Respiration, oxidative phosphorylation, and uncoupling protein in *Candida albicans*

**Abstract**

The respiration, membrane potential (Δψ), and oxidative phosphorylation of mitochondria *in situ* were determined in spheroplasts obtained from *Candida albicans* control strain ATCC 90028 by lyticase treatment. Mitochondria *in situ* were able to phosphorylate externally added ADP (200 µM) in the presence of 0.05% BSA. Mitochondria *in situ* generated and sustained stable mitochondrial Δψ respiring on 5 mM NAD-linked substrates, 5 mM succinate, or 100 µM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride plus 1 mM ascorbate. Rotenone (4 µM) inhibited respiration by 30% and 2 µM antimycin A or myxothiazole and 1 mM cyanide inhibited it by 85%. Cyanide-insensitive respiration was partially blocked by 2 mM benzohydroxamic acid, suggesting the presence of an alternative oxidase. *Candida albicans* mitochondria *in situ* presented a carboxyatractyside-insensitive increase of Δψ induced by 5 mM ATP and 0.5% BSA, and Δψ decrease induced by 10 µM linoleic acid, both suggesting the existence of an uncoupling protein. The presence of this protein was subsequently confirmed by immunodetection and respiration experiments with isolated mitochondria. In conclusion, *Candida albicans* ATCC 90028 possesses an alternative electron transfer chain and alternative oxidase, both absent in animal cells. These pathways can be exceptional targets for the design of new chemotherapeutic agents. Blockage of these respiratory pathways together with inhibition of the uncoupling protein (another potential target for drug design) could lead to increased production of reactive oxygen species, dysfunction of *Candida* mitochondria, and possibly to oxidative cell death.

**Introduction**

Candidiases are common infections of the skin, oral cavity, esophagus, gastrointestinal tract, vagina, and vascular system, and have become a major cause of mortality in immunocompromised patients, including those with AIDS (1-3) or debilitated in some other way (4). From 2001 to 2003, a total of 2803 *Candida* spp strains were isolated from various tissues of patients from the University of Campinas Hospital, UNICAMP, Brazil (5). The strains were identified and classified into the following species: *C. albicans*. 

**Key words**

- *Candida albicans*
- spheroplasts
- Mitochondria
- Respiratory chain
- Mitochondrial membrane potential
- Uncoupling protein

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that should be more general than that of UCP1 - the production of heat in brown adipose tissue of hibernating mammals (15). Evidence has been presented that the new UCPs are involved in cell defense against oxidative stress (22-27). They are able to induce mild uncoupling, thus increasing the rate of respiration and decreasing the production of reactive oxygen species (ROS) (24-27).

A UCP was also detected recently in *C. parapsilosis* (28). Therefore, it is of great interest to study *C. albicans* UCP in order to understand its role in the ability of these cells to resist stress. In the present study, we demonstrated that spheroplasts obtained from cultures of *C. albicans* in the middle of their exponential phase of growth possess intact mitochondria able to phosphorylate ADP and an uncoupling protein (CaUCP) homologous to the previously described *C. parapsilosis* UCP (CpUCP) (28).

**Material and Methods**

**Candida albicans**

*C. albicans* ATCC 90028 was obtained from the American Type Culture Collection. It is a quality control strain for antifungal susceptibility testing and was maintained in sterile water and Sabouraud Dextrose Agar (BD, Difco, Franklin Lakes, NJ, USA). The cell cultures were grown in YEPG medium (29) at 37°C and 200 rpm. To determine the inflexion point of exponential growth, samples were collected at 1-h intervals and culture density was determined spectrophotometrically at 530 nm. Experimental points were fitted using a logistic dose-response model and the maximum growth rate during the exponential phase was calculated as its power parameter.

**Spheroplast preparation**

One liter of culture was harvested by
centrifugation for the standard preparation (29) and cells were washed once with cold water and once with buffer A (1 M sorbitol, 10 mM MgCl₂, and 50 mM Tris-HCl, pH 7.8). Cells were resuspended in buffer A (3 ml/g of cells) supplemented with 30 mM dithiothreitol. After 15 min at room temperature with shaking, cells were harvested, resuspended in buffer A containing lyticase (1 mg/g of cells) and 1 mM DTT, and incubated at 30°C until about 90% of cells were converted to spheroplasts (~60 min). The reaction was stopped with an equal volume of ice-cold buffer A and spheroplasts were washed twice with buffer A. The protein concentration of the final suspension was determined by the biuret method (30) in the presence of 0.2% deoxycholate.

Isolation of mitochondria

The spheroplasts were resuspended in buffer B₁ (0.6 M mannitol, 1 mM EDTA, 0.5% BSA, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.4). Spheroplasts were broken mechanically using a Dounce homogenizer with a maximum of 10 strokes and cell debris were removed by centrifugation at 1,000 g for 10 min. Mitochondria were pelleted by 10-min centrifugation at 10,500 g and washed with buffer B₂ (0.6 M mannitol, 1 mM EDTA, 1% BSA, 10 mM Tris-HCl, pH 7.0). The last wash was made in buffer B₂ medium without BSA and EDTA (28) and mitochondrial protein concentration was determined by the biuret method (30).

