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Characterization of the mitochondrial respiratory pathways in *Candida albicans*

Eva J. Helmerhorst[a,b,*], Michael P. Murphy[b], Robert F. Troxler[a], Frank G. Oppenheim[a]

[a] *Goldman School of Dental Medicine, Boston University, 100 East Newton Street, Boston, MA 02118, USA*
[b] *MRC, Dunn Human Nutrition Unit, Hills Road, Cambridge, CB2 2XY, United Kingdom*

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Abstract

Candida albicans is an opportunistic oral pathogen. The flexibility of this microorganism in response to environmental changes includes the expression of a cyanide-resistant alternative respiratory pathway. In the present study, we characterized both conventional and alternative respiratory pathways and determined their ADP/O ratios, inhibitor sensitivity profiles and the impact of the utilization of either pathway on susceptibility to commonly used antimycotics. Oxygen consumption by isolated mitochondria using NADH or malate/pyruvate as respiratory substrates indicated that *C. albicans* cells express both cytoplasmic and matrix NADH–ubiquinone oxidoreductase activities. The ADP/O ratio was higher for malate/pyruvate (2.2±0.1), which generate NADH in the matrix, than for externally added NADH (1.4±0.2). In addition, malate/pyruvate respiration was rotenone-sensitive, and an enzyme activity assay further confirmed that *C. albicans* cells express Complex I activity. Cells grown in the presence of antimycin A expressed the cyanide-insensitive respiratory pathway. Determination of the respiratory control ratio (RCR) and ADP/O ratios of mitochondria from these cells indicated that electron transport from ubiquinone to oxygen via the alternative respiratory pathway was not coupled to ATP production; however, an ADP/O ratio of 0.8 was found for substrates that donate electrons at Complex I. Comparison of antifungal susceptibility of *C. albicans* cells respiring via the conventional or alternative respiratory pathways showed that respiration via the alternative pathway does not reduce the susceptibility of cells to a series of clinically employed antimycotics (using Fungitest®), or to the naturally occurring human salivary antifungal peptide, histatin 5.

Keywords: *Candida albicans*; Respiratory pathway; Mitochondrion

1. Introduction

ATP formation in respiring organisms is coupled to electron flow from reduced substrates through the respiratory chain to a terminal electron acceptor. In vertebrates, this pathway is cyanide-sensitive and involves NADH–ubiquinone (Q) oxidoreductase (Complex I), cytochrome bc_1 (Complex III) and cytochrome oxidase (Complex IV) as proton translocating oxidoreductases [1]. While in vertebrates, the respiratory chain complexes and the pathway of electron transport appear to be highly conserved, those of plants and fungi are more complex and flexible in nature [2]. Extensive research, particularly in the area of plant respiration, has led to the characterization of some of these alternative systems that include alternative NADH-Q oxidoreductases and alternative terminal oxidases (AOX) [3–5].

In mammals, the only known NADH-Q oxidoreductase is Complex I [6]. The active center of this complex is facing the mitochondrial matrix and is composed of at least 43 distinct subunits [6,7], at least five iron–sulfur clusters and a noncovalently bound FMN molecule [8]. In contrast to mammals, plants, fungi and bacteria contain other types of NADH-Q oxidoreductases that are distinct from Complex I [4]. Alternative NADH-Q oxidoreductases catalyze the same redox reaction as Complex I, but do not contribute to the generation of a transmembrane proton gradient and are rotenone-insensitive. In some microorganisms such as *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, Complex I is absent [9,10] and three alternative mitochondrial NADH-Q oxidoreductases transfer electrons from NADH to ubiquinone [3,4]. One of these faces the matrix

* Corresponding author. Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University, 700 Albany Street, 100 East Newton Street, Boston, MA 02118, USA. Tel.: +1-617-638-4916; fax: +1-617-638-4924.
E-mail address: helmer@bu.edu (E.J. Helmerhorst).

