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ORIGINAL PAPER

Inhibition of specific electron transport pathways leads to oxidative stress and decreased *Candida albicans* proliferation

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Abstract *Candida parapsilosis* mitochondria contain three respiratory chains: the classical respiratory chain (CRC), a secondary parallel chain (PAR) and an "alternative" oxidative pathway (AOX). We report here the existence of similar pathways in *C. albicans*. To observe the capacity of each pathway to sustain yeast growth, *C. albicans* cells were cultured in the presence of inhibitors of these pathways. Antimycin A and KCN totally abrogated yeast growth, while rotenone did not prevent proliferation. Furthermore, rotenone promoted only partial respiratory inhibition. Lower concentrations of KCN that promote partial inhibition of respiration did not inhibit yeast growth, while partial inhibition of respiration with antimycin A did. Similarly, AOX inhibitor BHAM decreased O_2 consumption slightly but completely stunted cell growth. Reactive oxygen species production and oxidized glutathione levels were enhanced in cells treated with antimycin A or BHAM, but not rotenone or KCN. These findings suggest that oxidative stress prevents *C. albicans* growth.

Keywords *Candida albicans* · Mitochondria · Alternative oxidase · Complex III · Coenzyme Q · Growth curve

Abbreviations AA: Antimycin A · AOX: Alternative oxidase· BHAM: Benzhydroxamic acid· CRC: Classical respiratory chain· DCF: Dicholofluorescein· DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid)· GSH: Reduced glutathione· GSSG: Oxidized glutathione. H_2DCF, 2: 7-dichlorodihydrofluorescein· PAR: Parallel respiratory chain· ROS: Reactive oxygen species· SHAM : Salicyl hydroxamic acid

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Introduction

The *Candida* genus is the main cause of fungal infections in humans (Segal, 2005), with *C. albicans* strains accounting for more than 50% of these infections. The toxicity of available antifungal agents and emergence of drug resistance are major disadvantages of current antifungal therapies (Ghannoum and Rice, 1999). Thus, development of new drugs and biological targets for their action is required. Since energy metabolism and redox state are potential targets for these drugs, it is important to study the organization of *C. albicans* respiratory chains and determine the effect of different electron transport pathways on reactive oxygen species (ROS) generation and cell viability.

While the organization of the vertebrate respiratory chain is well documented and electron transport follows a linear sequence, fungal respiration exhibits peculiar features and involves more complex and flexible pathways not yet fully elucidated (Guérin and Camougrand, 1994; Milani et al., 2001; see a schematic representation in Fig. 1). *C. parapsilosis* respiration is probably the best understood (Guerin and Camougrand, 1986; Guerin et al., 1989; Guerin and Camougrand, 1994; Milani et al., 2001). In this species, electron transport is similar, in several aspects, to plants. In both plants and *C. parapsilosis*, additional NADH dehydrogenases (internal and external, facing the mitochondrial matrix and intermembrane space) act in parallel with

