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Proton Re-uptake Partitioning between Uncoupling Protein and ATP Synthase during Benzohydroxamic Acid-resistant State 3 Respiration in Tomato Fruit Mitochondria*

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The yield of oxidative phosphorylation in isolated tomato fruit mitochondria depleted of free fatty acids remains constant when respiratory rates are decreased by a factor of 3 by the addition of *n*-butyl malonate. This constancy makes the determination of the contribution of the linoleic acid-induced energy-dissipating pathway by the ADP/O method possible. No decrease in membrane potential is observed in state 3 respiration with increasing concentration of *n*-butyl malonate, indicating that the rate of ATP synthesis is steeply dependent on membrane potential. Linoleic acid decreases the yield of oxidative phosphorylation in a concentration-dependent manner by a pure protonophoric process like that in the presence of FCCP. 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 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. The energy dissipative contribution to oxygen consumption is proposed to be equal to the protonophoric activity of plant 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.

Plant mitochondria contain two energy-dissipating systems: (i) electron carriers that dissipate redox energy instead of building a transmembrane proton electrochemical gradient ($\Delta\mu H^+$),[1] *i.e.* two (inner and outer) rotenone-insensitive NADH dehydrogenases and an alternative ubiquinol cyanide-resistant oxidase (AOX) (1); and (ii) a plant uncoupling mitochondrial protein (PUMP) that dissipates energy contained in the $\Delta\mu H^+$ through a free fatty acid (FFA)-activated H^+ cycling process (2, 3). These two energy-dissipating systems leading to the same final effect (*i.e.* a decrease in ATP synthesis) act at two different levels of the overall energy transduction pathway. Recently, it has been shown that PUMP and AOX must have different physiological functions in plant cells, as they are functionally connected by FFAs (*e.g.* linoleic acid, LA), which activate PUMP and drastically inhibit the AOX activity (4).

Measurements of respiration specifically sustained by ATP synthase, PUMP, or AOX activity were restricted to conditions in which one activity is functioning while the other two are blocked by specific inhibitors (5). These measurements do not reflect the true contribution of each pathway to the total state 3 respiration, as inhibition of one pathway inevitably affects the others. Indeed, inhibition of the cytochrome pathway by cyanide or myxothiazol increases concentration of ubiquinol that is the reduced substrate of AOX, thereby increasing the electron flux via AOX. Similarly, inhibition of ATP synthase by oligomycin increases the $\Delta\mu H^+$, which is the driving force of PUMP activity, thus increasing the activity of PUMP. The ADP/O method, based on the non-phosphorylating property of AOX with succinate (in the presence of rotenone) as oxidizable substrate, has been developed for the first time to determine the contributions of the cytochrome and AOX pathways in overall state 3 respiration of amoeba mitochondria (6). This method involves pair measurements of ADP/O ratios plus or minus benzohydroxamic acid (BHAM, an inhibitor of AOX) and measurements of overall state 3 respiration in the absence of inhibitors of ubiquinol-oxidizing pathways. It has provided the first description of the steady-state kinetics of the two ubiquinol-oxidizing pathways when both are active simultaneously and of electron partitioning between them when the steady-state rate of the quinone-reducing pathway is decreased.

Both PUMP and ATP synthase are able to consume $\Delta\mu H^+$ built up by the protonophoric oxidoreductases of the respiratory chain and may be considered as two branching pathways contributing to respiration through the cytochrome pathway. PUMP is the $\Delta\mu H^+$ energy-dissipating path, and ATP synthase is the $\Delta\mu H^+$ energy-conserving path. Preparation of green tomato fruit mitochondria fully depleted of FFA and observation of the decrease in ADP/O by LA addition have suggested calculation of the contribution of PUMP activity and ATP synthesis in state 3 using pair measurements of ADP/O ratios in the absence or presence of LA (7). The LA-induced energy-dissipating activity responsible for the decrease in ADP/O ratio was