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while the others face the cytoplasm and compensate for the absence of the malate/aspartate shuttle in this microorganism [11]. Unlike Complex I, alternative NADH-Q oxidoreductases, e.g. in *S. cerevisiae*, are single polypeptides of 53 or 58 kDa (external and internal NADH-Q oxidoreductase, respectively) and are devoid of iron–sulfur centers [11].

In addition to alternative NADH-Q oxidoreductases, it has been well established that higher plants, some fungi and protozoa can also express an alternative oxidase (AOX) that is reduced by electrons from the ubiquinol pool [12]. While AOX is biochemically consistent with a terminal oxidase, as it reduces oxygen to water, it is distinguished by its insensitivity to cyanide and antimycin A and its sensitivity to hydroxamic acids such as salicyl hydroxamic acid (SHAM) [3,5]. The nuclear encoded AOX found in most fungi (with the exception of *S. cerevisiae* and *Schizosaccharomyces pombe*) is 32–40 kDa in size, and indirect evidence suggests the presence of two iron centers [5]. While plant AOX differs from fungal AOX in its structure and allosteric control, both are induced by respiratory chain inhibitors that act downstream from coenzyme Q, or by oxidative stress, suggesting a universal role for AOX in plant and fungi in the protection against oxidative damage [13–18].

Research into fungal alternative respiration has been limited compared to plant alternative respiration, not only in terms of function but also in terms of identification of the respiratory chain components and their contributions to ATP formation. One species that has not been much investigated in this respect but is of considerable clinical importance is *Candida albicans*. *C. albicans* is a common resident of the human gastrointestinal tract that causes infections in immunocompromised patients [19]. It has long been known that alternative cyanide-insensitive respiration can be induced in *Candida* species [20–22], including *C. albicans*, upon ageing of cells [23] or upon exposure of cells to antimycin A or sodium cyanide [24]. Recently, Huh et al. [25,26] demonstrated that AOX in *C. albicans* is encoded for by two, tandemly arranged genes, designated *AOX1a* and *AOX1b*, encoding polypeptides of 379 and 365 amino acids, respectively. While *AOX1a* is constitutively expressed in low amounts, *AOX1b* expression is dependent on growth phase and can be induced by cytochrome oxidase inhibitors [26]. Only double knockout mutants of *AOX1b* are incapable of cyanide-insensitive respiration, indicating that this gene product confers cyanide-insensitive respiration in *C. albicans* [26].

Even though cyanide-insensitive respiration in *C. albicans* has been established and the genes responsible for the expression of the component(s) of this pathway have been identified, physiological characterization of the conventional and alternative respiratory pathways on a mitochondrial level is sparse [24,27]. In the present study, we applied conditions where either the conventional or the alternative pathway was active in *C. albicans* and used a combination of substrates, inhibitors and enzyme activity assays to investigate the contribution of the various redox systems to respiration and ATP formation. In addition, since expression of the alternative pathway occurs not only in response to antibiotics but also upon ageing of cells, conditions that are relevant in sites of *Candida* infection, the potential clinical implication of AOX induction in *C. albicans* was investigated by comparing antifungal susceptibility and germ tube formation in cells expressing either pathway.

2. Materials and methods

2.1. C. albicans growth conditions and spheroplast formation

C. albicans (ATCC 10231) was grown from Sabouraud dextrose agar (Difco, Detroit, MI) into Sabouraud dextrose broth (Difco)±10 μM antimycin A (Sigma, St. Louis, MO). After 16 h incubation, cells were subcultured 1:400 and early log phase cells were harvested 5 h later by centrifugation. Stationary phase cells were collected after 48 h incubation in Sabouraud dextrose broth. The pellets were washed in milliQ water and suspended in 1 ml buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 1.4 M sorbitol) per gram yeast pellet and treated with the β-1-3 glucanase zymolyase 100 T (Seikagaku America, Falmouth, MA) to form spheroplasts [28].

