20 Proton partitioning between ATP synthase and uncoupling protein during cytochrome pathway state 3 respiration in tomato fruit mitochondria

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Introduction

Tomato fruit (*Lycopersicon esculentum*) mitochondria contain two main Gibbs free-energy dissipating systems: (i) a cyanide-resistant alternative oxidase (AOX[1]) that dissipates redox Gibbs free-energy instead of building a transmembrane proton electrochemical gradient ($\Delta\mu H^+$) [1] and (ii) an uncoupling protein (LeUCP) that dissipates $\Delta\mu H^+$ through a free fatty acid (FFA)-activated H^+ cycling process [2]. These two Gibbs free-energy dissipating systems leading to the same final effect (i.e., a decrease in ATP synthesis yield) act at two different levels of the overall energy transduction pathway. Mechanism of uncoupling activity of UCP and role of FFA are still under debate: UCP could be a H^+ carrier activated by FFA [3] or UCP could be a FFA-anion carrier that allows a protonophoric cycle of FFA [4].

Measurements of respiration specifically sustained by either ATP synthase,

[1]Abbreviations: $\Delta\mu H^+$, proton electrochemical gradient; AOX, alternative oxidase; UCP, uncoupling protein; FFA, free fatty acids; LA, linoleic acid; BHAM, benzohydroxamate; $\Delta\Psi$, mitochondrial transmembrane electrical potential; V3, state 3 respiration; $\Delta\Psi 3$, membrane potential of state 3; Vcyt cons, contribution of ATP synthesis-sustained respiration; V_{UCP}, contribution of UCP activity-sustained respiration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MLNET, mosaic linear non-equilibrium thermodynamics.

LeUCP or AOX activity if restricted to conditions in which one activity is functioning while the other two are blocked by specific inhibitors [5] do not reflect the true contribution of each pathway to the total state 3 respiration since inhibition of one pathway inevitably affects the others.

Both, UCP and ATP synthase are able to consume $\Delta\mu H^+$ built up by the protonophoric oxido-reductases of the respiratory chain and may be considered as two branching pathways contributing to respiration through the cytochrome pathway. UCP is the Gibbs free-energy-dissipating path and ATP synthase is the Gibbs free-energy conserving path. Preparation of green tomato fruit mitochondria fully depleted of FFA and observation of the decrease in ADP/O by linoleic acid (LA) addition have suggested calculation of the contribution of LeUCP activity and ATP synthesis in state 3 using pair measurements of ADP/O ratios in the absence or presence of LA [6].

The present study supports the validity of the ADP/O method to determine the actual contributions of UCP (activated with various LA concentrations) and ATP synthase in the BHAM-resistant state 3 respiration of tomato fruit mitochondria fully depleted of FFA and describes how the two contributions vary when the rate of succinate dehydrogenase is decreased by succinate uptake limitation.

Materials and methods

Green tomato fruit mitochondria were isolated as described previously [5]. Oxygen uptake and mitochondrial transmembrane electrical potential ($\Delta\Psi$) were measured in 1.3 ml of incubation medium containing: 125 mM sucrose, 65 mM KCl, 0.33 mM EGTA, 1 mM $MgCl_2$, and 2.5 mM KH_2PO_4, 10 mM HEPES, pH 7.4, temp. 25°C. All measurements were made in the presence of 10 mM succinate, 1.5 mM BHAM, 170 μM ATP, 5 μM rotenone, 0–8 μM LA, and 0–35 mM *n*-butyl malonate.

Results

All experiments were performed in the presence of BHAM, to inhibit AOX activity, with succinate (plus rotenone) as oxidizable substrate. ADP/O ratio and state 3 respiration were measured during ADP pulses in the absence or presence of LA. LA increased the respiratory rate and decreased $\Delta\Psi$ in state 4 while it scarcely modified state 3 respiration (V3) and membrane potential ($\Delta\Psi$3). However, the ADP/O ratio was clearly lowered in the presence of LA suggesting UCP activation.

In order to describe how contributions of tomato UCP and ATP synthase change with variations in the state 3 respiration, the rate of the quinone-reducing pathway (succinate dehydrogenase) was decreased by *n*-butyl malonate, a non-penetrating competitive inhibitor of succinate uptake. V3 decreased with increasing concentrations of *n*-butyl malonate up to 35 mM inhibiting about 50% of respiration both

