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The contribution of uncoupling protein and ATP synthase to state 3 respiration in *Acanthamoeba castellanii* mitochondria★✪

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Mitochondria of the amoeba *Acanthamoeba castellanii* possess a free fatty acid-activated uncoupling protein (AcUCP) that mediates proton re-uptake driven by the mitochondrial proton electrochemical gradient. We show that AcUCP activity diverts energy from ATP synthesis during state 3 mitochondrial respiration in a fatty acid-dependent way. The efficiency of AcUCP in mitochondrial uncoupling increases when the state 3 respiratory rate decreases as the AcUCP contribution is constant at a given linoleic acid concentration while the ATP synthase contribution decreases with respiratory rate. Respiration sustained by this energy-dissipating process re-

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Abbreviations: AcUCP, uncoupling protein of *A. castellani*; BHAM, benzohydroxamate; BSA, bovine serum albumine; $\Delta\mu H^+$, proton electrochemical gradient; $\Delta\Psi$, mitochondrial transmembrane electrical potential; $\Delta\Psi 3$, membrane potential during state 3; FFA, free fatty acids; LA, linoleic acid; RCR, respiratory control ratio; V3, state 3 respiration; V_{AcUCP}, contribution of AcUCP activity-sustained respiration in V3; $V_{ATP\ synthase}$, contribution of ATP synthesis-sustained respiration in V3; UCP, uncoupling protein.

mains constant at a given linoleic acid concentration until more than 60% inhibition of state 3 respiration by *n*-butyl malonate is achieved. The present study supports the validity of the ADP/O method to determine the actual contributions of AcUCP (activated with various linoleic acid concentrations) and ATP synthase in state 3 respiration of *A. castellanii* mitochondria fully depleted of free fatty acid-activated and describes how the two contributions vary when the rate of succinate dehydrogenase is decreased by succinate uptake limitation.

Most mitochondria possess free energy-dissipating systems that decrease the yield of oxidative phosphorylation. Ubiquinol alternative oxidase dissipates redox energy in mitochondria of plants and some unicellulars (Siedow & Umbach, 2000). Uncoupling protein (UCP) dissipates proton electrochemical gradient energy ($\Delta\mu H^+$) in animal, plant and some fungal and protist mitochondria (Sluse & Jarmuszkiewicz, 2002). Their activities have the same final effect; that is a decrease of ATP production per oxygen consumed. Uncoupling proteins, forming a subfamily within the mitochondrial anion carrier protein family, dissipate $\Delta\mu H^+$ through a free fatty acid (FFA)-activated purine nucleotide-inhibited H^+ cycling process driven by membrane potential ($\Delta\Psi$) and ΔpH (both constituting $\Delta\mu H^+$). Indications that UCP is present in unicellulars are mainly based on functional studies and the cross-reactivity of an around 32 kDa mitochondrial protein with antibodies developed against plant UCP. In mitochondria of the nonphotosynthetic amoeboid protozoan *Acanthamoeba castellanii*, the action of UCP (AcUCP) has been shown to mediate FFA-activated, poorly purine nucleotide-inhibited H^+ re-uptake driven by $\Delta\mu H^+$ that in state 3 respiration can divert energy from oxidative phosphorylation (Jarmuszkiewicz *et al.*, 1999).

Both UCP and ATP synthase are able to consume $\Delta\mu H^+$ built up by the mitochondrial respiratory chain. They may be considered as two branching pathways: UCP as the $\Delta\mu H^+$ energy-dissipating path, and ATP synthase as the $\Delta\mu H^+$ energy-conserving path. The ADP/O method has been applied to calculate the contributions of UCP activity and ATP synthesis in state 3 respiration of tomato mitochondria, using pair measurements of ADP/O ratios in the absence or presence of various concentrations of linoleic acid (LA) (Jarmuszkiewicz *et al.*, 2000; Sluse *et al.*, 2000). In the present study, measurements in the presence of benzohydroxamate (BHAM) to exclude oxygen consumption by alternative oxidase and with an increasing concentration of *n*-butyl malonate (an inhibitor of succinate uptake) in order to decrease the rate of the quinone-reducing pathway (succinate oxidation) have been performed. They have allowed us to describe evolution of the UCP and ATP synthase contributions when state 3 respiration is decreased in *A. castellanii* mitochondria.