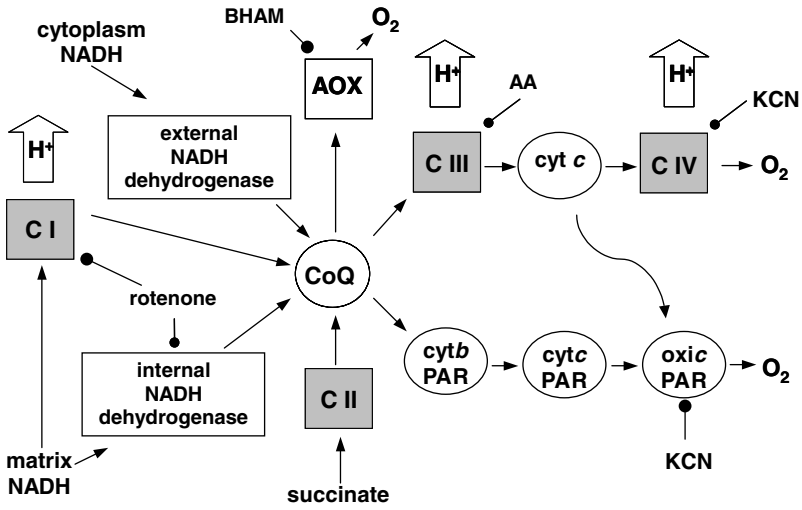

Fig. 1 Schematic representation of *C. albicans* electron transport. Electrons from NADH are transferred to proton-pumping complex I (C I) or external and internal NADH dehydrogenases. Electrons are then fed to coenzyme Q (CoQ), which can also be reduced by succinate-derived electrons channelled through complex II (C II). Electrons collected by CoQ can be directed to O_2 through AOX, PAR or the proton-pumping CRC, containing complex III (C III) cytochrome c (cyt c) and complex IV (C IV). Rotenone inhibits complex I and the internal NADH dehydrogenase. BHAM inhibits AOX, while AA and KCN inhibit complexes III and IV, respectively. KCN also inhibits the terminal PAR oxidase.

complex I (Guerin et al., 1989). These dehydrogenases do not translocate protons (Moller et al., 1993) and are only partially sensitive to rotenone. Both plants and fungi also present AOX, which branches electron transport at the level of coenzyme Q, reducing oxygen to water and avoiding transmembrane proton transport (Guerin and Camougrand, 1986; Siedow et al., 1995; Affourtit et al., 2001). AOX activity can be assessed using hydroxamic acids such as BHAM and SHAM as inhibitors (Guerin and Camougrand, 1986; Siedow et al., 1995; Affourtit et al., 2001). In addition to energy dissipation promoted by NADH dehydrogenases and AOX, both plants and *Candida* ssp present uncoupling proteins, which dissipate the electrochemical gradient, promoting higher respiratory rates (Vercesi et al., 1995; Jarmuskiewicz et al., 2000; Cavalheiro et al., 2004).

In contrast to plants, Guérin et al. (1989) observed that the oxidation of exogenous substrates such as NADH and NADPH by *C. parapsilosis* mitochondria occurs in part through a pathway distinct from the classical respiratory chain (CRC) or AOX, later characterized as a parallel electron transport chain (PAR, see Fig. 1). PAR is inhibited by amital and high concentrations of myxothiazol and KCN, but not antimycin A (Camougrand et al., 1986; Guérin, et al., 1989). Guérin and Camougrand (1994) propose a respiratory chain model in which PAR functions in parallel to CRC, allowing electron flux to be deviated upstream of complex III.

Milani et al. (2001) quantified the contribution of each of the three terminal oxidases and four routes for oxygen reduction in *C. parapsilosis* mitochondria using ADP/O ratios. They verified that CRC capacity is twice as large as AOX. On the other hand, PAR is only efficient when both CRC and AOX are blocked, leading to intense ubiquinone reduction. Indeed, PAR capacity is only one tenth of the maximal oxygen consumption rate. Interestingly, these authors found that electrons from cytochrome c in the CRC can be transferred to PAR's terminal oxidase, making electron transport even more branched and flexible in these cells. Reducing pathways are not additive, since their sum is larger than overall respiration, and the engagement of each electron transport component may be progressive according to the redox state of ubiquinone (Milani et al., 2001).

In the present study, we focus on respiratory pathways present in *C. albicans*, due to its importance as a pathogen, and study the role of each pathway to ensure yeast growth, in order to uncover possible targets for antifungal therapy. Our experimental findings suggest that partial inhibition of complex III of the CRC or inhibition of AOX prevent *C. albicans* growth by promoting oxidative stress, uncovering the importance of these pathways for yeast viability.

