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Cyanide-Resistant, ATP-Synthesis-Sustained, and Uncoupling-Protein-Sustained Respiration during Postharvest Ripening of Tomato Fruit[1]

Andrea Miyasaka Almeida[2], Wieslawa Jarmuszkiewicz[2], Hamid Khomsi, Paulo Arruda, Anibal E. Vercesi*, and Francis E. Sluse

Departamento de Patologia Clínica, Faculdade de Ciêancias Médicas (A.M.A., A.E.V.), and Centro de Biologia Molecular e Engenharia Genética (P.A.), Universidade Estadual de Campinas, Campinas, São Paolo, Brazil; Department of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61–701 Poznan, Poland (W.J.); and Laboratory of Bioenergetics, Center of Oxygen Biochemistry, Institute of Chemistry B6, University of Liège, Sart Tilman, B-4000 Liège, Belgium (H.K., F.E.S.)

Tomato (*Lycopersicon esculentum*) mitochondria contain both alternative oxidase (AOX) and uncoupling protein as energy-dissipating systems that can decrease the efficiency of oxidative phosphorylation. We followed the cyanide (CN)-resistant, ATP-synthesis-sustained, and uncoupling-protein-sustained respiration of isolated mitochondria, as well as the immunologically detectable levels of uncoupling protein and AOX, during tomato fruit ripening from the mature green stage to the red stage. The AOX protein level and CN-resistant respiration of isolated mitochondria decreased with ripening from the green to the red stage. The ATP-synthesis-sustained respiration followed the same behavior. In contrast, the level of uncoupling protein and the total uncoupling-protein-sustained respiration of isolated mitochondria decreased from only the yellow stage on. We observed an acute inhibition of the CN-resistant respiration by linoleic acid in the micromolar range. These results suggest that the two energy-dissipating systems could have different roles during the ripening process.

In addition to possessing multiple NAD(P)H dehydrogenases, the branched electron transport chain of plant mitochondria contains a CN- and antimycin-resistant AOX (Vanlerberghe and McIntosh, 1997; Wagner and Moore, 1997) that catalyzes the reduction of oxygen to water with electrons derived directly from ubiquinol, bypassing the energy-conserving sites (i.e. proton-translocating complexes III and IV) of the Cyt pathway. Since no protomotive force is generated during this reaction, electron flow through AOX appears to dissipate energy, decreasing ATP synthesis. The plant mitochondrial AOX is encoded by a nuclear gene(s) and consists of one to three proteins between 32 and 39 kD, depending on the plant and the tissue (McIntosh, 1994). AOX activity, which can be inhibited by hydroxamic acids such as BHAM, can be stimulated by α-keto acids such as pyruvate (Millar et al., 1993; Day et al., 1994). Moreover, the activity of AOX can be regulated by the reduction state of the enzyme; the covalently bound, oxidized dimer is much less active than the reduced form (Umbach and Siedow, 1993; Umbach et al., 1994).

In addition to AOX, some plant mitochondria contain another energy-dissipating system, PUMP (Vercesi et al., 1995, 1997), which has a molecular mass (32 kD for the protein purified from potato mitochondria) and activity profile similar to those from mammalian brown adipose tissue-uncoupling protein (Garlid et al., 1996; Skulachev, 1998). As a $\Delta\Psi$-dependent, anionic fatty acid uniporter (from the matrix to the outside), PUMP enables H^+ reentry into the matrix through a fatty acid-cycling process, bypassing ATP synthase and, as a consequence, dissipating the proton motive force (Jezek et al., 1996). In plant mitochondria, FFA (e.g. LA)-induced activity of PUMP is sensitive to inhibition by purine nucleotides, such as ATP and GTP, and by BSA, which removes fatty acids (Vercesi et al., 1995, 1997).

Tomato (*Lycopersicon esculentum*) fruit mitochondria display both CN-resistant respiration and PUMP activity as redox-dissipating and H^+-electrochemical gradient-dissipating systems, respectively (Flores and Chin, 1980; Jezek et al., 1997). Since the activity of these two dissipating pathways significantly affects the energy yield of the cell, it is possible that precise mechanisms are required to control the relative contributions of energy-dissipating and -conserving pathways to the total oxygen uptake in these mitochondria. Moreover, the presence of two energy-dissipating systems, AOX and PUMP, which yield essentially the same final effect (i.e. a decrease in the efficiency

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[2] These authors contributed equally to this work.

* Corresponding author; e-mail anibal@obelix.unicamp.br; fax 55–19–788–1118.

