt, 45% inhibition), respectively. The two lower lines illustrate the lack of transport in the presence of 66 μM phenylvaleric acid (line $PheC_5$), and the basal K^+ influx in the presence of 0.4% w/v BSA (line +BSA). The vesicle interior contained 75 mM tetraethylammonium sulfate, 75 mM tetraethylammonium-Tes, pH 7.2, 0.05 mM K_2SO_4, and 300 μM PBFI. The assay was typically conducted by mixing 25 μL of vesicles with 1.475 ml of 75 mM K_2SO_4 and 75 mM tetraethylammonium-Tes, pH 7.2. Transport was initiated by 1.3 μM valinomycin (val).

the former can interact with external ATP. Indeed, the presence of ATP on both sides produces nearly total inhibition (Ježek *et al.*, 1997a). The pattern of both processes (LA-induced H^+ efflux and LA anion uniport) were similar for the reconstituted green and red tomato PUMP (Table. I).

Table I. Typical Rates of Linoleic Acid-Induced H^+ Efflux in Proteoliposomes Containing PUMP Isolated from Mitochondria of Green and Red Tomatoes[a]

Conditions	LA-induced H^+ efflux (mM-s^{-1}) PUMP isolated from	
	Green tomatoes	Red tomatoes
Control	1.29	1.56
+ 4 mM GDP	0.81	1.00
+ 366 μM undecanesulfonate	0.13	0.20

[a] H^+ efflux was induced by 1.3 μM valinomycin added after 53 μM linoleic acid. The rates (average of duplicate measurements) are expressed in mM-s^{-1}. An equal amount of PUMP from each source was reconstituted (30 μg).

DISCUSSION

We have previously proposed that the existence of PUMP could help to explain various physiological events in plants (Vercesi *et al.*, 1995; Ježek *et al.*, 1996b, 1997a). PUMP is a mitochondrial, inner membrane protein that, in conjunction with its transport substrates (fatty acids), is able to finely regulate mitochondrial coupling. The discovery of PUMP in climacteric fruits such as tomatoes (Ježek *et al.*, 1997a), banana, mango, apple, strawberry, papaya, melon, pineapple, orange, pear, and peach (Ježek *et al.*, 1998), led us to hypothesize that the activation of PUMP could be associated with the respiratory burst known to occur during the ripening process of climacteric fruits (Kidd and West, 1925). We now demonstrate that, when green and red (ripe) tomato mitochondria were incubated in the absence of BSA, the PUMP activity was much more pronounced in red tomato mitochondria, as reflected by the additional coupling effects of BSA and ATP. Mitochondrial preparations obtained from tomatoes at an intermediate stage of maturation were slightly uncoupled, but developed a fully coupled state after the addition of BSA and ATP.

We have previously shown (Ježek *et al.*, 1997a) that, similarly to UCP1, PUMP leads to uncoupling by a fatty acid cycling mechanism, thus allowing for

the uniport of fatty acid anions, which are expelled from the matrix to the cytosol and return to the matrix in protonated form by flip-flop across the lipid bilayer, thereby carrying H^+ (Garlid *et al.*, 1996). Since free fatty acids are the true substrates for PUMP-mediated uncoupling, the results of Fig. 1 may be interpreted either in terms of a free fatty acid or PUMP deficiency in green tomatoes. The first possibility is in agreement with the finding that the free fatty acid content of tomatoes increases substantially during ripening (Galliard, 1980; Güçlü *et al.*, 1989; Rouet-Mayer *et al.*, 1995). The second possibility would imply that expression of PUMP is induced during tomato ripening. In this regard, we have demonstrated, using either intact mitochondria (Fig. 2) or a reconstituted system (Figs. 3 and 4), that LA-induced uncoupling and H^+ transport occur in both green and red tomatoes. Thus, we have shown that green tomato mitochondria, in a manner similar to mitochondria from brown adipose tissue, potato, and red tomato, can be uncoupled by LA via an ATP-sensitive mechanism and that the reconstituted PUMP from green tomatoes exhibits functional properties of red tomato PUMP. This is supported by our recent results showing that the addition of 10 μM linoleic acid to fatty acid depleted green tomato mitochondria purified in a self-generating Percoll gradient in the resting state stimulated respiration from 150 to 411 nmol of oxygen min-mg^{-1} of protein in parallel to a drop in $\Delta\Psi$ from 200 to 176 mV (Jarmuszkiewicz *et al.*, 1998). In addition, it was recently shown that the amount of immunologically detectable PUMP slightly decreases during tomato ripening (Almeida *et al.*, 1999).

The above results provide evidence that PUMP is present during different stages of tomato ripening and suggest that the higher concentration of free fatty acids (Güçlü *et al.*, 1989; Rouet-Mayer *et al.*, 1995; Galliard, 1980) may contribute to the lower degree of coupling in mitochondria isolated from red tomatoes. This hypothesis is strongly supported by the large extent of $\Delta\Psi$ increase induced by BSA alone in mitochondria from red tomatoes. The additional increase of $\Delta\Psi$ induced by ATP is attributable to the presence of remaining FA in the incubation medium, given the inability of BSA, even at a concentration of 0.5%, to completely remove the contaminant FA present in these mitochondrial preparations (Almeida *et al.*, 1999). These results may allow the speculation that this uncoupling activity may explain, at least in part, the observed oxygen burst and thermogenesis that occurs during tomatoes ripening (Andrews, 1995), and may be a mechanism to switch off ATP production associated with termination of the synthetic processes that occur during the final stages of seed formation and senescence (Ježek *et al.*, 1996b). Indeed, we have recently shown that LA-induced PUMP activity in state-3 respiring green tomato mitochondria can efficiently divert energy from ATP synthesis (Jarmuszkiewicz *et al.*, 1998). The observation that LA stimulates PUMP and inhibits AOx in the same concentration range (0–10 μM) (Sluse *et al.*, 1998) strongly indicates that these respiratory energy-dissipating pathways have different physiological roles and do not display maximum activity simultaneously. Therefore, the main function of PUMP could be the modulation of $\Delta\mu_{H^+}$, according to FA concentration, while the bypassing of the respiratory complexes III and IV by AOx, at high membrane potential, favors the cycling of cofactors necessary for biosynthesis and plant growth (Whelan, 1996).

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