Materials and methods

Cell cultures and mitochondrial isolation

C. albicans (American Type Culture Collection 90028) were cultured at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bacto peptone and 1% Bacto yeast extract) until the mid-exponential phase. Mitochondria were isolated and purified as described by Milani et al. (2001),

using lyticase to digest the cell wall then homogenizing the suspension and separating mitochondria through differential centrifugation. Protein concentrations were determined using the Biuret method (Gornall et al., 1949).

Cell growth measurements

Cells were incubated at 37°C with 200 rpm shaking in complete liquid medium, in the absence or presence of respiratory chain inhibitors, as indicated in the figures. Culture aliquots were collected each hour and the optical density of the suspension was measured at 530 nm using a Hitachi U-3000 spectrophotometer.

O_2 consumption measurements

Oxygen consumption was measured polarographically using a Clark-type electrode (Hansatech Instruments, Norfolk, England). Mitochondria were incubated in medium (28°C) containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1% bovine serum albumin, 5 mM K-malate, 5 mM K-pyruvate and 5 mMK-α-ketoglutarate. Cells were incubated in complete liquid medium at 37°C. Other additions are indicated in the figures.

Mitochondrial ROS release

ROS production was assessed at 28°C by following the oxidation of 2.7-dichlorodihydrofluorescein (H_2DCF) at excitation and emission wavelengths of 488 nm and 525 nm, respectively, using a F4500 Hitachi Fluorescence Spectrophotometer.

Oxidized glutathione measurements

The levels of oxidized glutathione were measured as described by Demasi et al. (2003). Intracellular glutathione was extracted by lysing the cells in 1 volume of glass beads and 2 volumes of 3.5% sulfosalicylic acid. The suspension was vortexed for 20 min at 4°C and centrifuged in a microcentrifuge. This procedure was repeated twice. Total glutathione and oxidized glutathione (GSSG) were determined by reaction with DTNB in the absence and presence of glutathione reductase and NADPH.

Data analysis

Traces shown are representative data of at least three similar experiments. Averages and standard errors (SEM) were determined from at least 3 repetitions. Differences between groups were considered statistically significant when $p < 0.05$, determined using one-way ANOVA conducted by OriginPro® 7.5.

Results and discussion

Electron transport pathways in *C. albicans* mitochondria

To assess the capacity of electron transport pathways in the *C. albicans* respiratory chain, we measured oxygen consumption in the presence of different respiratory inhibitors. As shown in Fig. 2, mitochondrial respiratory rates sustained by NADH-generating substrates greatly diminished after the addition of 4 μM antimycin A (AA), which blocks Complex III of the CRC but has no effect on PAR (Camougrand et al., 1991). Oxygen consumption was further inhibited by 10 mM KCN, an inhibitor of the terminal cytochrome oxidases of both CRC and PAR. The remaining cyanide-resistant respiration is completely abrogated after the addition of 2 mM BHAM, an AOX inhibitor. These results indicate that, in *C. albicans* mitochondria, oxygen can be reduced by three pathways: CRC, PAR and AOX, as observed previously in *C. parapsilosis* (Guerin and Camougrand, 1986; Guerin and Camougrand, 1994; Milani et al., 2001).

Contribution of electron transport pathways toward yeast proliferation

We investigated the capacity to ensure cellular growth of each electron transport pathway present in *C. albicans*. In

