Free fatty acids regulate the uncoupling protein and alternative oxidase activities in plant mitochondria

Francis E. Sluse[b], Andréa M. Almeida[1,a], Wieslawa Jarmuszkiewicz[c], Anibal E. Vercesi[a,*]

[a]*Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, CP 6111, 13083-970 Campinas, SP, Brazil*
[b]*Laboratory of Bioenergetics, Center of Oxygen Biochemistry, Institute of Chemistry B6, University of Liège, Sart Tilman, B-4000 Liège, Belgium*
[c]*Department of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61-701 Poznan, Poland*

Abstract Two energy-dissipating systems, an alternative oxidase and an uncoupling protein, are known to exist in plant mitochondria. In tomato fruit mitochondria linoleic acid, a substrate for the uncoupling protein, inhibited the alternative oxidase-sustained respiration and decreased the ADP/O ratio to the same value regardless of the level of alternative oxidase activity. Experiments with varying concentrations of linoleic acid have shown that inhibition of the alternative oxidase is more sensitive to the linoleic acid concentration than the uncoupling protein activation. It can be proposed that these dissipating systems work sequentially during the life of the plant cell, since a high level of free fatty acid-induced uncoupling protein activity excludes alternative oxidase activity.
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Key words: Alternative oxidase; Plant uncoupling mitochondrial protein; Mitochondrion; Linoleic acid; *Lycopersicon esculentum*

1. Introduction

In addition to the alternative oxidase (AOX) [1–3], some plant mitochondria contain another energy-dissipating system, namely a plant uncoupling mitochondrial protein (PUMP) [4,5]. The cyanide (CN)- and antimycin-resistant AOX, which bypasses the main cytochrome respiratory chain, catalyzes ubiquinol-oxygen oxido-reduction without H^+ release in the cytosol and thus dissipates redox potential energy [1–3]. AOX activity is inhibited by hydroxamic acids such as benzohydroxamate (BHAM) and can be allosterically stimulated by keto acids such as pyruvate (Pyr) [6,7]. In addition, the activity of AOX, existing in the inner mitochondrial membrane as an inactive covalently bound oxidized dimer or as a reduced active non-covalent dimer [8], may be regulated by reduction state of the enzyme [8,9].

The recently discovered plant uncoupling mitochondrial protein (PUMP) [4] exports anionic free fatty acids (FFA) across the inner mitochondrial membrane [10], in a manner similar to the mammalian brown adipose tissue uncoupling protein (UcP) [11–13]. Protonated fatty acids reenter into the mitochondrial matrix by diffusion [11,13]. By removing

the anionic FFA from the matrix back into the intermembrane space, PUMP enables H^+ reentry into the matrix through a fatty acid cycling process, thus bypassing the ATP synthase, and as a consequence dissipates the H^+ transmembrane electrochemical gradient [4,5]. Addition of FFA, such as linoleic acid (LA), to plant mitochondria results in mitochondrial uncoupling, while the presence of purine nucleotides, such as ATP or GTP which inhibit PUMP, and bovine serum album (BSA) which removes FFA, increases the membrane potential of these mitochondria [4,5,10]. Uncoupling by FFA, at least in animal mitochondria, is mediated not only by UcP but also by the ATP/ADP antiporter [14], the aspartate/glutamate antiporter [15], and the dicarboxylate carrier [16]. Therefore, by adding BSA together with GTP, not only PUMP can be inhibited but also the FFA transport activity of the above carriers provided that they participate in FFA-induced uncoupling in plant mitochondria.

Although the two energy-dissipating systems, AOX and PUMP, lead to the same final effect (i.e. a decrease in oxidative phosphorylation efficiency), they may have different physiological functions in plant cells. While activities of both proteins can counteract the imbalances between the supply of reducing substrates and the energy and carbon demand for biosynthesis [1,3,5], only PUMP can totally switch off chemiosmotic coupling. A puzzling question regarding the presence of these two energy-dissipating systems relates to a possible connection between their activities (shared regulation). Such a connection could shed light on the reasons for the existence of two energy-dissipating systems in plants (contrary to heterotrophic organisms) and how they interact to fulfil requirements of plant cell energy metabolism.