Mitochondrial membrane potential

The mitochondrial membrane potential (Δψ) of permeabilized cells was monitored by measuring the fluorescence spectrum of safranine O with a Hitachi F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at excitation-emission wavelength of 495-586 nm (31). All experiments were performed at 28°C in 2 ml of standard incubation medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM KH₂PO₄, and 1 mM MgCl₂, plus 1 mg mitochondrial protein.

Oxygen uptake

Oxygen uptake was measured polarographically at 28°C using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in 1.3 ml of standard incubation medium containing 1 mg mitochondrial protein (28).

Immunodetection of Candida albicans UCP

Fifty micrograms of total proteins extracted from spheroplasts prepared from cultures of C. albicans and C. parapsilosis (positive control) was separated on 10% polyacrylamide gel by standard SDS-PAGE. Protein bands were transferred to a nylon membrane (Hybond N) with a semi-dry blotting apparatus (Amersham Biosciences AB (Pharmacia) Uppsala, Sweden). The membrane was blocked overnight in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 10% (w/v) non-fat dry milk and incubated with the anti-AtPUMP1 polyclonal antibody (1:1000 dilution). After incubation with an anti-rabbit IgG alkaline phosphatase conjugate (1:5000 dilution), the membrane was developed in the dark in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 12.5 µM CSPD for 5 min. The bands were visualized by autoradiography and scanned with the Eagle-Eye photo documentation system (Eagle Eye Photo, Homer, AK, USA) (32).

Chemicals

ATP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, succinate, ethylene ethylenediaminetetraacetic acid, antimycin A, myxothiazol, carboxyatractyloside (CAT), BSA, linoleic acid, KCN, N,N,N',N’-tetramethyl-p-phenylenediamine dihydrochloride
In order to search for the presence of UCP and to examine its activity in these mitochondria we characterized the classical respiratory chain and its ability to sustain ∆ψ by oxidizing different respiratory substrates. Figure 1 shows that spheroplasts prepared from cell cultures grown to the middle exponential phase were permeable to mitochondrial substrates and inhibitors without affecting the functional integrity of the spheroplast mitochondria. These spheroplast mitochondria suspended in situ in reaction medium containing a cocktail of NAD-linked substrates generated and sustained a stable membrane potential and respiration rate of 43 nmol O₂ min⁻¹ mg protein⁻¹ (Figure 1). The subsequent inhibition of complex I by 4 µM rotenone resulted in a decrease of about 30% in the respiration rate (29 nmol O₂ min⁻¹ mg protein⁻¹) and ∆ψ. This suggests a significant contribution of this complex to ∆ψ generation, in agreement with the results obtained by Helmerhorst et al. (13). They found the presence of a rotenone-sensitive and a proton pumping NADH-Q oxidoreductase similar to complex I in C. albicans ATCC 10231. The addition of 5 mM succinate restored ∆ψ but total oxygen consumption level was unchanged (29 nmol O₂ min⁻¹ mg protein⁻¹). The increase in ∆ψ by succinate addition was certainly the consequence of an increased electron flux through the classic respiratory pathway (coupled respiration - the only one that increases ΔµH+) associated with some alteration in the partitioning of electrons among the three respiratory pathways (classic, parallel, and via alternative oxidase) present in Candida yeast (13), resulting in a decrease in electron flux through the uncoupled pathways. Therefore, the total respiration rate was not modified by the addition of succinate. Blockage of complex III by 2 µM antimycin A or 2 µM myxothiazol promoted collapse of ∆ψ and a reduction of more than 90% in respiration (4 nmol O₂ min⁻¹ mg protein⁻¹). Addition of 100 µM UCP, and lyticase were purchased from Sigma (St. Louis, MO, USA). Disodium 3-(4-methoxyspiro[1,2-dioethane-3,2’-(5’-chloro) tricyclo [3.3.1.13,7) decane]-4-yl] phenylphosphate (CSPD) was from Tropix (Applied Biosystems (Tropix), Foster City, CA, USA). All other reagents were of analytical grade.

Results and Discussion

In the present study we characterized mitochondrial bioenergetic pathways in C. albicans ATCC 90028 using a method previously applied to C. parapsilosis (29).
TMPD/1 mM ascorbate restored the respiration rate (28 nmol O₂ min⁻¹ mg protein⁻¹). Finally, inhibition of complex IV by 1 mM KCN was accompanied by a complete loss of Δψ and by a decrease of about 85% in respiration rate (6 nmol O₂ min⁻¹ mg protein⁻¹).