2.2. Isolation of mitochondria

Mitochondria were isolated from *C. albicans* spheroplasts as described previously [28]. In brief, *C. albicans* spheroplasts were collected by centrifugation (1000×*g* at 4 °C) and homogenized for 5 min on ice in 0.4 M sorbitol, 0.2% (w/v) bovine serum albumin (BSA; fraction V, Sigma) and 10 mM imidazole (pH 6.4) (Fisher, Fair Lawn, NJ), using a manual Potter-Elvehjem homogenizer. The homogenate was diluted with 1 vol. 1 M sorbitol, 25 mM KH_2PO_4, 4 mM EGTA (Sigma), 0.2% (w/v) BSA, and 10 mM imidazole (pH 6.4) and debris was pelleted by centrifugation (5 min at 1000×*g* at 4 °C). The supernatant was removed and mitochondria were pelleted by centrifugation (10 min at 12,000×*g* at 4 °C). The reddish pellet containing the mitochondria was suspended in 0.6 M mannitol, 2 mM EGTA, 0.2% (w/v) BSA, and 10 mM imidazole (pH 6.4), to an OD_{620} of ~15, and kept on ice. Protein concentrations in mitochondrial preparations were determined using the bicinchoninic acid (BCA) method with BSA as a standard [29]. *S. cerevisiae* mitochondria were isolated from wild type strain CEN.PK2-1C (MATa. Ura3, his3, leu2, trp1) [30] as described [31].

2.3. Measurement of oxygen consumption

Oxygen consumption was measured using a biological oxygen monitor model 5300 equipped with a 5331 standard oxygen probe (Yellow Springs Instruments, Ohio, USA) or

a Rank Brothers oxygen electrode and vessel (Rank Brothers, Bottisham, Cambridge, England). All experiments were performed at 30 °C in air-saturated buffer. Blastoconidia were suspended in 1 mM potassium phosphate buffer (pH 7.0) to an OD_{620} of 1.7±0.2. Spheroplasts were suspended in 1.2 M sorbitol, or 1.2 M sorbitol containing Sabouraud dextrose broth (30 g/l), to an OD_{620} of 1.7±0.2. Mitochondria were suspended in respiration buffer (0.65 M mannitol, 2 mM $MgCl_2$, 16 mM KH_2PO_4, 10 mM imidazole (pH 6.4)) to an OD_{620} of 0.15±0.05. This buffer does not contain BSA, and therefore AOX activity might be slightly underestimated due to inhibition of AOX by free fatty acids [32]. State 2, State 3 and State 4 respiratory rates were determined as described previously [2], using either 1 mM NADH (Sigma), 10 mM potassium succinate (Sigma) or a mixture of 2 mM malate (Sigma) and 10 mM pyruvate (Sigma), as respiratory substrates. ADP (Sigma) was used at a final concentration of 0.33 mM. For ADP/O ratio determination, the ADP concentration was determined from the absorbance at 259 nm (ε=15.4×10^3 M^{-1} cm^{-1}) and the consumed nanomoles of oxygen were determined based on the solubility of oxygen in buffered mitochondrial medium at 30 °C of 445 nmol O per milliliter [33]. The respiratory rates before addition of ADP (State 2), during phosphorylation (State 3) and after depletion of ADP (State 4) were used to calculate the ACR (State 3/State 2), the RCR (State 3/State 4) and ADP/O ratio.

