First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis*

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Abstract An uncoupling protein (UCP) was identified in mitochondria from *Candida parapsilosis* (CpUCP), a non-fermentative parasitic yeast. CpUCP was immunodetected using polyclonal antibodies raised against plant UCP. Activity of CpUCP, investigated in mitochondria depleted of free fatty acids, was stimulated by linoleic acid (LA) and inhibited by GTP. Activity of CpUCP enhanced state 4 respiration by decreasing $\Delta\Psi$ and lowered the ADP/O ratio. Thus, it was able to divert energy from oxidative phosphorylation. The voltage dependence of electron flux indicated that LA had a pure protonophoretic effect. The discovery of CpUCP proves that UCP-like proteins occur in the four eukaryotic kingdoms: animals, plants, fungi and protists.

Key words: Yeast mitochondrion; Uncoupling protein; *Candida parapsilosis*

1. Introduction

Mitochondria generate most of the aerobic cell's energy from the reduction of oxygen by their respiratory chain. However, these internal power plants release damaging reactive oxygen species arising from the non-catalyzed partial reduction of molecular oxygen at the level of complexes utilizing or producing ubiquinol [1,2]. Mitochondrial function [3] and mitochondrial proteins [4] are the first targets of such free radical side production. Every condition, which increases the reduced state of electron carriers of the respiratory chain, leads to an increase in reactive oxygen production. Conversely, processes that decrease the reduced state of electron carriers will decrease this damaging production [5].

The energy-dissipating systems such as alternative oxidase (AOX) and uncoupling protein (UCP) have been shown to act in this way in vitro [6,7] and in vivo [8]. AOX consumes ubiquinol independently to phosphate potential and UCP dissipates the proton electrochemical gradient ($\Delta\mu H^+$), allowing an increase of electron flux in the cytochrome pathway at the expense of ubiquinol. Therefore, these two energy-dissipating enzymes can be seen as endogenous protectors of mitochondria, able to prevent deterioration and energy deprivation of the cell. The cost of that protection is a decrease in oxidative phosphorylation yield [9,10].

After the emergence of eukaryotic cells, such intramitochondrial protectors should have appeared quite soon, and if so, they should presently be very widespread. AOX has been early recognized to exist not only in specialized thermogenic tissues of plants but also in plant non-thermogenic tissues and in a large variety of organisms like trypanosomes, fungi, amoeba and other microorganisms [10,11]. In contrast, evidence of the existence of UCP was restricted to mammal brown adipose tissue until 1995 when it was proved also to exist in plants [12]. More recently, several UCP-like proteins have been described in mammal non-thermogenic tissues [13–15], in fishes [16] and in a primitive amoeba, *Acanthamoeba castellanii* [17]. The presence of UCP in *A. castellanii* has allowed hypothesizing the presence of this protein in the whole eukaryotic world. However, no UCP had been shown to occur in the fungi kingdom until now.

The present study demonstrates the existence and characterizes function of an UCP in the parasitic non-fermentative yeast *Candida parapsilosis*.

2. Materials and methods

2.1. Strain and culture

C. parapsilosis CCT 3834 (ATCC 22019) was grown at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco)) until middle stationary phase.

2.2. Isolation of mitochondria

For standard preparation, 1 l of culture was harvested by centrifugation, and cells were washed once with cold water and once with A buffer (1 M sorbitol, 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.8). Cells were resuspended in A buffer (3 ml per g of cells) additionally containing 30 mM dithiothreitol (DTT). After a 15 min incubation at room temperature with shaking, cells were harvested by centrifugation, resuspended in A buffer containing lyticase (1 mg per g of cells) and 1 mM DTT and incubated at 30°C until about 90% of cells converted to spheroplasts (60–80 min). The digestion was stopped by the addition of an equal volume of ice-cold A buffer, and spheroplasts were washed with A buffer. The pellet was resuspended in B1

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Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CAT, carboxyatractyloside; CpUCP, uncoupling protein of *C. parapsilosis*; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acid; LA, linoleic acid; UCP, uncoupling protein; $\Delta\Psi$, mitochondrial transmembrane electrical potential

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buffer (0.6 M mannitol, 1 mM EDTA, 0.5% bovine serum albumin (BSA), 1 mM PMSF, 10 mM Tris–HCl, pH 7.4). Spheroplasts were mechanically broken using a Dounce homogenizer for a maximum of 10 up-and-down strokes. Cell debris was pelleted by centrifugation for 10 min at $1000 \times g$. Mitochondria were pelleted from the supernatant by 10 min centrifugation at $10\,500 \times g$ and washed with B2 buffer (0.6 M mannitol, 1 mM EDTA, 1% BSA, 10 mM Tris–HCl, pH 7.0). The presence of BSA in the medium allowed chelation of free fatty acid (FFA) from the mitochondrial suspension. The last washing was made in B2 medium without BSA and EDTA. The mitochondrial protein concentration was determined by the biuret method [18].