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; CN, cyanide; FCCP, cyanide *p*-trifluoromethoxyohenylhydrazone; FFA, free fatty acid; LA, linoleic acid; PUMP, plant uncoupling mitochondrial protein; $\Delta\Psi$, mitochondrial membrane potential.

of oxidative phosphorylation), raises the question as to whether they work in concert, simultaneously, or in a temporal sequence according to the particular physiological state of the plant cell. Fruit ripening may provide an interesting model with which to study this question. Indeed, it is well known that a respiratory burst and thermogenesis occur during the ripening of climacteric fruit such as tomato (Day et al., 1980; Kumar et al., 1990; Andrews, 1995; Cruz-Hernandez and Gomez-Lim, 1995), and until recently, both of these phenomena were attributed to AOX activity. However, the detection of PUMP in tomato fruit mitochondria has stimulated a reassessment.

The aim of this work was to evaluate the evolution of CN-resistant, ATP-synthesis-sustained, and PUMP-sustained respiration in isolated mitochondria from tomato fruit during postharvest ripening. Immunological identification of AOX and PUMP was also performed, together with an assessment of the alterations in the amounts of both proteins throughout the period of ripening.

MATERIALS AND METHODS

Tomato (*Lycopersicon esculentum* cv Petomech) plants were grown in a greenhouse at the Centro de Biologia Molecular e Engenharia Genética (Universidade de Estadual de Campinas, São Paulo, Brazil). Tomato fruits were harvested at a nearly developed green stage and stored at room temperature until they reached different stages of postharvest ripening, defined on the basis of fruit color: green, yellow, orange, and red.

Isolation of Mitochondria

Approximately 0.5 kg of tomatoes at the desired stage of ripening was sliced and homogenized (without seeds) in a domestic blender. The juice was immediately diluted to a final volume of 400 mL with medium containing 500 mM Suc, 0.2 mM EGTA, 4 mM Cys, and 40 mM Hepes, pH 7.8. During homogenization the pH was kept between 7.2 and 7.8 by adding 1 N KOH. After the homogenate was filtered through a layer of polyester, the crude mitochondria were isolated by conventional differential centrifugation (500*g* for 10 min; 12,300*g* for 10 min) and then washed twice in medium containing 250 mM Suc, 0.3 mM EGTA, and 10 mM Hepes, pH 7.2.

The mitochondria were then purified on a self-generating Percoll gradient using a method modified from that of Van den Bergen et al. (1994). Because of an increase in the density of the mitochondrial fraction during ripening of the fruit, different concentrations of Percoll in the gradient medium were used to improve the separation: 21% (v/v) for green and yellow tomato mitochondria and 25% and 28% for orange and red tomato mitochondria, respectively. The gradient medium contained 250 mM Suc, 0.3 mM EGTA, 10 mM Hepes, pH 7.2, and 0.5% (w/v) BSA. The presence of BSA in the medium allowed partial chelating of FFAs from the mitochondrial suspension. The gradient was centrifuged at 40,000*g* for 30 min. The mitochondrial layer was collected and washed three times in 250 mM Suc, 0.3 mM EGTA, and 10 mM Hepes, pH 7.2. Protein concentrations were determined by the biuret method (Gornall et al., 1949).

Measurement of Respiration

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) in 1.3 mL of standard incubation medium (25°C) containing 125 mM Suc, 65 mM KCl, 10 mM Hepes, pH 7.4, 0.33 mM EGTA, 1 mM $MgCl_2$, and 2.5 mM KH_2PO_4, with 0.4 to 0.5 mg of mitochondrial protein. All measurements were made in the presence of 10 mM succinate (plus 5 μM rotenone) as the oxidizable substrate. To ensure complete activation of succinate dehydrogenase, we added 0.18 mM ATP. Some state-4 measurements were performed in the presence of 2.5 μg oligomycin mL^{-1} incubation medium. For state-3 measurements, 0.17 mM (pulse) or 2 mM (saturating) ADP was supplied. Two millimolar BHAM inhibited the alternative pathway, and 1.5 mM KCN inhibited the Cyt pathway. PUMP activity was inhibited with 0.5% BSA and 1 mM GTP. We supplied 0.15 mM pyruvate and 1 mM DTT to activate the alternative pathway and LA (3.9 or 10 μM) to activate PUMP. Further details of the conditions are described in the figure legends.