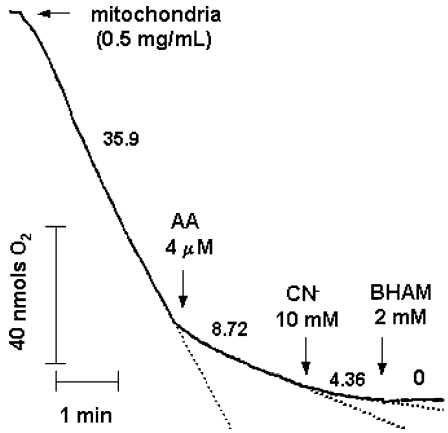

Fig. 2 *C. albicans* respiration includes AA, KCN and BHAM-sensitive pathways. Isolated *C. albicans* mitochondria (0.5 mg/mL) were added to 10 mM K-Hepes, pH 7.2, containing 125 mM sucrose, 65 mM KCl, 1 mM $MgCl_2$, 2.5 mM K_2HPO_4, 1% BSA, 5 mM K-malate, 5 mM K-pyruvate and 5 mMK-α-ketoglutarate. AA (4 μM), 10 mM KCN and 2 mM BHAM were added where indicated by the arrows. Numbers in parenthesis indicate O_2 consumption rates in nmols min^{-1} mg $protein^{-1}$. Dotted lines represent oxygen consumption in the absence of inhibitor addition. The results shown are representative of at least three independent and similar repetitions.

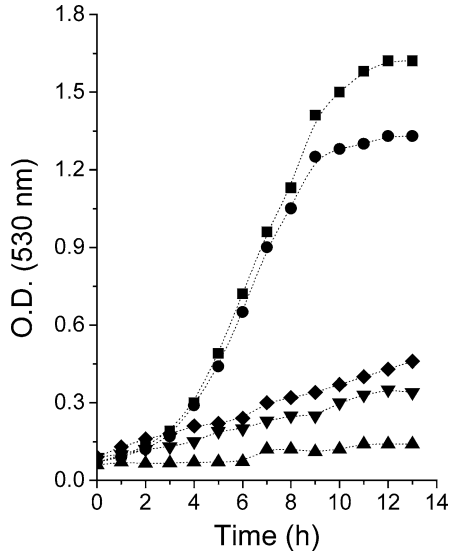

Fig. 3 Respiratory inhibitors limit *C. albicans* growth. *C. albicans* were cultured in complete liquid medium in the absence (control, ■) or presence of 2 mM rotenone (●), 2 mM AA (▲), 1 mM KCN (▼) or 2 mM BHAM (◆) as shown. Cell growth was .monitored by optical density (O.D.) measurements. The results are representative of at least three independent and similar repetitions.

aerobically cultured yeast (Fig. 3), both AA (7) and KCN (B) significantly prevented cell growth when compared to a control experiment (absence of inhibitors, ■), indicating the importance of the CRC for cell survival. The presence of 2 mM BHAM (◆) also inhibited growth, evidencing the importance of AOX in this process.

Surprisingly, the presence of 2 μM (●) rotenone did not completely prevent cellular growth, suggesting that Complex I function is not essential for *C. albicans* proliferation. *C. albicans* are probably able to oxidize cytoplasmatic substrates such as NADH, NADPH and glycerol 3-phosphate through external rotenone-insensitive dehydrogenases (Helmerhorst *et al.*, 2002; Joseph-Horne et al., 2001, see Fig. 1). These NADH dehydrogenases do not promote proton pumping, essential for ATP synthesis, as indicated by lower ADP/O ratios observed using external NADH versus matrix NADH sources (Helmerhorst *et al.*, 2002). The resulting reduction in oxidative phosphorylation may be the reason for the slight, but highly reproducible, decrease in cell proliferation observed in the presence of rotenone (Fig. 3, compare rotenone-containing trace, • to control, ■).

Next, we verified the effect on mitochondrial oxygen consumption of the high doses of respiratory inhibitors used to monitor cell proliferation in Fig. 3. We found that the addition of rotenone to isolated mitochondria was capable of promoting only a partial decrease in O_2 consumption rates (Fig. 4A), resulting in levels approximately 60% of those observed in mitochondria from cells grown in the absence of inhibitors. On the other hand, high doses of AA and KCN reduced O_2 consumption very significantly. The presence of 2 mM BHAM promoted a partial mitochondrial respiratory decrease (approximately 30%), similar to that observed in *C. parapsilosis* (Milani et al., 2001).