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[1] The abbreviations used are: $\Delta\mu H^+$, proton electrochemical gradient; AOX, alternative oxidase; PUMP, plant uncoupling mitochondrial 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_{PUMP}, contribution of PUMP activity-sustained respiration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; J_p, rate of ATP synthesis; J_o, respiratory rate; TPP^+, tetraphenylphosphonium.

attributed only to the PUMP activity (7). However, protonophoric action of FFA can be mediated not only by uncoupling protein but also by several members of the mitochondrial carrier family (8), mainly in a high energy state of mitochondria. This FFA-dependent H^+ re-cycling is a side function of these carriers and can be inhibited by their substrates or by their specific inhibitors. Thus, the LA-induced H^+ re-cycling could be mediated, at least in part, by ADP/ATP carrier, dicarboxylate carrier, and phosphate carrier (8), likely at high membrane potential ($\Delta\Psi$) but unlikely during phosphorylating respiration, where they are employed in the import of ADP, succinate, and phosphate, respectively. Furthermore, some pieces of evidence in favor of the main responsibility of PUMP in the LA-induced H^+ re-cycling have been obtained during study of post-harvest tomato fruit ripening (5). Indeed, a good correlation was established between PUMP protein concentration in the membrane and the effect induced by LA in state 4 and ADP/O. This makes PUMP the most important candidate to catalyze the LA-induced H^+ re-cycling in tomato mitochondria.

The present study supports the validity of the ADP/O method to determine the actual contributions of PUMP (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. Results show how efficiently PUMP activity can divert energy from oxidative phosphorylation and even more efficiently with decreasing respiration as the PUMP contribution is constant at a given LA concentration while the ATP synthase contribution decreases with respiration rate.

MATERIALS AND METHODS

Plant Material and Mitochondrial Isolation—Tomato plants (*Lycopersicon esculentum*) were grown in a greenhouse at the Centro de Biologia Molecular e Engenharia Genética. Tomato fruits were harvested at a green mature stage (but still completely green) and were used the same day. Mitochondria were isolated and purified on a self-generating Percoll gradient (21%) as described previously (4). The presence of 1% bovine serum albumin in isolation medium allowed chelating of FFA from the mitochondrial suspension and obtaining mitochondria fully depleted of FFA. For each mitochondrial preparation, full depletion of FFA was tested by measuring the effect of bovine serum albumin on the LA-induced respiration as described in Ref. 7. Mitochondrial protein concentration was determined by the Biuret method (9).

Oxygen Uptake and Membrane Potential—Oxygen uptake 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. Mitochondrial transmembrane electrical potential ($\Delta\Psi$) was measured under the same conditions as oxygen uptake, except that the standard incubation medium additionally contained 3 μM tetraphenylphosphonium (TPP^+), and a TPP^+-selective electrode was used as described by Kamo *et al.* (10). For calculation of the $\Delta\psi$ value, the matrix volume of tomato mitochondria was assumed as 2.0 μl $\times$ mg^{-1} protein.

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. ATP was added to ensure complete activation of succinate dehydrogenase. The ADP/O ratio was determined by an ADP pulse method with 220–240 nmol of ADP. The total amount of oxygen consumed during state 3 respiration was used for calculation of the ratio. Increasing concentrations of *n*-butyl malonate were used to decrease steady-state 3 respiration.

RESULTS

All experiments were performed in the presence of BHAM, to inhibit AOX activity, with succinate (plus rotenone) as oxidizable substrate. ADP/O ratios and state 3 respiration were measured during ADP pulses in the absence or presence of LA (Fig. 1). Pulse duration was defined with the help of $\Delta\Psi$ measurement. 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 PUMP activation.