SDS-PAGE and Immunoblotting of PUMP

Up to 80 μg of mitochondrial protein was solubilized in the sample buffer (Liu et al., 1977) containing 5% (w/v) SDS, 250 mM Tris-acetate, pH 7.4, 1.25 M Suc, 10 mM EDTA, 0.01% (v/v) bromphenol blue, and 0.5% β-mercaptoethanol and boiled for 4 to 5 min. SDS-PAGE was carried out in a manner similar to that of Laemmli (1979) using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel containing 4.5 M urea, followed by western blotting. After protein electrotransfer, the membranes (Hypobond N, Amersham) were blocked overnight at 4°C in 20 mM Tris-HCl, pH 7.4, containing 137 mM NaCl, 0.1% (v/v) Tween, and 5% (w/v) nonfat milk, and then incubated (1 h, 25°C) with diluted (1:1000) polyclonal antibodies against potato (*Solanum tuberosum*) PUMP. After incubation with an anti-rabbit IgG-alkaline phosphatase conjugate, the membranes were incubated for 10 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and a solution of chemiluminescent substrate (CSPD, Tropix, Bedford, MA) diluted 1:1000. The bands were detected by chemiluminescence.

SDS-PAGE and Immunoblotting of AOX Protein

Up to 150 μg of mitochondrial protein was solubilized in the sample buffer (5% [w/v] SDS, 60 mM Tris-HCl, pH 6.8, 10% glycerol, 0.004% [v/v] bromphenol blue, and 100 mM DTT) and boiled for 4 to 5 min. The mitochondrial samples were subjected to SDS-PAGE (12.5% nonurea gel), followed by western blotting (Umbach and Siedow, 1993). The antibodies developed against the AOX protein of *Sauromattum guttatum* (generously supplied by Dr. T.E. Elthon, University of Nebraska, Lincoln) were diluted 1:500. AOX bands

were visualized using a chemiluminescent substrate (see above).

RESULTS

Determination of the Respiration Sustained by Different Branches of the Plant Mitochondrial Respiratory Network

The scheme in Figure 1 shows the elements of the plant mitochondrial respiratory network that we investigated: two pathways transferring electrons to oxygen (i.e. the Cyt and AOX pathways) and three pathways consuming the proton electrochemical gradient built up by the Cyt pathway using succinate (plus rotenone) as the oxidizable substrate (i.e. ATP synthesis, PUMP activity, and H^+ leakage).

ATP-Synthesis-Sustained Respiration

Measuring the respiratory rates of isolated mitochondria with succinate (plus rotenone) as the substrate in the presence of BHAM, an inhibitor of AOX, and GTP plus BSA, inhibitors of PUMP, permitted determination of the activity of a mitochondrial network pathway involving respiratory complexes II, III, and IV and the ATP synthase and H^+ leak in states 3 (plus ADP) and 4 (no ADP). The state-3 respiration measurements were performed under two sets of conditions: (a) ADP-induced respiration (state 3) was initiated in the presence of BSA plus GTP, followed by the addition of BHAM (Fig. 2A), and (b) respiration was initiated in state 4 in the presence of GTP, BSA, and BHAM (Fig. 2B), followed by a pulse of 0.17 mM ADP (Fig. 2B). The state-4 respiration measurements were obtained after the added ADP had been consumed (Fig. 2B).

We pooled and averaged the results of the two approaches for obtaining state-3 rate measurements at each stage of ripening (Fig. 2C). We estimated the H^+ leak in state 4 by measuring respiration in the presence of GTP, BSA, BHAM, and oligomycin (Fig. 2C). The presence of oligomycin prevents respiration linked to ATPase activity and H^+ slip at the ATP synthase level. The H^+ leak is not measurable in state 3, where it is almost negligible because of its steep dependence on membrane potential (Nicholls, 1974; Nicholls and Ferguson, 1992). When the respiration sustained by ATP synthesis was plotted against the stage of ripeness (Fig. 2C), we found that it decreased up to the orange stage. This decline subsequently stabilized between the orange and red stages. State-4 respiration and H^+ leak-sustained respiration showed similar decreases with the stage of ripeness. The respiratory control ratio (state 3/state 4) remained almost constant (2.51 ± 0.16) throughout all stages. Thus, when both AOX and PUMP were inactive, the ATP-synthesis-sustained respiration in iso-