In this work we describe the functional connection between the AOX and PUMP energy-dissipating systems in mitochondria isolated from green tomato fruit. We show that LA, an abundant free fatty acid in plants, which activates PUMP, drastically inhibits the CN-resistant respiration mediated by AOX.

2. Materials and methods

Tomato plants (*Lycopersicon esculentum*) were grown in a greenhouse at the Centro de Biologia Molecular e Engenharia Genética, UNICAMP. Tomato fruits were harvested at a nearly developed stage (but still completely green) and were used the same day.

2.1. Isolation of mitochondria
Usually 0.5 kg of green tomatoes were sliced and homogenized in a domestic blender following removal of the seeds. The juice obtained was immediately mixed to a final volume of 400 ml with a medium containing 500 mM sucrose, 0.2 mM EGTA, 4 mM cysteine, and 40 mM HEPES, pH 7.8. During homogenization, the pH value was kept between 7.2 and 7.8 by adding 1 N KOH. After filtration of the

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*Corresponding author. Fax: (55) (19) 7881118.
E-mail: anibal@obelix.unicamp.br

[1]A.M.A. and W.J. contributed equally to this work.

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CN, cyanide; DTT, dithiothreitol; FFA, free fatty acids; LA, linoleic acid; PUMP, plant uncoupling mitochondrial protein; Pyr, pyruvate

homogenate through a layer of polyester cloth, crude mitochondria were subsequently isolated by conventional differential centrifugation (500×g for 10 min, 12 300×g for 10 min) and then washed twice in a medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES, pH 7.2.

The mitochondria were then purified on a 21% (v/v) self-generating Percoll gradient following method modified from Van den Bergen et al. [17]. Apart from Percoll, the gradient medium contained 250 mM sucrose, 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 FFA from the mitochondrial suspension. The gradient was centrifuged at 40 000×g for 30 min. The mitochondria layer was collected and washed three times in 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES, pH 7.2. Fully depleted of FFA mitochondria were obtained by using 1% (w/v) BSA in the last washing before Percoll gradient, during the Percoll gradient purification, and in the following first washing. With these mitochondria, the addition of GTP+BSA had no effect on the respiration in the presence of BHAM and oligomycin (not shown). Mitochondrial protein concentration was determined by the biuret method [18].

2.2. Measurements of respiration

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in 1.3 ml of standard incubation medium (25°C) containing 125 mM sucrose, 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 0.4–0.5 mg of mitochondrial protein. All measurements were made in the presence of 10 mM succinate (+5 μM rotenone) as oxidizable substrate. To ensure complete activation of succinate dehydrogenase, 0.17 mM ATP was added. State 4 measurements were performed in the presence of 2.5 μg of oligomycin/ml of incubation medium. For state 3 measurements, 0.17 mM (pulse) or 2 mM (saturating amount) ADP was supplied. The alternative pathway was inhibited with 2 mM BHAM, and the cytochrome pathway was inhibited with 1.5 mM KCN. PUMP activity was inhibited with 0.5% BSA and 1 mM GTP. The latter was also applied in measurements of CN-resistant respiration in state 3, in the presence of 2 mM ADP which could itself inhibit PUMP activity, to ensure total inhibition of PUMP and to keep the same experimental conditions for both state 3 and state 4. To activate the alternative pathway, 0.15 mM pyruvate and 1 mM dithiothreitol (DTT) were supplied, and to activate PUMP various concentrations of LA were supplied. Detailed conditions are described in the figure legends.