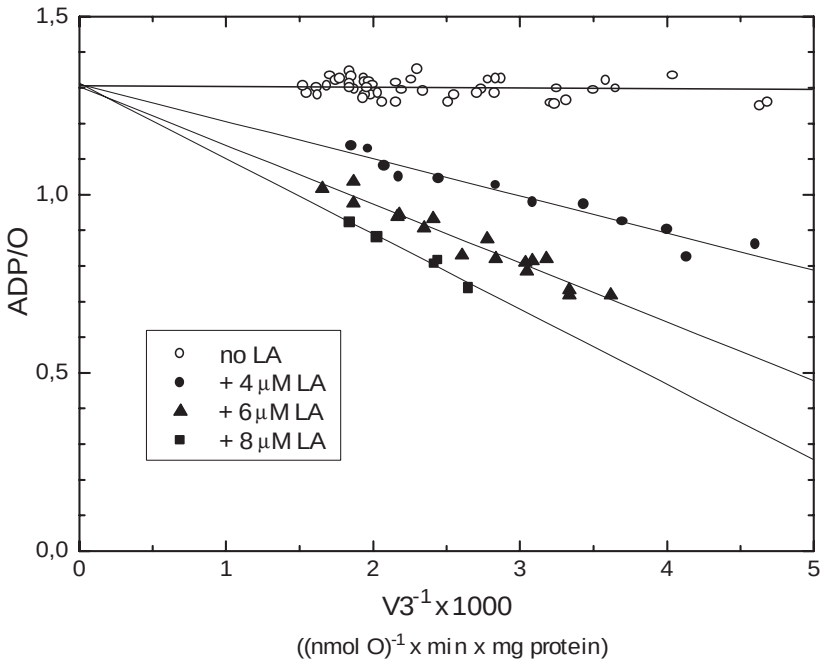

Fig. 20.1 Effect of decrease in state 3 respiration on the ADP/O ratio at different LA concentrations. Oxidation rate of succinate (+ BHAM) was gradually decreased by increasing concentrations of *n*-butyl malonate (5–35 mM). ADP/O ratios were determined in the presence of 0, 4, 6, and 8 μM LA. Data deal with 7 experiments (two with ± 4 μM LA, three with ± 6 μM LA, and two with ± 8 μM LA) with different rates of uninhibited state 3 respiration. Mean value of ADP/O ratio in the absence of LA is 1.294 ± 0.032 (S.D., $n = 50$). In the presence of LA, values of intercepts with ordinate axis (A) of the least square regression lines ($Y = A + BX$, where Y = ADP/O and $X = \mathrm{V3}^{-1} \times 10^3$) are: 1.310 ± 0.027 (S.D., $n = 12$), 1.304 ± 0.030 (S.D., $n = 17$), and 1.313 ± 0.051 (S.D., $n = 5$) for 4, 6, and 8 μM LA, respectively.

in the absence and presence of LA. No decrease in $\Delta\Psi 3$ linked to inhibition of respiration by *n*-butyl malonate or to LA addition was detected. The mean value of $\Delta\Psi 3$ obtained with different mitochondrial preparations and *n*-butyl malonate concentrations was 172 ± 5 mV (S.D., $n = 61$). In the absence of LA, the ADP/O ratio remained constant independent of V3 (Fig. 20.1). The mean value was 1.29 ± 0.03 (S.D., $n = 55$). In the presence of LA (4, 6, or 8 μM), the ADP/O ratio was lowered and decreased with decreasing V3 by *n*-butyl malonate. Linear relationships were obtained between the ADP/O and the reciprocal of V3, with ordinates at the origin independent of LA concentration and slopes increasingly negative as LA concentration was increased (Fig. 20.1). Such behavior is accounted for by eq. 20.1 where $(\mathrm{ADP/O})_{-\mathrm{LA}}$ is the ADP/O ratio in the absence of LA and V_{UCP} is the part of respiration sustained by tomato UCP activity.

$$\mathrm{ADP/O} = (\mathrm{ADP/O})_{-\mathrm{LA}} \times \left(1 - \frac{V_{\mathrm{UCP}}}{V3}\right) \tag{20.1}$$