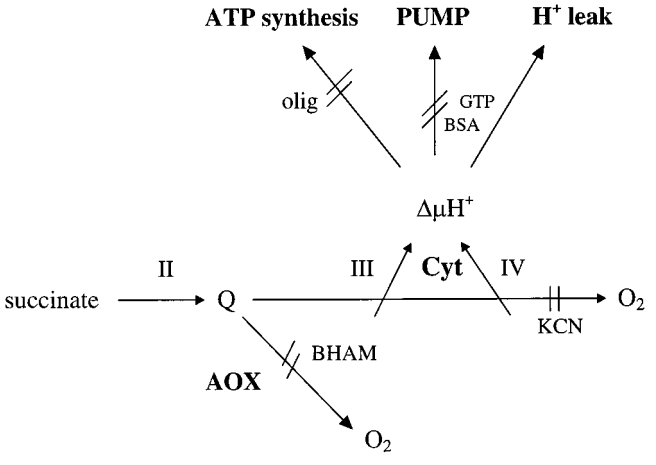

Figure 1. Respiratory network in plant mitochondria. This scheme shows the pathways of electron transport from succinate as the oxidizable substrate and three ways in which the proton electrochemical gradient ($\Delta\mu H^+$) is used. Inhibitors of the different complexes are shown. NADH oxidation in plant mitochondria and other ways of using $\Delta\mu H^+$ (e.g. transport of ions and metabolites) are not shown. olig, Oligomycin; Q, ubiquinone.

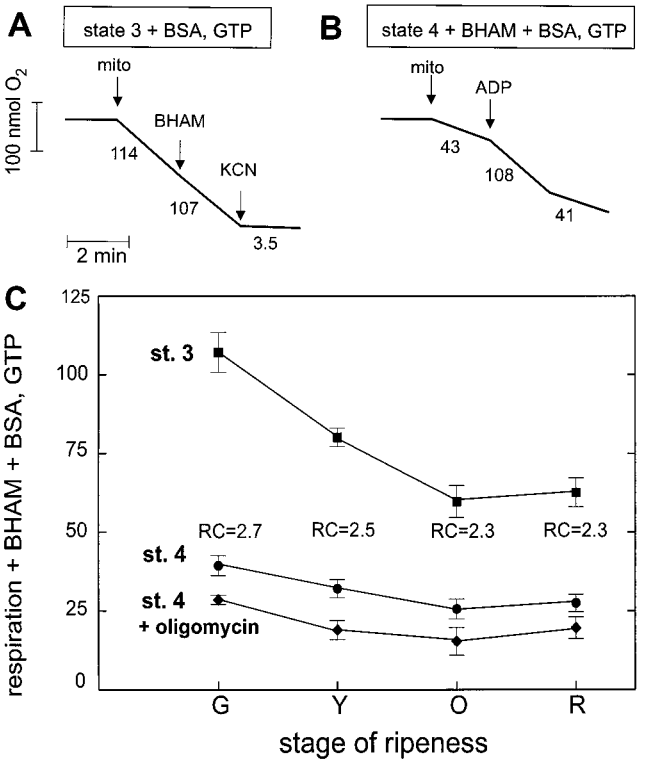

Figure 2. A and B, Determination of ATP-synthesis (plus H^+ leak)-sustained respiration (defined as [BHAM plus BSA and GTP]-resistant respiration) in states 3 (A) and 4 (B). State 4 (B) was measured after ADP exhaustion. Mitochondria (mito) were incubated in a standard reaction medium containing 0.5% BSA, 1 mM GTP, and 2 mM ADP (for state 3), as described in "Materials and Methods." Two millimolar BHAM, 0.17 mM ADP, and 1.5 mM KCN were added. The numbers on the traces refer to the O_2 consumption rates in nmol min^{-1} mg^{-1} protein. The respiration of green tomato mitochondria is shown as an example. C, ATP-synthesis (plus H^+ leak)-sustained respiration ([BHAM plus BSA and GTP]-resistant respiration) in state (st.) 3 (■), state 4 (●), and state 4 plus oligomycin (♦) in the four stages of tomato fruit ripeness (G, green; Y, yellow; O, orange; and R, red). Respiratory rates are in nmol O_2 min^{-1} mg^{-1} protein. The data are presented as the means ± SD of four independent experiments. The respiratory control values (RC) obtained from the average values for states-3 and -4 respiration at each stage of ripening also appear.

lated mitochondria decreased progressively throughout the period of ripening.

AOX-Sustained Respiration

CN-resistant, AOX-sustained respiration measured in the presence of PUMP and Cyt pathway inhibitors (GTP, BSA, and KCN, respectively) represents the activity of the electron transport pathway including complex II and AOX (in our experimental conditions with succinate plus rotenone). The addition of 1.5 mM KCN to isolated tomato fruit mitochondria respiring in state 3 (plus 2 mM ADP) or state 4 (plus oligomycin) resulted in similar CN-resistant respiratory rates (corrected for the low residual rates, 2–4 nmol O_2 min^{-1} mg^{-1} protein) (Fig. 3, A and B). In the presence of AOX activators (pyruvate and DTT), we observed an increase in CN-resistant respiration (Fig. 3, A and B; solid lines compared with dotted lines) using mitochondria isolated from green fruit as an example.