Drastic changes in Δψ after inhibition of complexes III and IV suggest the essential role of these complexes in energy conservation by C. albicans mitochondria. The CN⁻-insensitive respiration was inhibited by 2 mM benzohydroxamic acid, suggesting the presence of alternative oxidases, as previously described (8,13). In contrast, Helmerhorst et al. (13) reported that respiration by C. albicans ATCC 10231 cells was almost insensitive to 5 mM SHAM when grown in the absence of antimycin A. On the other hand, respiration by cells grown in the presence of 10 µM antimycin A was completely inhibited by 5 mM SHAM, indicating that the alternative pathway was the only pathway utilized by C. albicans mitochondria when the classical cytochrome pathway was inhibited at the cytochrome c oxidase level.

An uncoupling protein, another energy-dissipating system that can also modulate Δψ and the respiration rate, was recently identified in the fungus kingdom as CpUCP from C. parapsilosis (28). Interestingly, UCP is absent in Saccharomyces cerevisiae (33). In the present study, the existence of a CpUCP homologue (CaUCP) was also detected in C. albicans mitochondria by immunoblot analysis using anti-AtPUMP1 antibody (32) and CpUCP (line Cp) as a positive control. The immunoblot revealed 2 protein bands of about 32 kDa (Figure 2, lane Ca) in a sample of total protein extract from C. albicans cells. The existence of two bands in C. albicans suggests the presence of more than one UCP, as observed in mammals and plants (17,18,34,35).

Uncoupling proteins are known to be activated by free fatty acids (FFAs) and inhibited by ATP (36-39). Accordingly, in situ Δψ measurements in C. albicans mitochondria demonstrated an increase of Δψ after consecutive additions of 5 mM ATP and 0.5% BSA, which binds non-covalently FFAs (Figure 3A, trace b) as compared to control experiments without additions (trace a). The dotted line (c) shows the Δψ generated by C. albicans mitochondria in the presence of ATP and BSA from the beginning of the experiment. In contrast, the addition of linoleic acid caused a decrease in Δψ that was reversed by BSA (Figure 3B, trace e), reaching levels higher than control at the end (trace d), probably because BSA also binds the endogenous FFAs. These experiments were performed in the presence of 1 µM CAT to prevent the contribution of the ADP/ATP carrier to the FFA-induced H⁺ re-uptake through FFA anion translocation (23). Indeed, it is known that the ADP/ATP carrier in the absence of its substrates ADP and ATP can translocate FFA (23). In addition, the presence of glutamate, pyruvate, and malate/α-ketoglutarate prevented fatty acid anion transport through the corresponding carriers (23).

Accordingly, in experiments with isolated C. albicans mitochondria, the ADP/O
Figure 4. Effect of linoleic acid (LA) on oxidative phosphorylation. Candida albicans mitochondria (1 mg/ml) were added (as indicated by the arrow at “Mito”) to 1.3 ml standard incubation medium (28°C) with 5 mM substrate cocktail (malate, glutamate, pyruvate, and α-ketoglutarate) and 200 µM ADP in the absence or in the presence of 2 µM LA (+LA). The numbers on the traces indicate O2 consumption rates in nmol O2 min⁻¹ mg protein⁻¹. RC = respiratory control; ADP/O = ADP/O ratio.

Figure 5. ADP phosphorylation by Candida albicans spheroplast mitochondria. Spheroplasts (1.0 mg/ml) were incubated in reaction medium containing 5 mM substrate cocktail (malate, glutamate, pyruvate, and α-ketoglutarate), 0.05% BSA, and 5 µM safranine O. Spheroplasts (cells), 200 µM ADP, and 1 µM FCCP were added where indicated by the arrows. Dotted line represents a control experiment without ADP addition. FCCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

The observed uncoupling of oxidase phosphorylation caused by linoleic acid can be attributed to CaUCP. Figure 5 shows that, in the presence of ATP and BSA, C. albicans mitochondria can efficiently phosphorylate ADP in situ, as indicated by the transient ΔΨ decrease induced by the addition of ADP.

The presence of uncoupling protein in Candida ssp implies a role of this protein in yeast mitochondrial energy metabolism and raises the possibility of its involvement in cell protection against ROS overproduction. This protective role against ROS has been described for UCP2 (22) and UCP3 (25). Mitochondria from underexpressing mice had significantly higher levels of oxidative damage than wild-type controls (26). In plants, leaf discs of transgenic tobacco plants overexpressing AtPUMP1 showed an increase in the tolerance to oxidative stress promoted by exogenous hydrogen peroxide compared to wild-type control plants (24).

The presence in C. albicans of an alternative electron transfer chain (13) and alternative oxidase (39) absent in animal cells offers exceptional targets for the design of new chemotherapeutic agents. Blockage of these respiratory pathways and/or inhibition of the uncoupling protein (another target for drug design) could lead to mitochondrial dysfunction, increased production of ROS, and possibly to cell death.

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