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting of UCP

A different amount of mitochondrial protein was solubilized in the sample buffer containing 1% (w/v) SDS, 0.1 M Tris–HCl, pH 6.8, 10% glycerol, 0.005% (v/v) bromophenol blue and 0.5% β-mercaptoethanol, and boiled for 4–5 min. Electrophoresis (SDS–PAGE) was carried out using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel containing 4.5 M urea, followed by Western blotting as described before [17,19]. Antibodies against UCP protein of potato (*Solanum tuberosum*) (generously supplied by Dr. P. Arruda) were used at a dilution of 1:1000. The bands were detected by chemiluminescence (Amersham ECL system).

2.4. Oxygen uptake and membrane potential

Oxygen uptake was measured polarographically using a Clark-type electrode (Yellow Springs Instruments) in 1.3 ml of standard incubation medium (28°C) containing: 125 mM sucrose, 65 mM KCl, 10 mM HEPES pH 7.2, 2.5 mM KH_2PO_4 and 1 mM $MgCl_2$, with 0.4–0.5 mg of mitochondrial protein. The membrane potential of mitochondria was measured under the same conditions as oxygen uptake (additionally with 3 μM tetraphenylphosphonium, TPP^+) using a TPP^+-specific electrode according to Kamo et al. [20]. For calculation of the mitochondrial transmembrane electrical potential ($\Delta\Psi$) value, the matrix volume of yeast mitochondria was assumed as 2.0 μl/mg protein. All measurements were made in the presence of 1.5 mM benzohydroxamate (BHAM). Details of measurements are included in the legends of figures.

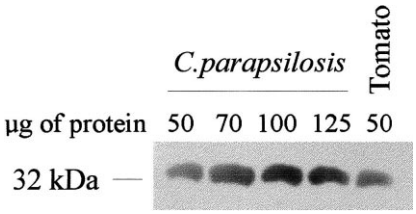

Fig. 1. Immunological identification of CpUCP protein in yeast *C. parapsilosis* mitochondria. Detection of UCP in mitochondria isolated from green tomato fruit (control) is also shown. Primary antibodies were raised against potato UCP. The amount of protein loaded on lane is indicated.

3. Results

3.1. Immunological detection of C. parapsilosis UCP (CpUCP)

Polyclonal antibodies developed against potato UCP cross-react widely not only with various plant tissues or plant species [21] but also with the UCP of a primitive soil ameboid protozoan, *A. castellanii* [17]. These antibodies were used in total mitochondrial proteins to evidence the presence of UCP in *C. parapsilosis* (CpUCP). A single protein band with an appropriate similar molecular mass (32 000 Da) was revealed in green tomato fruit (control) and *C. parapsilosis*, indicating cross-reactivity of plant antibodies with the fungi protein (Fig. 1). A similar band was observed with monoclonal antibodies developed against *Arabidopsis thaliana* protein (not shown).

3.2. Determination of coupling parameters in C. parapsilosis mitochondria

In addition to the classical respiratory chain, *C. parapsilosis* possesses an alternative pathway, which differs from the AOX