MATERIAL AND METHODS

Cell culture and mitochondrial isolation. The soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz *et al.* (1997). Trophozoites of the amoeba were collected between 44–48 h following inoculation at the middle exponential phase (at a density of about 5–6 x 10^6 cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described earlier (Jarmuszkiewicz *et al.*, 1997). The presence of 0.4% bovine serum albumin (BSA) in isolation media allowed FFA to be chelated from the mitochondrial suspension and mitochondria fully depleted of FFA to be obtained. For each mitochondrial preparation, full depletion of FFA was tested by measuring the effect of BSA on the LA-induced respiration as described by Jarmuszkiewicz *et al.* (1998). Mitochondrial protein concentration was determined by the biuret method.

Oxygen uptake and membrane potential. Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge, U.K.) oxygen electrode or a Hansatech oxygen electrode in 1.4 ml or 2.7 ml (respectively) of standard incubation medium (25°C) containing: 120 mM KCl, 20 mM Tris/HCl pH 7.4, 3 mM KH_2PO_4, and 0.5 mM $MgCl_2$, with 1–1.7 mg of mitochondrial protein. Membrane potential of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al.* (1979). For calculation of the $\Delta\Psi$ value the matrix volume of amoeba mitochondria was assumed as 2.0 μl/mg protein.

All measurements were made in the presence of 7 mM succinate, 1.5 mM benzohydroxamate (BHAM), 170 μM ATP, 4 μM rotenone, 0–12.6 μM LA, and 0–30 mM *n*-butyl malonate. The ADP/O ratio was determined by the ADP pulse method with 250–500 nmoles of ADP. The total amount of oxygen consumed during state 3 respiration was used for calculation of the ratio. A prepulse of ADP (80 μM) was always applied before the main pulse to ensure that a true state 4 had been achieved and to activate succinate dehydrogenase by the produced ATP. Increasing concentrations of *n*-butyl malonate were used to decrease steady-state 3 respiration. Measurements of $\Delta\Psi$ allowed fine control of the duration of state 3 respiration.

RESULTS AND DISCUSSION

State 3 respiratory rates and ADP/O ratios were measured during ADP pulses in the absence or presence of different concentrations of LA. Figure 1 shows an example of the effect of 9.1 μM LA on the coupling parameters in isolated *A. castellanii* mitochondria. In resting state (state 4), LA increased the respiratory rate and decreased $\Delta\Psi$. On the other hand, LA scarcely modified phosphorylating

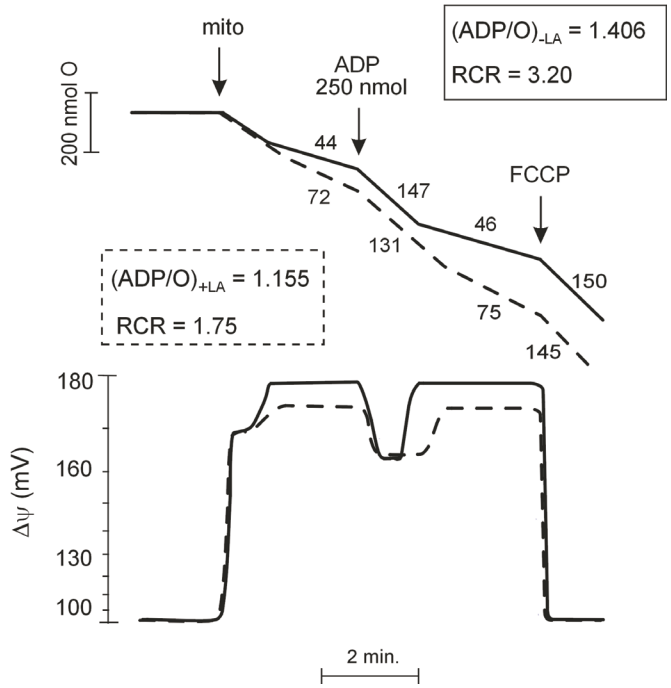

Figure 1. The effect of LA on coupling parameters of *A. castellanii* mitochondria.

Mitochondria (mito) were incubated in the presence of 7 mM succinate, 170 μM ATP, 4 μM rotenone, 1.5 mM BHAM, in the absence (soild line) or presence of 9.1 μM LA (dashed line). After the ADP pulse, respiration was uncoupled and membrane potential was collapsed by 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). RCR, respiratory control ratio. Numbers on the traces refer to O_2 consumption rates in nmol O_2 x min^{-1} per mg protein.