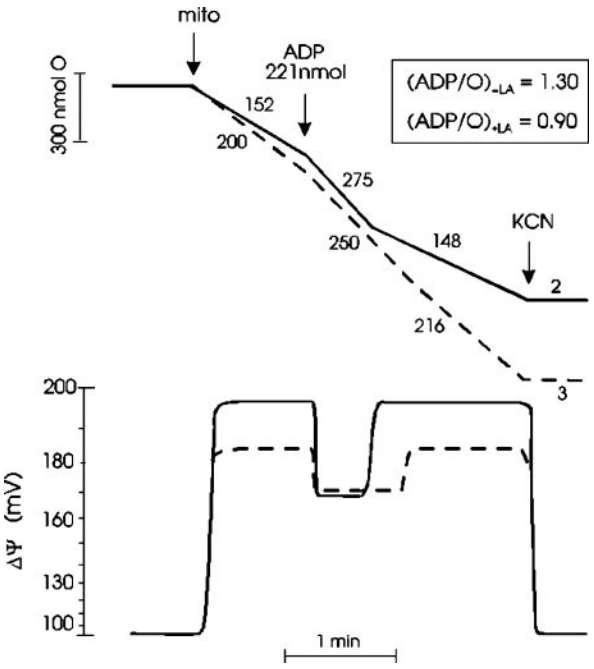

FIG. 1. **Effect of LA on coupling parameters of tomato fruit mitochondria.** Mitochondria (*mito*) were incubated in the presence of 10 mM succinate, 170 μM ATP, 5 μM rotenone, 1.5 mM BHAM, 30 mM *n*-butyl malonate, in the absence (*solid line*) or presence of 4 μM LA (*dashed line*). After the ADP pulse, respiration was inhibited and membrane potential was collapsed by 1.5 mM KCN. *Numbers* on the *traces* refer to O_2 consumption rates in nanomoles of oxygen atom $\times$ min^{-1} $\times$ mg^{-1} protein.

In order to describe how contributions of PUMP 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. A typical example obtained from a single mitochondrial preparation is shown in Table I. V3 decreased with increasing concentrations of *n*-butyl malonate up to 35 mM, inhibiting about 50% of respiration both in the absence and presence of LA. In the presence of 4 μM LA, V3 was lower compared with the value obtained at the same *n*-butyl malonate concentration in the absence of LA (roughly 10% inhibition). $\Delta\Psi$3 was almost unchanged by LA addition; however, a slight (around 2 mV) increase was systematically observed. Decrease in $\Delta\Psi$3 linked to inhibition of respiration by *n*-butyl malonate was not detected. The mean value of $\Delta\Psi$3 obtained with a set of different mitochondrial preparations and *n*-butyl malonate concentrations was 172 $\pm$ 5 mV (S.D., n = 61). In the absence of LA, the ADP/O ratio remained constant independent of V3 or respiratory control ratio. The mean value was 1.29 $\pm$ 0.03 (S.D., n = 55). In the presence of LA, the ADP/O ratio was lower and decreased with increasing concentration of *n*-butyl malonate. Thus, the lowering of electron supply to the cytochrome pathway amplified the decrease in ADP/O induced by LA. This suggests that the relative contribution of the PUMP-sustained respiration was increasing with increasing concentration of *n*-butyl malonate.

Calculation of the respective contributions to respiration in steady-state state 3 of ATP synthesis, which consumes $\Delta\mu H^+$ with energy conservation (Vcyt cons), and of PUMP activity, which consumes $\Delta\mu H^+$ with energy dissipation (V_{PUMP}), by using Equations 1 and 2 implies several conditions.

TABLE I

Effect of decrease in state 3 respiration on membrane potential and ADP/O ratio in the presence or absence of 4 μM LA

Assay conditions are described under "Materials and Methods." Oxidation of succinate (+ BHAM) in the presence or absence of 4 μM LA was gradually decreased by increasing concentrations of n-butyl malonate (10–35 mM). Data deal with a single mitochondrial preparation.