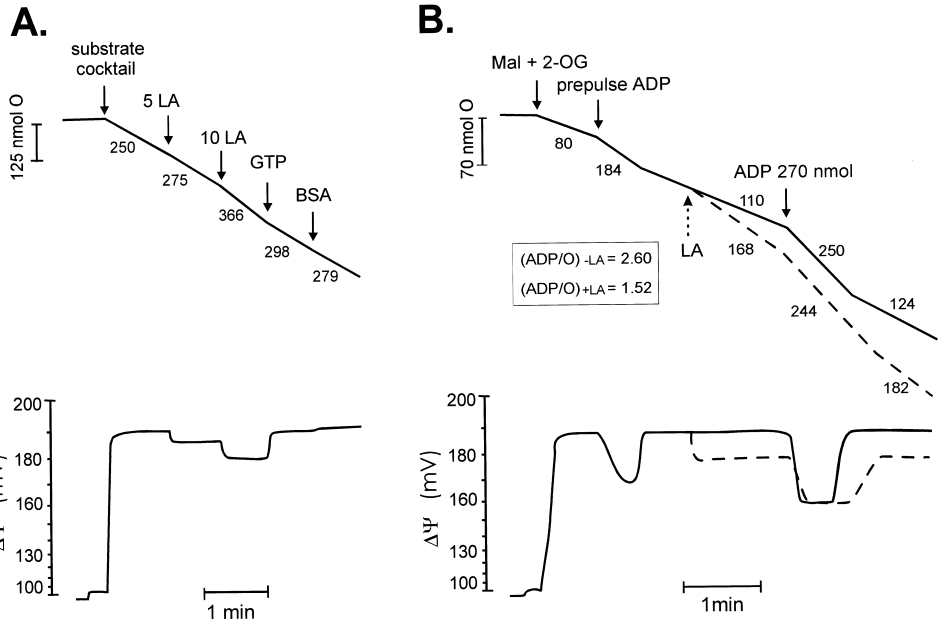

Fig. 2. Influence of LA on respiratory rates, coupling parameters and $\Delta\Psi$ of *C. parapsilosis* mitochondria depleted of FFAs. A: Mitochondria (0.5 mg of protein) were incubated in the presence of 1.5 mM BHAM, 1 μM CAT. Substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH); 5 or 10 μM LA; 2 mM GTP; and 0.5% BSA were added where indicated. B: Mitochondria (0.5 mg of protein) were incubated in the presence of 1.5 mM BHAM and in the presence (solid lines) or absence (dotted lines) of 15 μM LA. Additions: malate (5 mM)+2-oxoglutarate (5 mM), 120 μM (prepulse) and 207 μM ADP (pulse). The total amount of oxygen consumed during state 3 respiration was used for calculation of the ADP/O ratio. Numbers on the traces refer to O_2 consumption rates in nmol O/min/mg protein. Membrane potential changes are shown in mV.

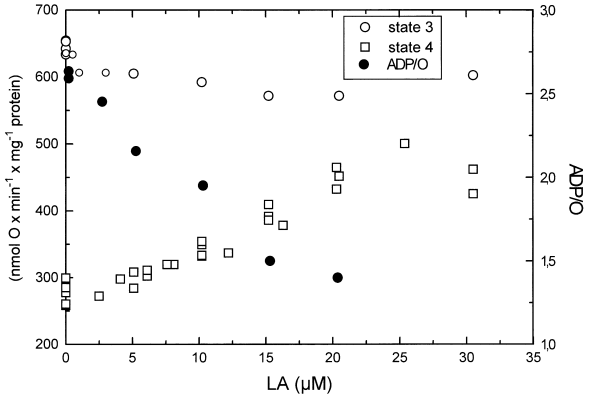

Fig. 3. Concentration dependence of LA effect on phosphorylating respiration (state 3), resting respiration (state 4) and ADP/O ratio. Effect of LA on respiratory rates (left abscissa axis, open symbols) were measured with mitochondria incubated with substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH), 1.5 mM BHAM, and 1 μM CAT. State 3 respiration was measured in the presence of 207 μM ADP or 2 mM ADP (saturating conditions). Increasing concentrations of LA (1–30 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 concentrations. Effect of LA on ADP/O ratio (right abscissa, solid symbols) was measured with 5 mM malate+5 mM 2-oxoglutarate as oxidizable substrates as described in Fig. 2B.

well described in plants and some microorganisms. This second respiratory chain, fully energy-dissipating, parallels the classical one and has specific components: external NADH dehydrogenase, quinone (Qx), cytochrome *b*, cytochrome c_{alt} and alternative terminal oxidase sensitive to salicylhydroxamate. Two possible sites of electron partitioning with the main pathway exist, one upstream of complex III and the second at the level of the two cytochromes *c* [22]. Such network complexity leads to a wide range of ADP/O values and respiratory rates according to the reducing substrate and the electron route even in the presence of salicylhydroxamate. In a representative experiment, the ADP/O ratios were 1.08, 2.55, 0.79 and 0.49 and the state 3 rates were 276, 258, 594 and 428 nmol O/min/mg protein for malate+pyruvate, malate+2-oxoglutarate, substrate cocktail (malate+pyruvate+succinate+NADH) and NADH alone, respectively.