It is interesting to note that, although rotenone has a larger effect on mitochondrial respiration than BHAM (Fig. 4A), it affects cell proliferation only slightly, while BHAM strongly prevents cell growth (Fig. 3). This indicates that not overall respiratory rates, but the specific respiratory pathway inhibited, determine cell growth. In order to compare the contribution of each electron transport pathway component equally, we titrated concentrations of each inhibitor in order to obtain O_2 consumption levels similar to those observed in the presence of BHAM (results not shown). In isolated mitochondria, we found that 0.5 μM rotenone, 15 nM AA and 0.8 μM KCN decreased electron transport to levels equal to those observed with saturating BHAM concentrations (Fig. 4A).

We then tested the effect of these inhibitor concentrations on respiration in intact *C. albicans* cells (Fig. 4B). The low doses of rotenone and KCN resulted in decreases in cellular O_2 consumption similar to those observed with BHAM. On the other hand, 15 nM AA inhibited cellular respiration quite significantly, and a lower concentration of 7.5 nM was used to promote respiratory inhibition similar to that observed with the other drugs.

From the values obtained in Fig. 4B, yeasts were cultured in the absence and presence of low concentrations of drugs capable of promoting comparable respiratory inhibitions. Under these conditions, 7.5 nM AA completely inhibited cellular growth (Figs. 5 and 7), evidencing the importance of functional Complex III for *C. albicans* proliferation. As noted previously, BHAM (◆) also prevented growth, demonstrating the role of AOX in this process. On the other hand, inhibition of Complex I, promoted by rotenone (●), or complex IV, promoted by KCN (▼), were not sufficient to inhibit yeast proliferation. Since the inhibition of respiration was partial in all cases, prevention of cell growth cannot be ascribed to the lack of oxidative phosphorylation, which would, in fact, be more efficient in the presence of BHAM (Milani et al., 2001). Instead, we hypothesized that changes in proliferation were related to altered mitochondrial ROS release.

ROS production and increased glutathione oxidation are enhanced by complex III or AOX inhibition

ROS are naturally generated by mitochondrial respiration. An important source of these species is coenzyme Q, since semiquinone radicals formed during its redox cycle can donate an electron to O_2, leading to the production of superoxide radicals and other ROS (see Kowaltowski and Vercesi, 1999, for review). Indeed, we observed that *C. albicans* mitochondria present an increase in steady-state ROS production, measured through DCF fluorescence increments (Rothe and