n-Butyl malonate	V3		ΔΨ3		ADP/O	
	−LA	+LA	−LA	+LA	−LA	+LA
mM	*nmol O × min^{-1} × mg^{-1} protein*		*mV*			
0	542	538	171	173	1.345	1.136
10	435	408	174	177	1.352	1.045
20	352	324	174	176	1.325	0.976
27.5	308	271	173	175	1.297	0.924
35	279	242	175	175	1.320	0.782
0	538	508	174	177	1.333	1.129

$$\text{Vcyt cons} = \text{V3} \times \frac{\text{ADP/O}}{(\text{ADP/O})_{-\text{LA}}} \quad \text{(Eq. 1)}$$

$$V_{PUMP} = \text{V3} - \text{Vcyt cons} \quad \text{(Eq. 2)}$$

$(ADP/O)_{-LA}$ is the ADP/O ratio in the absence of LA. Indeed, it assumes that: (*a*) $\Delta\mu H^+$ dissipation occurs only through PUMP activity and then $(ADP/O)_{-LA}$ may be considered as an intrinsic stoichiometry including the intrinsic H^+/O ratio of the respiratory chain and the intrinsic P/H^+ ratio of ADP phosphorylation; (*b*) LA has no direct effect on the intrinsic stoichiometries through interactions with the complexes of the oxidative phosphorylation; and (*c*) LA has no indirect effect on the intrinsic stoichiometries through electron fluxes or ΔΨ modification. Assumptions *b* and *c* are required because of the use of $(ADP/O)_{-LA}$ in Equation 1, which is supposed to be applied in the presence of LA as well as in its absence. These assumptions must be made because the intrinsic stoichiometries of the enzymatic machinery are not necessarily fixed due to possible slippage (see "Discussion"). At almost constant ΔΨ3 (Table I), $(ADP/O)_{-LA}$ did not vary with V3, indicating that condition *a* and part of condition *c* (*i.e.* stoichiometry-flux independence) are valid. Nevertheless, a possible occurrence of $\Delta\mu H^+$ dissipation (H^+ leak) in the absence of PUMP activity will be considered under "Discussion." Furthermore, as LA effect on ΔΨ3, if any, was very small (Table I), it is possible that condition *c* is fully valid. Lack of direct interaction between LA and the redox proton pumps in the presence of rotenone has already been evidenced (10), supporting part of assumption *b*.

Further support in favor of assumptions *b* and *c* arises from the analysis of the ADP/O ratio behavior during titration with *n*-butyl malonate in the presence of LA. For this purpose, Equations 1 and 2 have been rearranged and combined, leading to Equation 3.

$$\text{V3} \times \text{ADP/O} = (\text{ADP/O})_{-\text{LA}} \times (\text{V3} - V_{PUMP}) \quad \text{(Eq. 3)}$$

This equation predicts a linear relationship between the rate of ATP synthesis (V3 × ADP/O) and V3 provided that V_{PUMP} remains constant when V3 is varied by *n*-butyl malonate addition (at fixed concentration of LA). This linearity was verified with 4, 6, and 8 μM LA (Fig. 2). In the absence of LA, the straight line intersects the abscissa axis at a point not significantly different from the origin in accordance with the $(ADP/O)_{-LA}$ constancy. With increasing LA concentration, the abscissa axis intercept (V_{PUMP}) was shifted to the right but the slope ($(ADP/O)_{-LA}$) was not modified. Thus the set of *parallel straight lines* shown in Fig. 2 strongly suggests that LA has no effect on stoichiometries of the H^+ translocating enzymes employed in the oxidative phosphorylation and that the H^+ conduction rate via PUMP does not change with respiration rates. Several mitochondrial preparations used in this study were not identical regarding V3 value at a given condition (concentrations of LA and *n*-butyl malonate). Nevertheless, it was established that they have V_{PUMP} contributions, at the same LA concentration, close enough to allow their gathering in the present analysis.