Substrate conditions were chosen to lead to the highest ADP/O value (malate+2-oxoglutarate) and to the highest respiratory rates (substrate cocktail) in order to describe the specific effects of linoleic acid (LA) in *C. parapsilosis* mitochondria (Fig. 2A,B). Simultaneous measurements of oxygen consumption and $\Delta\Psi$ in the presence of BHAM were performed with mitochondria depleted of endogenous FFA by isolation in the presence of BSA (see Section 2). State 4 respiration (plus carboxyatractyloside (CAT), plus BHAM) was stimulated by the successive addition of LA (+47% at 10 μM LA) with a parallel decrease in $\Delta\Psi$ from 190 to 180 mV (−5%) (Fig. 2A). The addition of 2 mM GTP partly reversed stimulation of respiration and restored $\Delta\Psi$. The subsequent addition of BSA slightly decreased respiration. Presence of both, GTP and BSA, totally cancelled the LA-induced stimulation of respiration. This stimulation in state 4 suggests the existence of a FFA-linked H^+ re-uptake in *C. parapsilosis* mitochondria. The equality between starting state 4 respiratory rate and the GTP+BSA-inhibited rate confirmed the absence of endogenous FFA in mitochondria. The ADP/O ratios and state 3 respiration were measured during ADP pulses (Fig. 2B) in the absence or presence of LA. Pulse duration was defined with the help of $\Delta\Psi$ measurements. LA (15 μM) increased the state 4 respiratory rate and decreased $\Delta\Psi$ in state 4 while not modifying respiration and $\Delta\Psi$ in state 3. Respiratory control and ADP/O were clearly lowered in the presence of LA, suggesting the LA-induced dissipation of the H^+ electrochemical gradient possibly through CpUCP.

3.3. Concentration dependence of LA effect

State 4 (with 1 μM CAT) and state 3 respiration were measured in the presence of BHAM, substrate cocktail and increasing concentration of LA (Fig. 3). State 4+CAT respiratory rates increased with LA concentration up to 85% of state 3 rates that remained almost constant. The same behavior of respiratory rates (increase in state 4+CAT, no increase in state 3) was observed in the other substrate conditions (not shown).

The effect of LA concentration on ADP/O ratios was determined with malate+2-oxoglutarate as oxidizable substrates. A decrease in the ADP/O ratio was observed with an increasing LA concentration up to 20 μM (Fig. 3). The effect of LA was also checked on the ADP/O ratios with the other substrates. LA decreased the ADP/O ratios in every condition in the following manner in a representative experiment: from 2.59 to 1.4 at 20 μM LA (with malate+2-oxoglutarate), from 0.89 to a value lower than 0.5 at 5 μM LA (with substrate cocktail), from 1.09 to 0.68 at 20 μM LA (with malate+pyruvate) and from 0.5 to a non-measurable value with NADH. These results mean that the LA-induced H^+ recycling can efficiently divert energy from oxidative phosphorylation in state 3 respiration even if the state 3 respiratory rates are not modified.

3.4. Voltage dependence of electron flux

Fig. 4 reports the relation between $\Delta\Psi$ and respiratory rate measurements with substrate cocktail and in the presence of

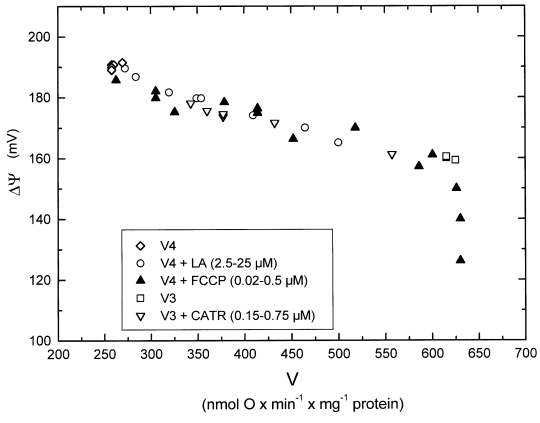

Fig. 4. Relation between $\Delta\Psi$ and mitochondrial respiration in the presence of BHAM. All measurements were made in the presence of substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH), 1.5 mM BHAM. State 4 (V4) was measured in the presence of 1 μM CAT. State 3 (V3) was reached by the addition of 2 mM ADP. (◇) State 4, (○) state 4 with increasing concentrations of LA (2.5–25 μM), (▲) state 4 with increasing concentration of FCCP (0.02–0.5 μM), (□) state 3, and (▽) state 3 with increasing concentration of CAT (0.15–0.75 μM).