With the stage of ripeness, the activity of the CN-resistant pathway measured with isolated mitochondria decreased from the green to the orange stage and then stabilized, both in the absence and presence of DTT and pyruvate (50% decrease for both, Fig. 3C). The relative activation by DTT and pyruvate remained constant in the four stages of tomato fruit ripeness (approximately 100% stimulation). However, when PUMP was activated by a low concentration of exogenously added LA (3.9 μM) in the absence of PUMP inhibitors, we observed a strong decrease (70%–80% for all stages) in the CN-resistant respiration, plus DTT and pyruvate (Fig. 3C). Thus, either the activation of PUMP by LA or the presence of LA itself strongly inhibited CN-resistant respiration in isolated tomato fruit mitochondria. On the contrary, in state-3 respiration (in the presence of BHAM) we observed neither inhibition nor stimulation of the Cyt pathway by LA, although PUMP was working under these conditions (Jarmuszkiewicz et al., 1999).

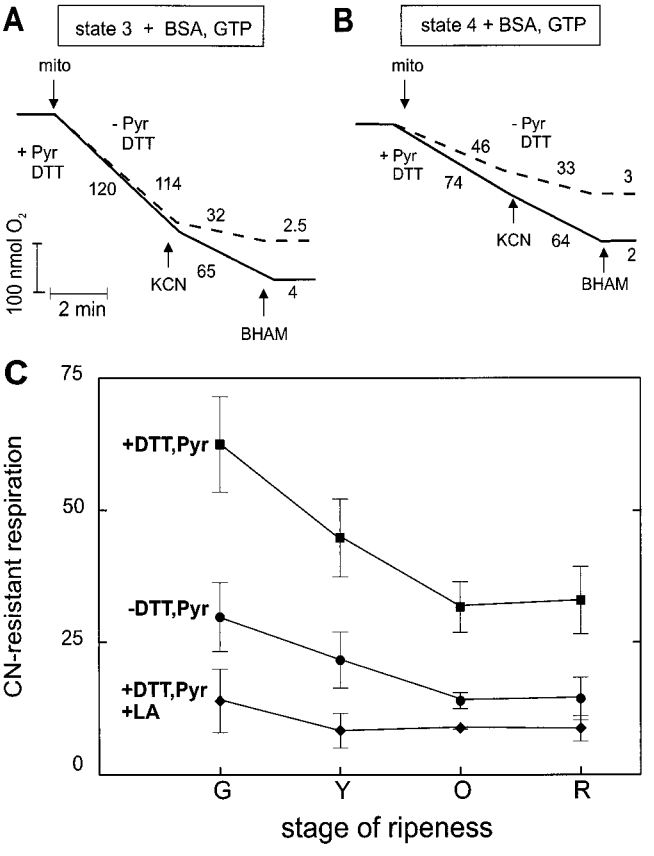

Figure 3. Determination of AOX-sustained respiration (defined as [KCN plus BSA and GTP]-resistant respiration) in state 3 (A) and state 4 (B) in the presence and absence of DTT and pyruvate (Pyr). Mitochondria (mito) were incubated in a standard reaction medium in the presence of 0.5% BSA, 1 mM GTP, and 2 mM ADP (state 3) or 2.5 μg oligomycin mL^{-1} incubation medium (state 4), as described in "Materials and Methods"; 1.5 mM KCN and 2 mM BHAM were added. The numbers on the traces refer to the O_2-consumption rates in nmol min^{-1} mg^{-1} protein. The respiration of green tomato mitochondria is shown as an example. C, CN-resistant respiration (BSA plus GTP) in the absence (●) and presence (■) of DTT and pyruvate in the four stages of tomato fruit ripeness (G, green; Y, yellow; O, orange; and R, red). The effect of 3.9 μM LA in the presence of DTT and pyruvate (minus BSA minus GTP) is shown (◆). Respiratory rates are in nmol O_2 min^{-1} mg^{-1} in states 3 and 4 from four to six independent experiments.