As V_{PUMP} values (see legend of Fig. 2) were almost proportional to the LA concentration, the apparent K_m for LA, higher than 8 μM, cannot be evaluated. Indeed, higher LA concentration have not been investigated owing to the difficulty to safely determine ADP/O ratios using the ADP-pulse method when the respiratory control ratio decreases below 1.2. An apparent K_m (equal to 83 μM) expressed in total concentration of LA added to the bulk medium has been determined with PUMP reconstituted into liposomes (11). Unfortunately, the LA solubility into liposome and mitochondrial membrane prevents a straightforward comparison between K_m values for the two systems. On the other hand, the saturation observed for the LA-induced non-phosphorylating respiration ($S_{0.5}$ = 10 μM) (7) did not reflect actual saturation of PUMP activity as ΔΨ decreased with increasing concentrations of LA (5).

Data obtained in the presence of carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) at low concentration (0.03 μM) exhibited the same feature as observed with LA (Fig. 2), suggesting a similar type of uncoupling action (see "Discussion"). Additionally, neither V3 nor ΔΨ3 was modified by 0.03 μM FCCP (data not shown), which only decreased the rate of ATP synthesis at a given V3 (Fig. 2).

DISCUSSION

Yield of the Oxidative Phosphorylation in the Absence of Uncoupler (and of AOX Activity)—Measured ADP/O ratios are expected to be lower than an ideal value corresponding to the highest mechanistic stoichiometries, H^+/O of the proton pumps of the respiratory chain (in this study, the ubiquinol-cytochrome *c* oxidoreductase complex III and the cytochrome *c* oxidase complex IV), and P/H^+ of ATP synthase plus ADP/ATP carrier. This means incomplete coupling that may be attributed to the passive permeability of the inner mitochondrial membrane to protons (leak) and to intrinsic uncoupling within the redox pumps or the ATP synthase (slip) as reviewed in Ref. 12. Leaks and slips depend on ΔΨ and values of pH inside and outside of mitochondria. However, leaks are usually supposed to act independently of the way ΔΨ and pH values have been reached in contrast to slips because slips are part of the kinetic mechanisms of oxidative phosphorylation enzymes. However, as membrane permeability to ions is likely influenced by membrane proteins (13), the proton leak may also be under the direct influence of the respiratory enzymes' activity (14). This view does not seem to be supported by experiment (15).

Modulation of electron fluxes using inhibitors is expected to influence the relative contributions of uncoupling processes and of energy conservation processes, and consequently to modify the ADP/O ratio. A simplified theoretical approach (non-equilibrium thermodynamic approach) (16) predicts that a lin-