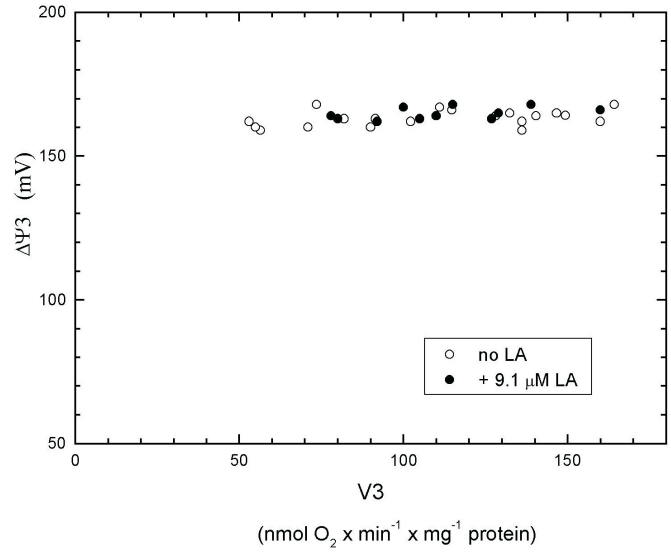

Figure 2. Constancy of membrane potential in state 3 respiration.

Assay conditions as described in Material and Methods. Oxidation rate of succinate in the presence or absence of 9.1 μM LA was gradually decreased by increasing concentrations of *n*-butyl malonate (2–30 mM). Data from 3 experiments are shown. The mean value of $\Delta\Psi 3$ in the absence of LA is 163.2 ± 2.8 (S.D., n = 20), in the presence of 9.1 μM LA is 164.8 ± 2.2 (S.D., n = 11). The mean value of $\Delta\Psi 3$ (± LA) is 163.8 ± 2.7 (S.D., n = 31).

state 3 respiration (V3) and membrane potential ($\Delta\Psi 3$). As a consequence, the ADP/O ratio and respiratory control ratio (RCR) were clearly lowered in the presence of LA suggesting activation of AcUCP (i.e. an increased LA-induced AcUCP-mediated H^+ re-uptake sustaining part of state 3 respiration).

In order to describe how the contributions of AcUCP and ATP synthase change with variations in state 3 respiration, the rate of the ubiquinone-reducing pathway (succinate dehydrogenase) was decreased by *n*-butyl malonate, an inhibitor of succinate uptake. Pair measurements of ADP/O ratios and respiratory rates in the absence or presence of a given LA concentration were performed with increasing concentrations of *n*-butyl malonate (up to 30 mM). V3 decreased with increasing concentrations of the inhibitor both when AcUCP was activated (plus LA) and not (no LA) (Fig. 2). $\Delta\Psi 3$ was almost unchanged by LA addition. However, like in tomato mitochondria (Jarmuszkiewicz *et al.*, 2000), a slight increase was systematically observed in the presence of LA. The mean value of $\Delta\Psi 3$ (± 9.1 μM LA) obtained with different mitochondrial preparations and *n*-butyl malonate concentrations was 163.8 ± 2.7 (S.D., n = 31). Figure 3 shows the effect of three concentrations of LA on the ADP/O ratio plotted against the reciprocal of state 3 respiration which was varied with *n*-butyl malonate. In the absence of LA, the ADP/O ratio remained constant when V3 was decreased (up to about 65%) and the mean value was 1.403 ± 0.021 (S.D., *n* = 31) (Fig. 3). This constancy is one of the requirements for

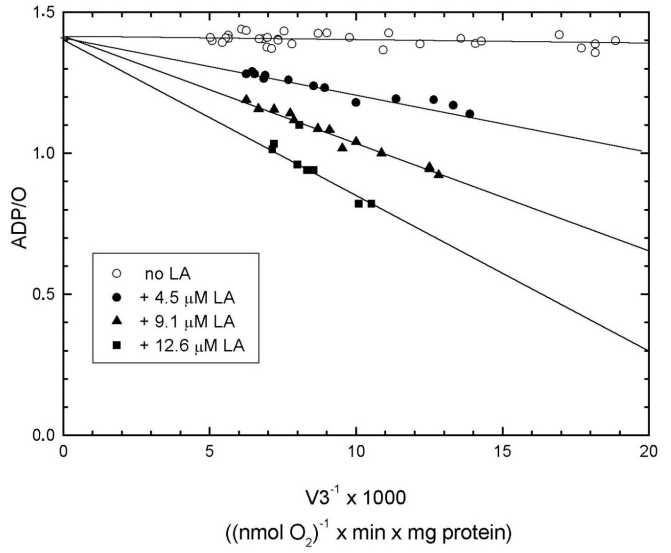

Figure 3. The effect of different LA concentrations on the ADP/O ratio plotted *versus* 1/V3.