2.4. NADH dehydrogenase activity assay

Mitochondria from *C. albicans* or *S. cerevisiae* (10 and 35 mg protein/ml, respectively) were freeze-thawed once, collected by centrifugation (16,000×*g* for 5 min at 25 °C) and diluted 10-fold in cold milliQ water. Mitochondria were homogenized (3×20 s at 13,000 rpm/min on ice) in an Ultra-turrax T 25 basic blender (IKA-Werke, Staufen, Germany) to disrupt the membranes. Disrupted mitochondrial preparations were collected by centrifugation (5 min at 16,000×*g* at 25 °C) and suspended in 0.6 M mannitol, 2 mM EGTA, 0.2% (w/v) BSA, and 10 mM imidazole (pH 6.4) to approximately 5 mg protein/ml. NADH dehydrogenase activity in *C. albicans* or *S. cerevisiae* mitochondrial preparations (final concentrations 77 and 11 μg protein/ml, respectively) was determined by measurement of NADH oxidation at 340 nm (ε=6.22×10^3 M^{-1} cm^{-1}) in buffer containing 250 mM sucrose (BDH laboratory supplies, England), 1 mM EDTA (BDH), 50 mM Tris–HCl (Sigma), pH 7.4, 300 nM antimycin A (Sigma), 2 mM KCN (Fluka Chemie, Buchs, Switzerland), 0.15 mM Coenzyme Q_1 (Sigma), and 0.1 mM NADH (Sigma). Rotenone (Sigma) was used at a final concentration of 10 μg/ml.

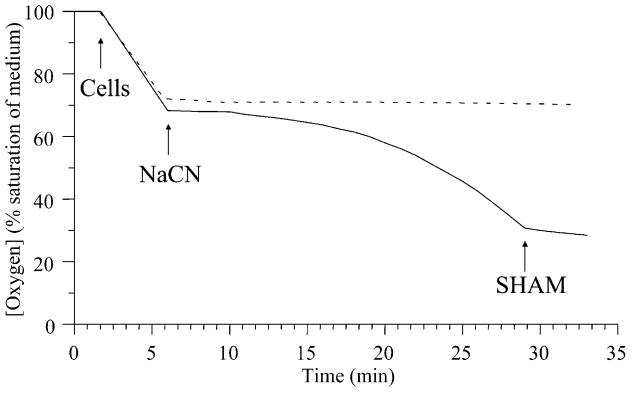

Fig. 1. Induction of the alternative respiratory pathway in *C. albicans*. Oxygen consumption was measured with an oxygen electrode at 30 °C. Spheroplasts from log phase *C. albicans* cells were suspended in 1.2 M sorbitol (nutrient-free buffer, dashed line) or in 1.2 M sorbitol containing Sabouraud dextrose broth (30 g/l, nutrient-rich buffer, solid line), final OD_{620} 1.7, sodium cyanide (0.66 mM) or SHAM (5 mM) were added where indicated.

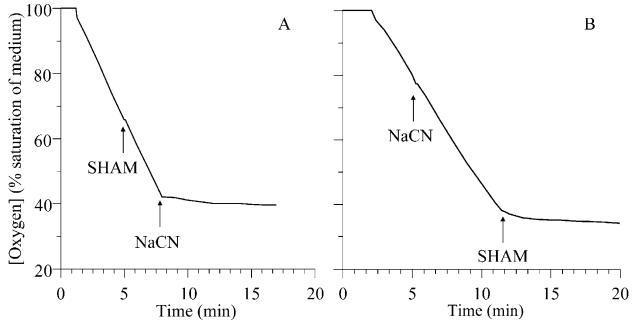

Fig. 2. Respiration by log phase *C. albicans* cells grown in the absence (A) or presence (B) of antimycin A. *C. albicans* were grown to log phase in Sabouraud dextrose broth±antimycin A (10 μM). Oxygen consumption of cells suspended in 1 mM potassium phosphate (pH 7.0), OD_{620} 1.7, was measured with an oxygen electrode at 30 °C. Where indicated, sodium cyanide (0.66 mM) and SHAM (5 mM) were added.