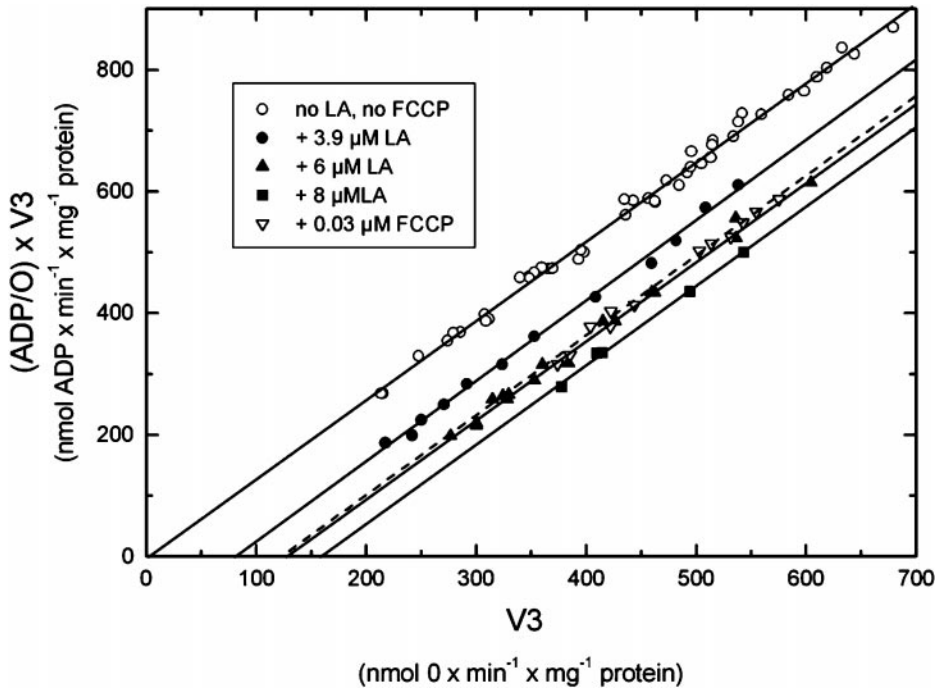

FIG. 2. **Relation between rate of phosphorylation (V3 × ADP/O) and rate of oxygen uptake in state 3 respiration (V3) at different concentrations of LA or with FCCP.** Assay conditions are described under "Materials and Methods." Oxidation rate of succinate (+ *BHAM*) was gradually decreased by increasing 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 14 experiments (7 without LA, 2 with ± 4 μM LA, 3 with ± 6 μM LA, and 2 with ± 8 μM LA,) with different rates of uninhibited state 3 respiration. The *dashed line* deals with 0.03 μM FCCP. For the *solid lines*, values of intercepts with *abscissa axis* (V_{PUMP}) are: 3 ± 5 (S.D., n = 50), 82 ± 7 (S.D., n = 12), 126 ± 8 (S.D., n = 12), and 159 ± 14 (S.D., n = 17) (all in nanomoles of O × min^{-1} × mg^{-1} protein) for 0, 4, 6, and 8 μM LA, respectively. Value of intercepts with *abscissa axis* for 0.03 μM FCCP is 130 ± 14 (nanomoles of O × min^{-1} × mg^{-1} protein) (S.D., n = 12). Slopes of the regression lines are: 1.304 ± 0.019, 1.319 ± 0.024, 1.302 ± 0.021, and 1.304 ± 0.043 for 0, and increasing concentrations of LA, and 1.329 ± 0.038 for 0.03 μM FCCP.

ear relationship between the phosphorylation rate (J_p = V3 × ADP/O) and the respiration rate (J_o = V3) can be obtained. The slope (dJ_p/dJ_o) is either an upper or a lower limit of the mechanistic P/O ratio according to the step sensitive to the inhibitor used for the titration (17, 18). If respiration with succinate is inhibited with malonate (a competitive inhibitor of the succinate dehydrogenase), the ADP/O is expected to decrease and dJ_p/dJ_o is expected to be a lower limit of the mechanistic stoichiometry. Results obtained with rat liver mitochondria seem to be in agreement with the theoretical prediction (18). According to the same simplified theoretical approach, ADP/O constancy such as observed with succinate (+ rotenone) when electron fluxes are changed by *n*-butyl malonate in tomato fruit mitochondria (mean ADP/O value = 1.29 ± 0.03 S.D., n = 55; this work) and in amoeba *Acanthamoeba castellanii* mitochondria (mean ADP/O value = 1.41 ± 0.07 S.D., n = 98) (6) would mean that the measured ratio is the optimal mechanistic stoichiometry *i.e.* that leaks and slips are negligible. Such an interpretation is questionable owing to experimental results in favor of higher stoichiometry with coupling sites 2 (complex III) and 3 (complex IV). Indeed, an ideal value of 1.5, significantly higher than the ADP/O obtained for succinate with tomato fruit mitochondria, has become generally accepted. For instance, an ideal value close to 2 for oxidative phosphorylation with succinate has been determined in rat liver mitochondria (19) and ADP/O ratio of 2.5 has been observed with pyruvate in yeast mitochondria in which coupling site 1 (complex I) is absent (20). Therefore, it cannot be excluded that in our experiments, leaks and/or slips are not negligible and, surprisingly, remain in a constant ratio with oxygen consumption during titration with *n*-butyl malonate. However, a proper modeling of such behavior is lacking so far. In contrast, when titration concerns complex III (*e.g.* inhibition with antimycin A) instead of an upstream step, it has been shown that ADP/O constancy (or increase) may be due to a compensatory (or dominating) effect of the decrease in the relative contribution of slip within the titrated pump (21). ADP/O constancy using antimycin A or cyanide as inhibitors was also observed with yeast mitochondria (20).