BHAM in five conditions: (1) in state 4+CAT (◇), (2) in state 4+CAT with increasing LA concentration (from 2.5 to 25 μM) (○), (3) in state 4+CAT with increasing carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) concentration (0.02–0.5 μM) (▲), (4) in state 3 (□) and (5) in state 3 with increasing CAT concentration (0.15–0.75 μM) (▽). The maximum uncoupled respiration (at ≥0.2 μM FCCP) was 630 nmol O/min/mg protein, i.e. not higher than the state 3 respiratory rate (615–625 nmol O/min/mg protein), suggesting that ATP synthase activity is not a limiting factor in the oxidative phosphorylation rate with these oxidizable substrates (substrate cocktail). On the contrary, maximum respiratory rate measured with 25 μM LA (30 μM led to a decrease in rate) was lower (500 nmol O/min/mg protein) compared to FCCP- (fully uncoupled) and ADP- (fully coupled) induced respiratory rates, indicating that maximum electron flux was not reached with the LA-induced H^+ recycling system. However, a set of conditions (state 3 with increasing CAT concentration, state 4 with both FCCP and LA increasing concentrations) constituted a single force-flow relationship, indicating that LA had a pure protonophoretic effect like the artificial protonophore, FCCP.

4. Discussion

The results reported in this paper support the existence of the UCP-like protein in mitochondria from the pathogenic yeast *C. parapsilosis*. The immunodetection of CpUCP with antibodies raised against the UCP from potato mitochondria indicates a close molecular relationship not only with plant UCP but also with amoeba protein [17].

C. parapsilosis mitochondria fully depleted of endogenous FFA exhibit a stimulation of state 4 respiration linked to the addition of LA as well as a decrease in $\Delta\Psi$ in state 4, suggesting the existence of a FFA-induced H^+ re-uptake.

A single force-flow relationship is observed for state 4 respiration enhanced by LA or FCCP as well as for state 3 respiration progressively inhibited by CAT. Therefore, modulation of $\Delta\Psi$ either by CAT (increase in $\Delta\Psi$), by H^+ permeabilization with FCCP or by LA addition exerts the same control of the flow (oxygen consumption rate). These results prove the exclusive protonophoric effect of LA: namely that LA does not act directly on the respiratory chain activity (no intrinsic uncoupling or slips).

Stimulation of state 4 by LA is observed in the presence of CAT, an inhibitor of adenylic carrier. In such conditions, this carrier cannot participate in the FFA-induced H^+ re-uptake through FFA anion translocation. In state 4, respiration stimulated by 10 μM LA is inhibited by 2 mM GTP (60% of the LA-induced stimulation) and $\Delta\Psi$ lowered by LA is restored by GTP. As purine nucleotides are known to inhibit UCP-like proteins, it is likely that CpUCP is involved in the protonophoric uncoupling effect of LA either by LA^- translocation [23] or by LA-stimulated H^+ transport [24]. It seems that the sensitivity of CpUCP is similar to that of UCP in potato [25] and of UCP2 in mammals [24] ($K_i \approx 1$ mM).

Although as with tomato and amoeba *A. castellanii* mitochondria, in *C. parapsilosis* mitochondria, LA does not stimulate respiration in state 3 (which is probably limited by the rate of electron supply to the respiratory chain), its uncoupling effect is evidenced by a decrease in ADP/O ratio. Thus, the apparent insensitivity of state 3 respiration to LA is accompanied by an important decline in energy conservation by oxidative phosphorylation. It can be concluded that *C. parapsilosis* mitochondria possess a UCP-like protein (CpUCP) with uncoupling properties similar to those of the other members of the UCP family.

The discovery of UCP in *A. castellanii* [17], an ameboid protozoan positioned in the molecular phylogenetic tree of eukaryotes on a branch basal to the divergence points of plants, animals and fungi [26,27], has allowed us to hypothesize its existence in the whole eukaryotic world and to propose its emergence, as specialized proteins for H^+ recycling very early during phylogenesis [17]. The evidence presented in this paper of the existence of an UCP-like protein in the yeast *C. parapsilosis* demonstrates that the UCP family occurs also in the fungi kingdom even if absent from *Saccharomyces cerevisiae*. Moreover, *C. parapsilosis* also possesses a redox energy-dissipating respiratory chain quite complex when compared to plant and *A. castellanii* AOX. Thus, the presence of two energy-dissipating systems, that may alter the coupling between respiration and ATP synthesis, can be used to maintain a balance between supply of reducing substrates and energy and carbon demand for biosynthesis in the *Candida* cells; this could also hold true for every non-thermogenic tissue and monocellular [28]. Moreover, these two energy-dissipating systems can be postulated to take part in the defense system against reactive oxygen species in the living cell by decreasing the reducing power and the local oxygen concentration [29] and also to play a role in cell or tissue aging as shown in fruit ripening [19].

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