2.5. Antifungal susceptibility testing

C. albicans cells were grown directly from the ATCC stock (10231) onto Sabouraud dextrose agar (Difco) for 48 h at 30 °C. Cells were cultured in triplicate in 100 ml Sabouraud dextrose ±10 μM antimycin with each culture derived from a different colony. After 16-h incubation, each culture was subcultured 1:400 in 100 ml Sabouraud Dextrose Broth, and incubated for 5 h until the cells reached the logarithmic growth phase. Cells were then collected by centrifugation (5 min at 6000×*g* at 4 °C) and suspended in milliQ water to an OD_{620} of 0.47 (which approximates 2.5 McFarland units). Antifungal susceptibility was assessed with Fungitest® (BioRad, Marnes La Coquette, France) according to the manufacturer's instructions, but with a slightly higher inoculum density as proposed by Davey et al. [34]. Fungitest® includes six antifungal agents, each at two concentrations: amphotericin B (2 and 8 μg/ml), 5-fluorocytosine (2 and 32 μg/ml), fluconazole (8 and 64 μg/ml), itraconazole and ketoconazole (0.5 and 4 μg/ml) and miconazole (0.5 and 8 μg/ml). The sensitivity of the cells to the oral antifungal peptide, histatin 5 [35] was investigated

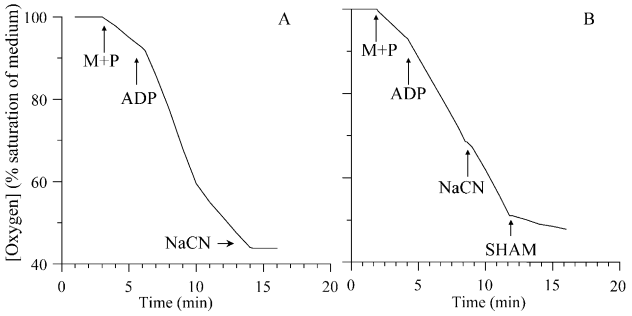

Fig. 3. Respiratory activity of mitochondria isolated from *C. albicans* grown in the absence (A) or presence (B) of antimycin A. Mitochondria were suspended in respiration buffer (OD_{620} 0.15) and oxygen consumption was measured by an oxygen electrode at 30 °C. Where indicated, malate/pyruvate (M+P, 2 and 10 mM, respectively), ADP (0.33 mM), or SHAM (5 mM) were added.

in a killing assay essentially as previously described [36]. In brief, log phase cells grown in Sabouraud dextrose broth ±10 μM antimycin A were washed in milliQ water, suspended in 1 mM potassium phosphate buffer (pH 7.0), and exposed to various concentrations of histatin 5 (American Peptide Company, Sunnyvale, CA) for 1.5 h at 37 °C. Cell viability was assessed by colony formation on Sabouraud Dextrose Agar after 48 h incubation at 30 °C.

2.6. Induction of germ tube formation

In Sabouraud dextrose broth, *C. albicans* grow as blastoconidia. To induce the formation of germ tubes, logarithmic phase *C. albicans* blastoconidia, expressing the conventional or the alternative respiratory pathway, were suspended in horse serum (Sigma) for 3 h at 37 °C. Germination was assessed qualitatively by light microscopy.

3. Results

3.1. Induction of the alternative respiratory pathway under non-growth conditions

The alternative respiratory chain in *C. albicans* is induced by inhibitors such as antimycin A and cyanide, which inhibit cytochromes bc_1 and aa_3, respectively [24]. To verify the inducibility of this pathway in the ATCC 10231 *C. albicans* strain, blastoconidia or spheroplasts were suspended in nutrient-free buffer, or in nutrient-rich buffer (Fig. 1). In both nutrient-free and nutrient-rich buffers, *C. albicans* spheroplasts respired at similar rates, indicative of respiration on intracellular energy stores. After 5-min incubation, cyanide, an inhibitor of cytochrome oxidase, was added, which abolished respiration in both buffers. In the nutrient-rich buffer, the alternative, cyanide-insensitive respiratory chain was induced within 10–20 min, and respiratory rates 50±6% (n=4) of the original respiratory rate were reestablished. This respiration was inhibited by salicylhydroxamic acid (SHAM), a specific inhibitor of the alternative respiratory pathway [37]. The alternative pathway could not be induced in nutrient-free medium.