In conclusion, the yield of oxidative phosphorylation of isolated tomato fruit mitochondria remains constant in the our experimental conditions but the exact meaning of this constancy is unclear. Nevertheless, it allows for the determination of the contribution of an energy-dissipating pathway by ADP/O measurement as has already been made for AOX in amoeba (6). This will be discussed further for the LA-induced uncoupling.

Another aspect of interest is that no decrease in $\Delta\Psi$ (main part the proton motive force) is observed in state 3 when the respiratory rate is decreased by *n*-butyl malonate to around 50% ($\Delta\Psi 3$ = 172 ± 5, S.D., n = 43). This means that the rate of ATP synthesis, which decreases with inhibition of respiration, is steeply dependent on $\Delta\Psi$, at least as strongly as reported for rat liver mitochondria (22, 23).

Yield of the Oxidative Phosphorylation in the Presence of Uncouplers (LA or FCCP)—Uncouplers stimulate the rate of respiration and decrease $\Delta\Psi$ in state 4. They also decrease the yield of oxidative phosphorylation in state 3 (ADP/O). Such effects of uncouplers may arise from introducing an additional leak pathway (protonophoric uncoupling) or increasing slippage proportion when they bind to a redox proton pump or to ATP synthase (intrinsic uncoupling). Decoupling of localized proton circuits may also be considered (24).

FCCP is usually considered as a typical protonophoric uncoupler, although it might also have some direct effect on the redox pumps (21, 25). In the rationale of non-equilibrium thermodynamics, the effect of increased permeability to protons on the J_p *versus* J_o dependence obtained when the electron supply

to the redox pumps is modulated (lower limit titration) should be a decrease in slope and an increase in the intercept with abscissa (linear extrapolation at $J_p = 0$). This has been observed with rat liver mitochondria (18). However, in this experiment, the effect of FCCP on the slope was relatively small compared with that on the intercept. From these data, extrapolation to zero leak provided a P/O stoichiometry of 1.76 with succinate as oxidizable substrate. Luvisetto *et al.* (26) have reported that FCCP effect in lower limit titration plot of J_{K^+} *versus* J_o (determination of q+/O lower limit at sites II and III in rat liver mitochondria) is only a shift to the right with negligible change in slope. The same behavior observed in upper limit titration has been simulated using the kinetic model (slippy pump) of Pietrobon and Caplan (27) for a protonophoric ohmic leak inducer. In contrast, this model predicts that an intrinsic uncoupler (slip inducer) would modify the slope. In fact, lack of slope modification in lower limit titration appears to be a safe indication that two conditions are fulfilled: (i) no modification in the intrinsic stoichiometry of the oxidative phosphorylation by added agent, and (ii) no steep sensitivity of the protonophoric activity to $\Delta\Psi$ variation during the titration.

Thus, it may be concluded from the data of Fig. 2 that (i) both FCCP (0.03 μM) and LA (up to 8 μM) decrease the yield of oxidative phosphorylation in tomato fruit mitochondria by a pure protonophoric process; (ii) contribution sustained by the LA-induced protonophoric activity remains almost constant during titration with *n*-butyl malonate; and so (iii) ADP/O measurements allow calculation of this contribution. The inability of LA and FCCP to increase V3 suggests that, in state 3, respiration is likely at a maximum value limited by the electron supply to the cytochrome pathway (7). Insensitivity of $\Delta\Psi$3 to LA as well as to *n*-butyl malonate is likely due to the kinetics of ATP synthesis. However, a small decrease in V3 accompanied by a small increase in $\Delta\Psi$3 (Fig. 1, Table I), if significant, may suggest that LA inhibits ATP synthesis, probably by inhibiting the ADP/ATP carrier (28) or interacting with the ATP synthase. In any case, slip induction seems to be excluded due to the absence of a decrease in dJ_p/J_o for all used concentrations of LA (Fig. 2). Pure protonophoric effect of LA is in agreement with the conclusion reached by Schönfeld *et al.* (29) regarding FFA-induced uncoupling in rat liver mitochondria and contradicts the occurrence of slip induction in the redox proton pumps (21, 25, 30).