PUMP-Sustained Respiration

The LA-induced PUMP activity of purified tomato fruit mitochondria isolated on a Percoll gradient containing 0.5% BSA was measured in state-4 respiration in the presence of BHAM and oligomycin (Fig. 4). Using the slopes of the respiratory rates shown in Figure 4A, we were able to calculate the extent of PUMP stimulation by 10 μM LA as the difference between steady-state respiration rates (slope 2 minus slope 1). The respiratory rate in the presence of LA (slope 2) represents the activity of the mitochondrial pathway that includes complexes II, III, and IV, as well as the PUMP and H^+ leak, in state 4. In these conditions, the H^+ leak was probably negligible because of the LA-induced drop in $\Delta\Psi$ to a value close to that of state 3 ($\Delta\Psi$ of state 3 = 170 mV; $\Delta\Psi$ of state 4 + 10 μM LA = 174 mV). The subsequent addition of 0.5% BSA and 1 mM GTP inhibited PUMP activity and led to a respiratory rate sustained only by the H^+ leak (slope 3).

The difference between the LA-stimulated respiratory rate and the GTP/BSA-inhibited respiratory rate (slope 2 minus slope 3) could correspond to the total PUMP-sustained respiratory rate if the addition of LA did not modify the H^+ leak, which is unlikely because of the drop in $\Delta\Psi$. Therefore, using this calculation the total PUMP-sustained respiratory rate can only be underestimated. The respiratory rate that preceded the addition of LA (slope 1) represents the H^+ leak-sustained respiration and a possible "endogenous" PUMP activity attributable to the presence of remaining FFAs in the mitochondrial suspension after isolation in the presence of 0.5% BSA. The difference between slope 1 and slope 3 yielded an estimate of endogenous PUMP activity. Accordingly, total PUMP activity was equal to the LA-stimulated activity plus endogenous PUMP activity. As shown in Figure 4A, in green tomato

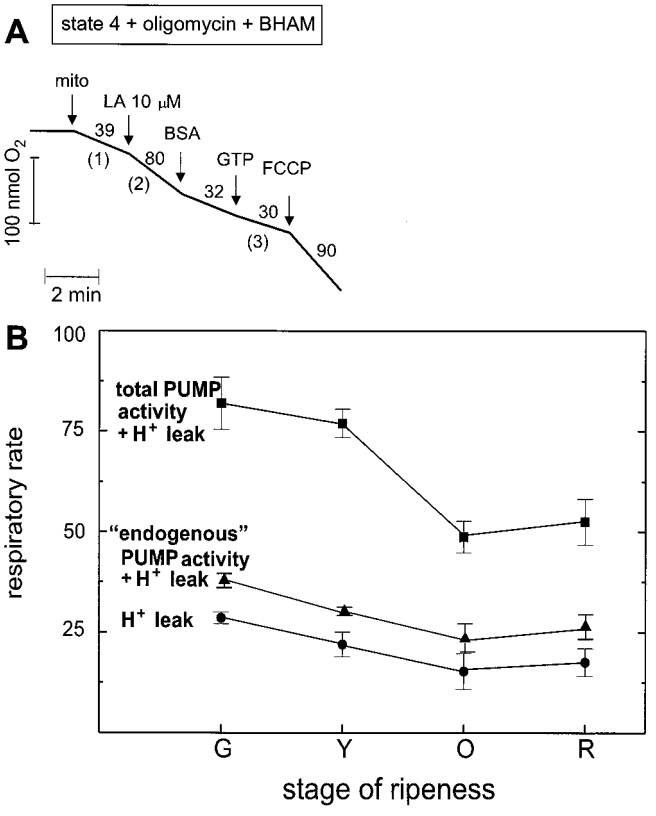

Figure 4. A, Determination of PUMP-sustained respiration. Mitochondria (mito) were incubated in a standard reaction medium in the presence of 2.5 μg oligomycin mg^{-1} protein and 2 mM BHAM, as described in "Materials and Methods"; 10 μM LA, 0.5% BSA, 1 mM GTP, and 1 μM FCCP were added. The numbers on the traces refer to the O_2-consumption rates in nmol min^{-1} mg^{-1} protein. The numbers in parentheses refer to the slopes used to calculate the total PUMP activity and stimulation by LA shown in B. The respiration of green tomato mitochondria is shown as an example. B, PUMP-sustained respiration in the four stages of ripeness (G, green; Y, yellow; O, orange; and R, red). ■, Total PUMP activity plus H^+ leak (respiration after the addition of LA; slope 2 from A); ▲, endogenous PUMP activity plus H^+ leak in the absence of LA (respiration before the addition of LA; slope 1 from A); ●, H^+ leak (respiration after the addition of BSA and GTP; slope 3 from A). Respiratory rates are in nmol O_2 min^{-1} mg^{-1} protein. The data are presented as the means ± SD from three to four independent experiments.

mitochondria the addition of 1 μM FCCP stimulated respiratory rates more than 10 μM LA. We did not observe this difference between LA-stimulated and FCCP-stimulated respiration in the other stages of tomato ripening (data not shown).