3. Results and discussion

CN-resistant respiration was measured in purified tomato fruit mitochondria partially depleted of FFA with succinate (plus rotenone) as oxidizable substrate, displaying no significant difference for both state 3 and state 4 respiration (taking into account the absolute values of the CN-resistant respiratory rates as shown in Table 1). Four incubation conditions were used: (a) in the presence of the PUMP inhibitors, GTP and BSA, of which BSA is the most effective by chelating substrates of PUMP (FFA), (b) in the presence of GTP and

BSA plus the AOX activators, DTT (reductant) and Pyr, (c) in the presence of DTT and Pyr plus a substrate of PUMP, 3.9 μM LA and (d) in the presence of DTT and Pyr. When PUMP was blocked, a significant stimulation of CN-resistant respiration (taking into account both the absolute values and the percent of total respiration) was observed in the presence of DTT and Pyr, similar in both states 3 and 4. On the other hand, the addition of 3.9 μM LA instead of GTP and BSA, dramatically decreased CN-resistant respiration. In the absence of PUMP inhibitors or PUMP substrate, CN-resistant respiration in the presence of DTT and Pyr had an intermediary value, which could be explained by the presence of a small amount of endogenous FFA in the mitochondrial suspension. These observations clearly indicated an inhibitory effect of LA on the AOX activity stimulated by DTT and Pyr.

The coupling state of the phosphorylating mitochondria was determined in four conditions (Table 2): (a) in the presence of inhibitors of AOX (BHAM) and PUMP (GTP/BSA), (b) in the presence of GTP/BSA plus AOX activators (DTT/Pyr), (c) in the presence of 3.9 μM LA (PUMP substrate) and DTT/Pyr, and (d) in the presence of 3.9 μM LA and BHAM. State 3 respiration was lower in the presence of BHAM compared to when AOX was active (plus DTT/Pyr) with PUMP activity either inhibited (by GTP/BSA) or slightly stimulated (plus LA). This was also the case for state 4 respiration with GTP/BSA but not in the presence of LA. The ADP/O ratio, which quantifies the efficiency of oxidative phosphorylation, was optimal (=1.43) in the presence of inhibitors of both dissipating systems, decreased when AOX was activated by DTT/Pyr (=1.29) and fell significantly to 0.95 when LA was also present (i.e. when both AOX and PUMP were activated). However, when AOX was inhibited by BHAM, the ADP/O ratio in the presence of LA remained almost the same (= 1.01). These results suggest that either the efficiency of oxidative phosphorylation in the presence of LA is independent of the activity of AOX, or that AOX is inhibited by a low concentration of LA.

CN-resistant respiration and LA-induced BHAM-resistant respiration represent part of AOX and PUMP activity, respectively. These activities were measured for various LA concentrations in fully depleted of FFA tomato mitochondria as described in the legend to Fig. 1. CN-resistant respiration (Fig. 1) in the presence of DTT with (■) or without (●) Pyr decreased with the increasing concentration of LA. Fifty percent inhibition was reached for both situations at an LA concentration of 3–4 μM. On the other hand, LA-induced respiration increased with the increasing concentrations of LA and 50% maximal stimulation was reached at 10.3 μM

Table 1

Effect of various incubation conditions on CN-resistant respiration in mitochondria isolated from green tomato fruit

Conditions	CN-resistant respiration			
	State 3 (nmol O min^{-1} mg^{-1} protein)	(% of total respiration)	State 4 (nmol O min^{-1} mg^{-1} protein)	(% of total respiration)
+GTP+BSA	92 ± 13	21 ± 3	86 ± 14	55 ± 8
+GTP+BSA+DTT+Pyr	218 ± 45	44 ± 9	212 ± 29	97 ± 13
+3.9 μM LA+DTT+Pyr	56 ± 14	14 ± 3	46 ± 8	20 ± 4
+DTT+Pyr	131 ± 16	28 ± 4	122 ± 15	62 ± 9