Proton Re-uptake Partitioning—Contribution of respiration leading to ATP synthesis (Vcyt cons) with the same yield (probably not the highest possible but constant) as in the absence of uncoupler, and contribution of respiration sustained by dissipative H^+ re-uptake (V_{PUMP}) have been calculated using the ADP/O values. This dissipative contribution to oxygen consumption is equal to the electrogenic transport activity of PUMP (H^+ uptake (Ref. 31) or LA^- exit (Refs. 3 and 32)) divided by the intrinsic H^+/O of the cytochrome pathway, prevailing in our experimental conditions. The relative contribution of PUMP in oxygen consumption (V_{PUMP}/V3) is also the relative contribution of PUMP in proton re-uptake. With 4 μM LA, this relative contribution varies from about 15% in the absence of *n*-butyl malonate to about 40% in the presence of 35 mM *n*-butyl malonate. The relative PUMP activity increases with LA concentration at a given *n*-butyl malonate concentration and with *n*-butyl malonate concentration at a given LA concentration at the expense of ADP phosphorylation.

Simultaneous Presence of PUMP and AOX in Tomato Fruit Mitochondria—Heat production is an obvious physiological role of AOX and uncoupling protein in specialized plant and animal thermogenic tissues, *e.g.* in spadices of *Araceae* (33) and in brown adipose tissue (8), respectively. Their discovery in non-thermogenic tissues and protists has stimulated search for other functions of non-coupled respiration and uncoupling such as decreasing formation of reactive oxygen species (8, 34–36).

Our results on tomato fruit mitochondria indicate that AOX and PUMP probably do not have interchangeable roles despite the fact that both activities lead to decrease in ATP synthesis and to increase in energy dissipation. Indeed, expression of AOX and PUMP proteins does not change in a parallel manner during post-harvest ripening of tomato (5). Furthermore, FFAs (like LA), which activate PUMP drastically inhibit AOX (4). Therefore, it seems that the importance of the AOX activity *in vivo* in relation to that of PUMP, decreases with ripening during which FFA content increases (37, 38). Results described in this paper point out an additional difference between PUMP and AOX. Namely, it has been shown that in the presence of BHAM (AOX inactive) and at a fixed concentration of LA, the ADP/O ratio decreases when state 3 respiration of isolated mitochondria is decreased by succinate uptake inhibition (Table I), the PUMP activity remains constant, while the rate of ATP synthesis decreases (Fig. 2). When the same experiment is performed in the absence of BHAM (AOX active) and in the absence of LA (PUMP inactive), the ADP/O ratio rapidly increases, when V3 decreases, up to that value obtained when neither AOX nor PUMP are active (39). Such behavior has also been observed with soybean cotyledon mitochondria (40) and amoeba mitochondria (6). It is due to the steep decrease in AOX activity when steady-state proportion of quinol (AOX substrate) is decreased (6, 41). Thus, AOX and PUMP, which both decrease the ADP/O, have completely different kinetic behavior since AOX is highly sensitive to the redox state of the quinone pool that has no effect on PUMP. This leads to opposite effects of a ubiquinol level change on the yield of the oxidative phosphorylation according to whether AOX or PUMP is active. This further supports the view that AOX and PUMP have different roles *in vivo*.

In conclusion, we have demonstrated that the LA-induced PUMP activity very efficiently diverts energy from oxidative phosphorylation in state 3 respiration and that this diversion increases with LA concentration and relatively increases with the decreasing of electron flux.

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