With the stage of ripeness, the total PUMP-sustained respiration plus the H^+ leak-sustained respiration measured with isolated mitochondria markedly decreased (approximately 40%) between the yellow and orange stages and then stabilized, displaying no distinct ripening burst in respiration (Fig. 4B). On the contrary, H^+ leak-sustained respiration and endogenous PUMP activity-sustained respiration remained almost constant between the yellow and orange stages of ripeness.

When ATP synthase and AOX were blocked in isolated mitochondria, the respiration allocated to PUMP activity in the presence of 10 μM LA fell significantly between the yellow and orange stages of tomato fruit ripeness.

Immunological Analysis of AOX and PUMP

Immunoblotting of the total mitochondrial proteins of tomato fruit allowed immunological detection of AOX and PUMP in this fruit for the first time (to our knowledge). Monoclonal antibodies developed against AOX of *S. guttatum* cross-reacted with a single protein band of approximately 36 kD (Fig. 5). This protein corresponded to a low-molecular-mass monomeric form of AOX detected under reducing conditions (in the presence of DTT) in other plant mitochondria (McIntosh et al., 1994). On the other hand, antibodies developed against potato PUMP revealed a single protein band with a molecular mass of approximately 32 kD (the same as for the isolated protein found by Jezek et al. [1997]). These results indicated clearly that both proteins were simultaneously present in tomato fruit mitochondria.

The level of immunologically detectable AOX protein decreased with ripening, from the green stage (Fig. 5) forward, and paralleled the decrease in CN-resistant respiration in isolated mitochondria (Fig. 3). The AOX protein band was still present in the orange and red stages, as visualized after longer film exposure. Changes in PUMP levels with the stage of tomato fruit ripening were less pronounced, and a decrease in the amount of the 32-kD band occurred at the yellow stage (Fig. 5) and beyond, as was also the case for the total PUMP activity measured in mitochondria from the different stages of tomato ripening (Fig. 4).

DISCUSSION

We obtained the results reported here using Percoll-purified mitochondria depleted of FFAs in tomato fruit in four different stages of postharvest ripening (green, yellow, orange, and red). The aim was to measure the LA-

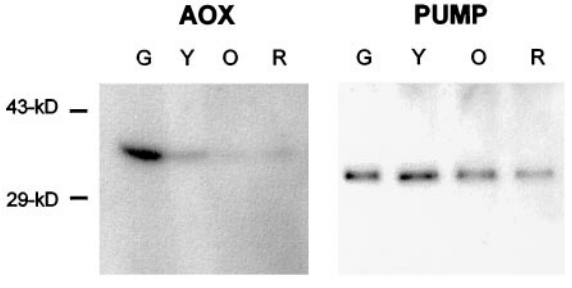

Figure 5. Immunoblot analysis of tomato total mitochondrial proteins from fruits at different stages of ripeness (G, green; Y, yellow; O, orange; and R, red). Proteins were electrophoresed, transferred to nitrocellulose, and reacted with monoclonal antibodies against the *S. guttatum* AOX (left) or with polyclonal antibodies against the potato PUMP (right), as described in "Materials and Methods." The total mitochondrial protein load was 150 μg for AOX detection and 30 μg for PUMP detection. The molecular mass markers appear on the left.

induced PUMP-sustained respiration when both ATP synthesis and AOX activity were blocked by oligomycin and BHAM, respectively. An LA concentration of 10 μM was chosen because it caused optimal stimulation of state-4 respiration (plus oligomycin and BHAM) at a mitochondrial protein concentration of 0.4 mg mL^{-1} (Sluse et al., 1998). Higher LA concentrations had adverse side effects on the mitochondrial membrane properties (data not shown). We also measured the ATP-synthesis-sustained respiration and CN-resistant respiration to compare the evolution of the three activities in isolated mitochondria during tomato fruit ripening. In addition, the immunological analysis of both PUMP and AOX proteins allowed investigation of the evolution of their levels in mitochondria during fruit ripening.

When the activities of AOX and PUMP were excluded by inhibition, ATP-synthesis-sustained respiration decreased markedly from the green to the orange stage and then stabilized until the red stage (Fig. 2), whereas the respiratory control remained almost constant throughout the period of tomato fruit ripening. Thus, purified mitochondria isolated in the presence of 0.5% BSA, which chelates endogenous FFAs from the mitochondrial suspension, displayed almost unaltered coupling properties during fruit ripening, whereas the ATP synthesis activity (measured as ATP-synthesis-sustained respiration) decreased from the green mature stage to the orange stage.