Assay conditions as in Section 2. CN-resistant respiration (+1 mM KCN) was measured in the presence of 1 mM GTP, 0.5% BSA, 1 mM DTT, 0.15 mM Pyr, and 3.9 μM LA (as indicated). CN-resistant respiration rates are expressed in nmol O min^{-1} mg^{-1} mitochondrial protein or as a percent of the total respiratory rate, before the addition of KCN. Data are mean values ± S.D. of four determinations.
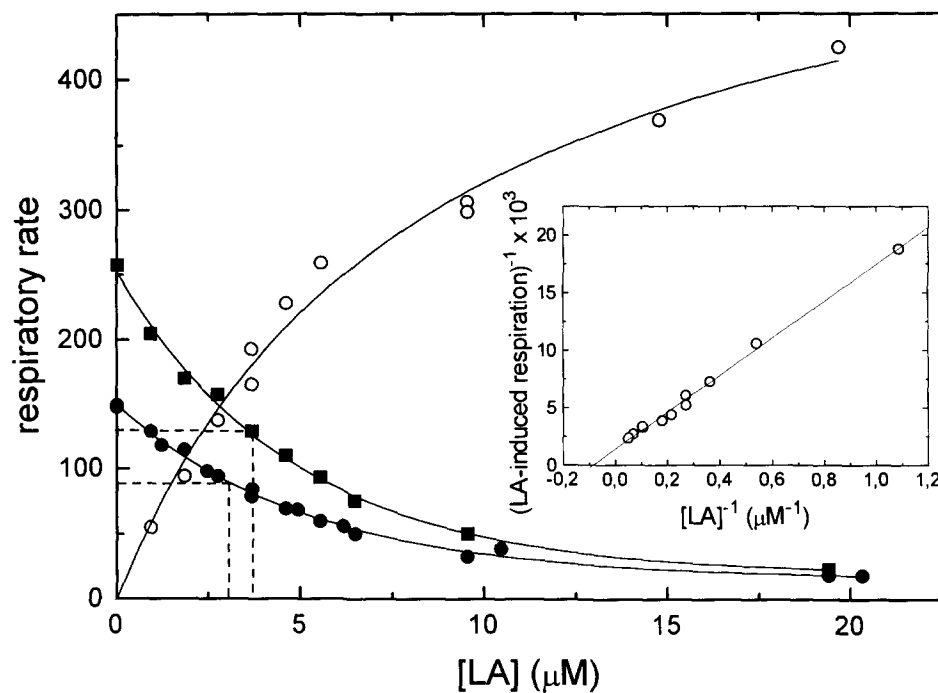
Fig. 1. CN-resistant respiration and LA-induced respiration versus LA concentration. Fully depleted FFA mitochondria (see Section 2) were incubated with 10 mM succinate, 5 μM rotenone, 2.5 μg of oligomycin/mg protein, and 0.17 mM ATP (state 4 respiration). CN-resistant respiration (+1 mM KCN) was measured in the presence of 1 mM DTT (●) or in the presence of 1 mM DTT plus 0.15 mM Pyr (■). LA-induced respiration (○) was measured in the presence of 2 mM BHAM. Increasing concentrations of LA (1.2-20 μM) were obtained by successive additions when the steady-state respiration rate was achieved. Several oxygen traces were needed to cover the full investigated range of LA concentration. LA concentrations producing 50% inhibition ($S_{0.5}$) of CN-resistant respiration are indicated by the dashed vertical lines. Values of respiratory rates are expressed in nmol O min^{-1} mg^{-1} mitochondrial protein. Inset: Double reciprocal plot of LA-induced respiration versus LA concentration. The linear regression was made ($y = A+BX$), where $R = 0.997$, $A = 1.51 \pm 0.16$, and $B = 15.53 \pm 0.42$. A^{-1} gives the apparent V_{max} (663 nmol O min^{-1} mg^{-1} protein) and B/A gives $S_{0.5}$ for LA-induced respiration (i.e. LA concentration that produces 50% stimulation) and corresponds to 10.3 μM.