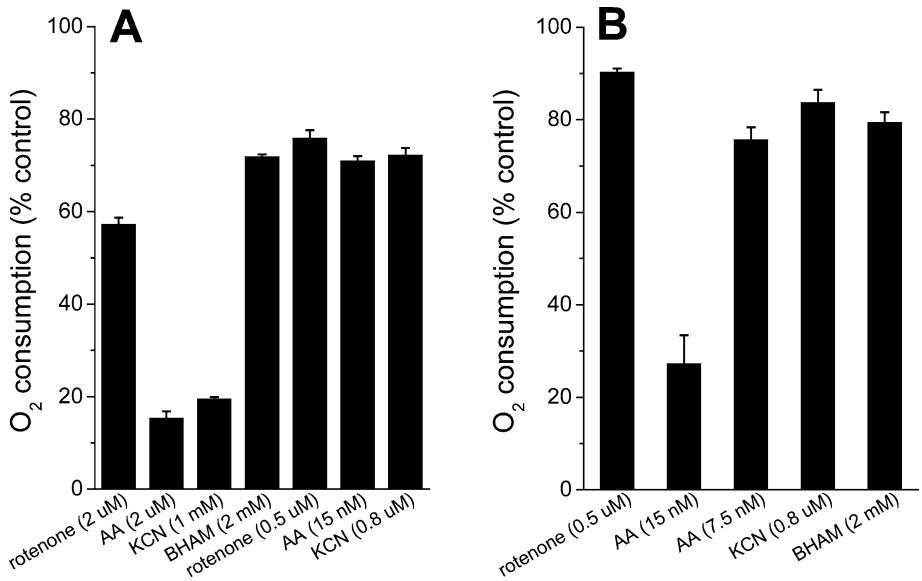

Fig. 4 Respiratory effects of different respiratory inhibitors. O_2 consumption rates were measured in isolated *C. albicans* mitochondria (Panel A) under conditions similar to those described for Fig. 2, in the presence of the respiratory inhibitors at the concentrations indicated. In Panel B, inhibitors were added to intact cells suspended in complete liquid medium, and O_2 consumption was determined after 10 min incubation. The results are averages ± SEM O_2 consumption relative to untreated controls of at least three independent repetitions. All respiratory rates in treated mitochondria and cells were significantly different from control (100%) rates.

Valet, 1990), when treated with AA (Fig. 6A). On the other hand, rotenone and KCN did not significantly enhance DCF oxidation. This indicates that superoxide radical generation from complex I stimulated by the presence of rotenone in *C. albicans* is not as substantial as in some other tissues (Kowaltowski and Vercesi, 1999; Turrens, 2003), probably due to competition between different NADH dehydrogenases. On the other hand, the low doses of KCN used in our experiments probably favoured the inhibition of CRC, but not PAR, which presents smaller sensitivity to KCN (Milani *et al.*, 2001). As a result, electrons from cytochrome c in CRC can still flow to PAR cytochrome c oxidase (see Fig. 1), preventing electron accumulation and leakage leading to ROS formation. Unlike KCN, AA inhibits complex III directly, leading to a more reduced state of coenzyme Q, a major source of superoxide radicals in many tissues (Kowaltowski and Vercesi, 1999). AOX inhibition should present a similar effect, since this enzyme also receives electrons directly from coenzyme Q. Unfortunately, we were not able to measure ROS release in mitochondria incubated in the presence of BHAM or SHAM due a reaction between these compounds and the probe (results not shown).

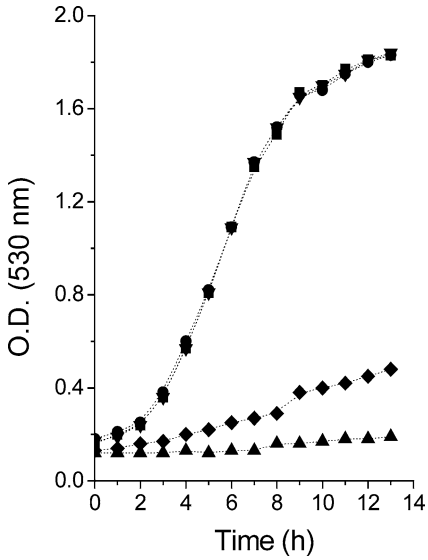

Fig. 5 AOX and partial complex III inhibition prevent *C. albicans* proliferation. *C. albicans* were cultured under conditions similar to Fig. 3 in the absence (control, ■) or presence of 0.5 mM rotenone (●), 7.5 nM AA (▲), 0.8 mM KCN (▼) or 2 mM BHAM (◆), as shown. Cell growth was monitored by O.D. measurements. The results are representative of at least three independent and similar repetitions.

As a second parameter to evaluate redox state in the presence of respiratory inhibitors, we measured oxidized over reduced glutathione levels (GSSG/GSH). This measurement was conducted in cells grown in the presence of respiratory inhibitors, allowing for an evaluation of cellular redox state changes as a result of alterations in mitochondrial ROS release. Fig. 6B shows that cells treated with AA or BHAM presented significant increases in oxidized glutathione levels. Although rotenone or KCN also slightly increased GSSG/GSH levels relative to controls, the effect was not statistically significant.