Assay conditions as in Material and Methods. Oxidation rate of succinate was gradually decreased by increasing concentrations of *n*-butyl malonate (2–30 mM). ADP/O ratios were determined in the presence of 0, 4.5, 9.1, and 12.6 μM LA. Data from 3 experiments are shown. Mean value of ADP/O ratio in the absence of LA is 1.403 ± 0.021 (S.D., n = 31). 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 = $1/V3 \times 10^3$) are: 1.391 ± 0.014 (S.D., n = 13), 1.429 ± 0.014 (S.D., n = 13), and 1.422 ± 0.053 (S.D., n = 8) for 4.5, 9.1, and 12.6 μM LA, respectively.

the validity of the ADP/O method and has been already verified with tomato mitochondria (Jarmuszkiewicz *et al.*, 2000). In the presence of LA, the ADP/O ratio was lowered and decreased with decreasing V3 (increasing *n*-butyl malonate concentration). Thus, the lowering of the electron supply to the cytochrome pathway amplified the decrease in ADP/O induced by LA, suggesting an increasing relative contribution of the AcUCP-sustained respiration to the overall state 3 respiration. At a given V3, the higher the LA concentration was the lower ADP/O ratio was observed, thereby indicating a higher AcUCP contribution (Fig. 3). A linear relationship was obtained between ADP/O and the reciprocal of V3, with identical ordinates at origin (independently of the LA concentration) and slopes which increased negatively when LA concentration was increased. In the presence of LA, the ordinates at the origin (obtained using linear regression, see legend of Fig. 3) were not significantly different for the mean ADP/O value in the absence of LA. Such behaviour, observed also for tomato mitochondria (Jarmuszkiewicz *et al.*, 2000; Sluse *et al.*, 2000), indicates that (i) at a fixed LA concentration, V_{UCP} remains constant during titration by *n*-butyl malonate, and (ii) LA does not affect the intrinsic stoichiometry of oxidative phosphorylation (ratio of H^+/O and H^+/ADP).

The contributions of ATP synthesis and AcUCP activity to state 3 respiration were calculated from the pair measurements of ADP/O and V3 in the absence or presence of LA (as described by Jarmuszkiewicz *et al.*, 2000, see also legend of Fig. 4) and plotted against V3 in order to emphasize their behaviour during titration of respiration by *n*-butyl malonate. As a result, at a fixed LA concentration, the AcUCP activity remained constant and ATP synthesis decreased linearly with decreasing state 3 respiration (Fig. 4). Using higher concentrations of LA, UCP activity increased at the expense of ADP phosphorylation without an increase in respiration. Namely, at a given V3, increasing LA decreased the ATP synthase contribution and increased the AcUCP activity contribution. These results show how efficiently the UCP activity can divert energy from oxidative phosphorylation, especially when state 3 respiration is progressively inhibited.

The protonophoric action of FFA can be mediated not only by UCP but also, at least in part, by several members of the mitochondrial carrier family such as the ADP/ATP, dicarboxylate, aspartate/glutamate, and phosphate carriers (Andreyev *et al.*, 1989; Wieckowski & Wojtczak, 1997; Samartsev *et al.*, 1997; Žačková *et al.*, 2000). However, this FFA-dependent mitochondrial uncoupling, being a side function of these carriers, is ex-

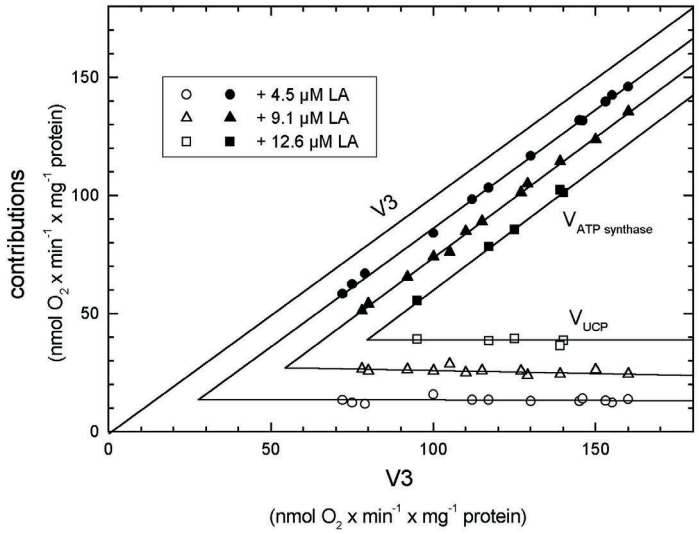

Figure 4. The partitioning of state 3 respiratory rate (V3) between uncoupling protein activity (V_{UCP}) and ATP synthesis ($V_{ATP\ synthase}$) at different LA concentrations.