(Fig. 1, inset). The apparent maximal rate of LA-induced respiration as calculated from the linear regression equation of the data in Fig. 2 was 663 nmol O min^{-1} mg^{-1} protein. The difference between the two CN-resistant respiration curves in Fig. 1 (■ and ●, with and without Pyr, respectively) could be plotted as a percent of the respiration without Pyr versus LA concentration up to 10 μM (Fig. 2). The stimulatory effect of Pyr (on average, about $59 \pm 8\%$, S.D.) on AOX activity (in the presence of DTT) was not significantly affected by the LA concentration. This suggests that the inhibitory effect of LA on CN-resistant respiration is not related to the binding of Pyr to the allosteric site of AOX. As a result, there is no competition between LA and Pyr for the allosteric activator binding site. The above experiments using increasing concentrations of LA describe for the first time how an in-

crease in FFA level affects both of the energy-dissipating systems, but in opposite directions. It seems that AOX activity is more sensitive to the inhibitory effect of LA than PUMP activity is to activation by LA, as indicated by the respective $S_{0.5}$ concentrations (i.e. LA concentrations that lead to 50% inhibition of CN-resistant respiration or to 50% stimulation of LA-induced respiration, respectively) (Fig. 1).

These results are important because they show how a highly regulated enzyme such as AOX can be progressively switched off by an increase in the FFA level in plant cells. They also shed light on the respective roles of each energy-dissipating system. Indeed, AOX and PUMP never seem to work simultaneously at their maximal activity, and it may be that when PUMP reaches high activity, AOX is already switched off. We suggest that these two energy-dissipating enzymes work se-

Table 2
Effect of various incubation conditions on the phosphorylating respiratory rate and coupling parameters

Conditions	Coupling parameters			
	State 3 (nmol O min^{-1} mg^{-1} protein)	State 4 (nmol O min^{-1} mg^{-1} protein)	RC	ADP/O
+GTP+BSA+BHAM	435 ± 33	160 ± 13	2.72 ± 0.22	1.43 ± 0.03
+GTP+BSA+DTT+Pyr	557 ± 42	355 ± 19	1.57 ± 0.12	1.29 ± 0.10
+3.9 μM LA+DTT+Pyr	449 ± 45	305 ± 14	1.47 ± 0.14	0.95 ± 0.13
+3.9 μM LA+BHAM	385 ± 35	297 ± 10	1.30 ± 0.12	1.01 ± 0.03

Assay conditions as in Section 2. State 3 respiration was measured during ADP pulse (0.17 mM), and state 4 respiration was determined after the phosphorylation step. The concentrations used were: 1 mM GTP, 0.5% BSA, 1 mM DTT, 0.15 mM Pyr, 3.9 μM LA and 2 mM BHAM (where indicated). Values of respiratory rates are expressed in nmol O min^{-1} mg^{-1} mitochondrial protein. Data are mean values ± S.D. of four determinations.
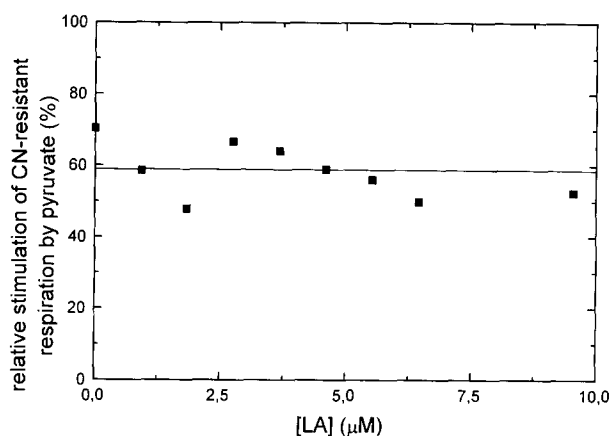
Fig. 2. Stimulation of CN-resistant respiration by pyruvate versus concentration of LA (0–10 μM). Assay conditions as in Fig. 1. Pyruvate stimulation is shown in relative values (as percent of unstimulated (−Pyr) respiration). The horizontal line indicates the average stimulation by pyruvate (59%).

quentially. Thus AOX would be active mainly during high biosynthetic activities in the cell (e.g. during plant growth and development), thereby providing safety balance between redox potential, phosphate potential, and biosynthesis demands, whereas PUMP would start working in the post-growing stage when the FFA concentration increases [19,20], thereby providing a mechanism for heat generation via a decrease in the efficiency of oxidative phosphorylation in parallel with the termination of biosynthetic processes.

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