Taken together, our results indicate that *C. albicans* present branched respiratory chains similar to other *Candida* species. We also found that partial respiratory inhibition at the level of complex III of the CRC or prevention of AOX

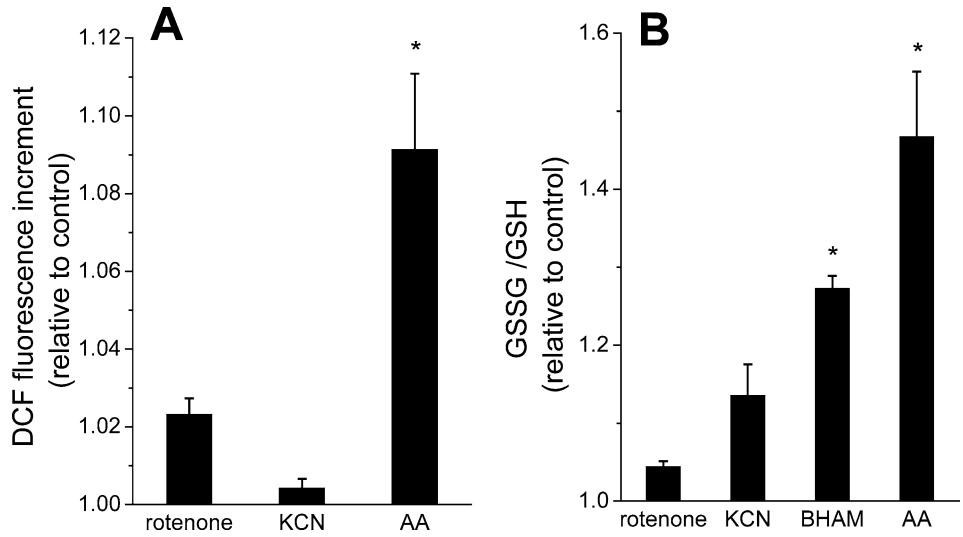

Fig. 6 AOX and complex III inhibition lead to oxidative stress in *C. albicans*. In Panel A, isolated mitochondria were incubated under conditions similar to those in Fig. 2, in the presence of 10 μM H_2DCF and 0.5 μM rotenone, 0.8 μM KCN or 7.5 nM AA. DCF fluorescence increments were determined between 5 and 20 min and normalized to increments in fluorescence of untreated mitochondria. In Panel B, cellular GSSG/total glutathione was determined after 45 min incubation of cells in the presence of 0.5 μM rotenone, 0.8 μM KCN, 7.5 nM AA or 2 mM BHAM, as indicated. Values are means ± SEM of 3 independent experiments, relative to control. $^*p < 0.05$ versus control.

activity leads to oxidative stress and inhibition of cellular proliferation. We hypothesize that oxidative stress occurs under these conditions due to the reduction of coenzyme Q. Indeed, partially reduced coenzyme Q, formed during physiological electron transfer, is a free radical (the semiquinone radical) capable of generating superoxide radicals within the respiratory chain (Kowaltowski and Vercesi, 1999).

Our results suggest that complex III and AOX may be attractive targets for therapeutic interventions designed to control infection by *C. albicans*. AOX is particularly interesting in this sense, since it is not expressed in mammals and can thus be targeted more readily by drugs without large side effects. AOX may have an important role as a regulator for fungal energy metabolism, promoting tricarboxylic acid cycle function even in the presence of high ATP/ADP ratios and substituting the need for a fermentative pathway (Veiga *et al*., 2000). In addition, this enzyme is capable of preventing mitochondrial ROS release, as suggested by our results (Fig. 6) and demonstrated in plant tissue (Popov et al., 1997). Our results indicate that the uniquely branched electron transport chain in C. albicans has a critical role in the maintenance of cellular integrity, and that disruptions in electron flow leading to coenzyme Q reduction are associated with oxidative stress and decreased proliferation. Indeed, the ability to regulate individual complexes and partition electron flux among CRC, PAR and AOX may be the reason for the success of *Candida* ssp as infections agents, allowing these cells to modify their energetic and redox status in order to ensure survival.

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