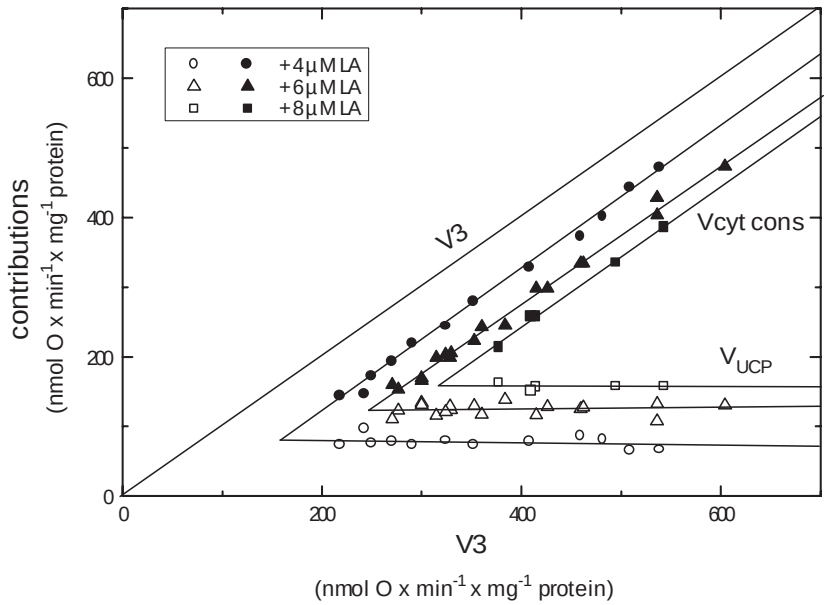

Fig. 20.2 Partitioning of state 3 respiratory rate between uncoupling protein activity (V_{UCP}) and ATP synthesis (Vcyt cons) at different LA concentrations. Assay conditions as in Fig. 20.1. Contributions, V_{UCP} and Vcyt cons, were calculated according to eqns. 20.2 and 20.3. Mean values of V_{UCP} are: 78±8 (S.D., n = 12), 125±8 (S.D., n = 17), 157±4 (S.D., n = 5) for 4, 6, 8 μM LA, respectively.

Such relationship supports the view (see Discussion) that: (i) at fixed LA concentration, V_{UCP} remains constant during the titration by n-butyl malonate (this means that plant UCP activity is not strongly dependent on $\Delta\Psi 3$ which, in fact, remains essentially constant), and (ii) LA does not affect the intrinsic stoichiometry ($(ADP/O)_{-LA}$) of oxidative phosphorylation (this means that LA decreases the yield of the oxidative phosphorylation by a pure protonophoric process). Then, the respective contributions to respiration in steady-state state 3 of ATP synthesis, which consumes $\Delta\mu H^+$ with Gibbs free-energy conservation (Vcyt cons) and of tomato UCP activity, which consumes $\Delta\mu H^+$ with Gibbs free-energy dissipation (V_{UCP}) can be calculated by using eqs. 20.2 and 20.3.

$$V\text{cyt cons} = V3 \times \frac{(ADP/O)}{(ADP/O)_{-LA}} \tag{20.2}$$

$$V_{UCP} = V3 - V\text{cyt cons} \tag{20.3}$$

In Fig. 20.2, the calculated contributions Vcyt cons (eq. 20.2) and V_{UCP} (eq. 20.3) were plotted versus the total rate of respiration (V3) in order to emphasize their behaviour during titration of respiration by n-butyl malonate: the constancy of V_{UCP} (at fixed LA concentration) and the linear decrease in Vcyt cons. It is clear that this behaviour cannot be extrapolated without caution.

Discussion

The yield of oxidative phosphorylation in isolated tomato fruit mitochondria depleted of free fatty acids remains constant when respiratory rates was decreased by a factor of 3 by the addition of *n*-butyl malonate. However, the constant ADP/O value (1.3) is lower than the generally accepted ideal stoichiometric value (1.5) indicating incomplete coupling. This can be easily explained on the basis of the mosaic protonic coupling hypothesis [7] if the inhibitor is assumed to eliminate coupling units (that would include the succinate translocator) and if the proton back leakage outside the coupling units is negligible. Then the ADP/O ratio (lower than the mechanistic one because proton back leakage occurs inside the coupling unit) remains constant. Furthermore, this model accounts for the fact that the rate of phosphorylation varies while $\Delta\mu H^+$ remains almost constant [7]. Our results (Fig. 20.1, eq. 20.1) imply that LA would act exclusively outside the coupling units. On the basis of the MLNET approach of delocalized chemiosmosis [8–10], $\Delta\mu H^+$ constancy would result from a very high value for the factor (Lp) of the ATP synthesis flux force relationship. Additionally, a reaction slip in the redox H^+ pumps (not modified by LA) might explain the rather low ordinate intercept value of the straight lines in Fig. 20.1. Whatever is the approach, it may be concluded that linoleic acid decreases the yield of oxidative phosphorylation by a pure protonophoric process (i.e., LA is not a slip inducer nor a decoupler of localized proton circuit) and that ADP/O measurements allow calculation of the part of respiration leading to ATP synthesis and the part of respiration sustained by the dissipative H^+ re-uptake induced by linoleic acid. Respiration sustained by this Gibbs free-energy dissipating process remains constant at a given LA concentration until more than 50% inhibition of state 3 respiration by *n*-butyl malonate is achieved (Fig. 20.2). The dissipative contribution to oxygen consumption is proposed to be equal to the protonophoric activity of tomato uncoupling protein divided by the intrinsic H^+/O of the cytochrome pathway. It increases with linoleic acid concentration taking place at the expense of ADP phosphorylation without an increase in the respiration.

Acknowledgments

This work was supported by grants from the Belgian (FNRS), Brazilian, and Polish (KBN) Agencies.

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