AOX-sustained respiration of isolated mitochondria diminished from the green to the orange stage and then remained unchanged. The sensitivity of AOX to stimulators (pyruvate and DTT) did not change with ripening (Fig. 3). The decreased AOX activity was accompanied by a decrease in the amount of 36-kD protein (Fig. 5). These results suggest a parallel between AOX activity and the amount of AOX protein, even if a level of immunodetectable protein appears to decrease more sharply (8-fold, when measured densitometrically) compared with the activity. A similar lack of proportionality between the amount of protein loaded on a gel lane and detectable bands has already been reported for an immunological analysis of AOX (Umbach and Siedow, 1993). Nevertheless, our results indicate a clear regulation of this enzyme activity through a decrease in protein expression during postharvest tomato fruit ripening. In mango, another climacteric fruit, the levels of AOX were constant throughout most of the ripening process and increased in ripe fruit; this increase was correlated with an increase in CN-resistant respiration (Kumar et al., 1990; Cruz-Hernandez and Gomez-Lim, 1995). The dissimilarity between these two fruits may indicate a difference in climacteric behavior, including differences in AOX activity.

In isolated mitochondria the total PUMP-sustained respiration in the presence of 10 μM LA (plus residual endogenous FFAs) was almost the same in the green and yellow stages and then decreased by 40% in the orange and red stages (Fig. 4). The changes in the amounts of immunologically detectable PUMP (Fig. 5), a 30% decrease as measured densitometrically (data not shown), paralleled the decrease in potential PUMP activity observed during tomato ripening. This is the first report, to our knowledge, that analyzes PUMP expression during the ripening of a climacteric fruit. We must point out that the measured PUMP activity could be much lower than the actual PUMP protein capacity in the membrane, because mitochondria cannot respire faster. This could be the case in the yellow, orange, and red stages, in which the total PUMP plus H^+-leak activities (Fig. 4) are close to the ATP-synthesis-sustained activities (Fig. 2). Therefore, a decrease in the capacity of the respiratory chain could explain a decrease in the measured PUMP activity. On the contrary, in the green stage, state-3 respiration (Fig. 2) and FCCP-uncoupled respiration (Fig. 4A) were higher than the total (PUMP-plus H^+-leak-sustained) respiration (Fig. 4B), indicating that the respiratory chain capacity was higher than the PUMP capacity.

Our results on the respiratory activities measured with isolated mitochondria (sustained by ATP synthesis, PUMP, and AOX activities) and their evolution during tomato fruit ripening were restricted to situations in which one of the activities was functioning while the other two were blocked. Even if these measurements do not reflect the true contributions of the three pathways to the overall state-3 respiration (because any change in one inevitably affects the others), they do allow relative comparisons. Thus, the complex II→ATP synthase pathway, the complex II→AOX pathway, and the complex II→H^+ leak pathway showed the same evolution during ripening (i.e. a progressive decrease with fruit ripening until stabilization at the orange stage). On the other side, the complex II→PUMP pathway displayed little or no decrease at the beginning of ripening and was followed by a distinct decrease between the yellow and orange stages. However, because the changes in the three activities were very similar, the question arises as to whether they could be due to a single event, such as a decrease in succinate dehydrogenase activity, which would affect both oxidizing pathways (Cyt and AOX pathways) in a similar way. Indeed, a decrease in the respiratory chain activity, when supplied with electrons from complex II, could be responsible for decreases in the three respirations (i.e. ATP synthesis-, PUMP-, and AOX-sustained respirations), in combination with changes in AOX and PUMP contents.

Further studies are necessary to determine the functional connection between the two energy-dissipating systems, PUMP and AOX, in plant mitochondria, particularly during periods such as fruit ripening, when the content of FFAs increases significantly (Güçlü et al., 1989; Rouet-Mayer et al., 1995). The importance of this is indicated by the acute inhibition of CN-resistant respiration by exogenous LA (in the micromolar range) in isolated tomato mitochondria (Sluse et al., 1998). The inhibitory effect of LA on CN-resistant respiration was also found in mitochondria of *Arum maculatum* (Kay and Palmer, 1985) and *Hansenula anomala* (Minagawa et al., 1992). The opposite effect of added LA on PUMP and AOX activities observed in isolated mitochondria could indicate their different roles during the ripening process in tomato fruit, because a progressive increase in cytosolic FFAs could progressively activate PUMP but at the same time inhibit AOX (Sluse at al., 1998).

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