Assay conditions as in Fig. 3. As described by Jarmuszkiewicz *et al.* (2000), $V_{ATP\ synthase}$ is equal to V3 x $(ADP/O)_{+LA}/(ADP/O)_{-LA}$ and V_{UCP} is equal to V3 – $V_{ATP\ synthase}$. Mean values of V_{UCP} for different concentrations of LA are: 13.4 ± 1.1 (S.D., n = 12), 25.8 ± 1.3 (S.D., n = 12), 37.5 ± 4.5 (S.D., n = 8) for 4.5, 9.1, and 12.6 μM LA, respectively.

clusively observed in the high-energy state of mitochondria (at high $\Delta\Psi$ like in state 4) and can be inhibited by their substrates or by their specific inhibitors. It is highly unlikely that this unspecific uncoupling takes place during phosphorylating respiration where the particular carriers are employed in the import of ADP, succinate or phosphate. Thus, the first candidate to catalyze the LA-induced H^+ re-cycling observed in amoeba mitochondria in state 3 measurements is AcUCP.

In isolated *A. castellanii* mitochondria depleted of FFA, the yield of oxidative phosphorylation (ADP/O ratio) remained constant when the respiratory rate was decreased by a factor of 3 by the addition of *n*-butyl malonate. This constancy allows the contribution of the LA-induced energy-dissipating pathway to be determined by the ADP/O method. Linoleic acid decreases the yield of oxidative phosphorylation in a concentration dependent manner by a pure protonophoric process (i.e., LA is not a slip inducer, see Jarmuszkiewicz *et al.*, 2000). Therefore, it can be concluded that in *A. castellanii* 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 LA that can be attributed to AcUCP activity. Thus, the ADP/O method first verified in tomato mitochondria (Jarmuszkiewicz *et al.*, 2000) can be applied widely in different mitochondria including those of unicellulars. Most of the investigations on the activity and regulation of uncoupling proteins in isolated mitochondria are performed only in state 4 (nonphosphorylating) respiration which implicates a high phosphate potential (no ADP) and a high reducing power (slow respiratory substrate oxidation). Under these experimental conditions, ubiquinone is highly reduced, production of superoxide by mitochondria is high and $\Delta\mu H^+$ is maximal (high membrane potential). The present work describes an experimental approach that allows the boundaries of the high energy status of mitochondria to be crossed so as to focus on regulation of UCP activity in state 3 respiration, thus during ADP phosphorylation.

REFERENCES

Andreyev AY, Bondareva TO, Dedukhova VI, Mokhova EN, Skulachev VP, Tsofina LM, Volkov NL, Vygodina TV. (1989) *Eur J. Biochem.*; **182**: 585–92.

Jarmuszkiewicz W. (2001) *Acta Biochim Polon.*; **48**: 145–55.

Jarmuszkiewicz W, Wagner AM, Wagner MJ, Hryniewiecka L. (1997) *FEBS Lett.*; **411**: 110–14.

Jarmuszkiewicz W, Almeida AM, Sluse-Goffart CM, Sluse FE, Vercesi AE. (1998) *J Biol Chem.*; **273**: 34882–6.

Jarmuszkiewicz W, Sluse-Goffart CM, Hryniewiecka L, Sluse FE. (1999) *J Biol Chem.*; **274**: 23198–202.

Jarmuszkiewicz W, Almeida AM, Vercesi AE, Sluse FE, Sluse-Goffart CM. (2000) *J Biol Chem.*; **275**: 13315–20.

Kamo N, Muratsugu N, Hongoh R, Kobatake Y. (1979) *J Membr Biol.*; **49**: 105–21.

Sluse FE, Jarmuszkiewicz W. (2002) *FEBS Lett.*; **510**: 117–20.

Sluse FE, Jarmuszkiewicz W, Almeida AM, Vercesi AE, Sluse-Goffart CM. (2000) In *Animating the Cellular Map.* Hofmeyr J-H, Rohwer JM, Snoep JL, eds, pp 135–40. Stellenbosch University Press, Stellenbosh, South Africa.

Samartsev VN, Mokhova EN, Skulachev VP. (1997) *FEBS Lett.*; **412**: 251–7.

Siedow JN, Umbach AL. (2000) *Biochim Biophys Acta.*; **1459**: 432–9.

Wieckowski M, Wojtczak L. (1997) *Biochem Biophys Res Commun.*; **232**: 414–7.

Žačková M, Krämer R, Ježek P. (2000) *Int J Biochem Cell Biol.*; **32**: 499–508.