3.2. Induction of the alternative respiratory pathway under growth conditions

To investigate further the alternative respiratory pathway, cells were grown to log phase in the presence of 10 μM antimycin A and compared with control cells grown in the absence of antimycin A (Fig. 2). The respiratory rates of log phase cells grown with or without antimycin A were comparable (27±7.0 and 33±1.0 nmol O/min/OD_{620}, respectively). However, the percent cyanide sensitivities were quite different, 1.7±2.5% and 99±1% inhibition, respectively. Respiration by control cells was insensitive to SHAM (Fig. 2A) while respiration by cells grown in the presence of antimycin was almost completely inhibited by SHAM (Fig. 2B). The insensitivity of the antimycin-grown cells to cyanide in the absence of SHAM indicated that the alternative pathway is the only pathway utilized. Likewise, it can be concluded from results in Fig. 2A that log phase cells grown in the absence of cyanide only respire through the cytochrome oxidase pathway. In contrast to log phase control cells, the respiration of stationary phase control cells was largely resistant to cyanide (11.6±0.9% inhibition of respiration by cyanide). These findings are in accordance with previous reports showing that ageing of *C. albicans* cultures induces alternative respiration [23] and that under these conditions, both respiratory pathways coexist (Ref. [23] and Helmerhorst et al., unpublished observations).

Table 1
ACR, RCR, and ADP/O values of mitochondria respiring through the conventional and alternative respiratory pathway

Conventional pathway				Alternative pathway			
Substrate	ACR[a]	RCR[b]	ADP/O	Substrate	ACR	RCR	ADP/O
NADH	2.1±0.2[c]	2.4±0.2	1.4±0.2	NADH	n.d.[d]	n.d.[d]	0
Succinate	2.4±0.1	1.6±0.1	1.4±0.2	Succinate	n.d.[d]	n.d.[d]	0
Mal+Pyr	3.3±0.5	2.3±0.3	2.2±0.1	Mal+Pyr	1.6±0.2	1.3±0.1	0.8±0.1

[a] ACR, acceptor control ratio, ratio of the respiratory rates in State 3/State 2.
[b] RCR, respiratory control ratio, ratio of the respiratory rates in State 3/State 4.
[c] Values represent the mean±S.D. (n>4).
[d] When no increase in respiration was observed upon addition of ADP, ACR and RCR values did not apply (n.d., not determined).

Table 2
Rotenone sensitivity of mitochondria isolated from log phase and stationary phase *C. albicans* cells

Respiratory substrate	Log phase mitochondria	Stationary phase mitochondria
NADH	108.0±1.6[a]	n.d.
Malate+pyruvate	4.2±3.3	4.1±4.1
Succinate	97.0±5.1	n.d.

[a] Values are expressed as percentage of State 2 respiration, prior to the addition of rotenone, and represent the mean±S.D. of three measurements.

3.3. Properties of mitochondria expressing the conventional or the alternative respiratory pathway

To study the properties of mitochondria in which either the conventional or the alternative respiratory pathway was active, cells were grown to log phase in broth with or without antimycin A. Oxygen consumption by isolated mitochondria was assessed using malate/pyruvate as the respiratory substrates. They generate NADH within the mitochondrial matrix (Fig. 3), which donates electrons to an NADH-Q oxidoreductase facing the matrix. Both cyanide-sensitive (Fig. 3A) and cyanide-resistant (Fig. 3B) mitochondria oxidized malate/pyruvate, and, upon addition of ADP, respiratory control was observed. Both cyanide-sensitive and cyanide-insensitive mitochondria also oxidized NADH, which cannot cross the mitochondrial inner membrane, indicating NADH-Q oxidoreductase activity facing the cytoplasmic side (Table 1). From a series of experiments (n>4), the RCR and ADP/O ratios were determined for cyanide-sensitive and cyanide-resistant mitochondria using various respiratory substrates (Table 1). Typical respiratory rates observed with mitochondria from control cells and antimycin-grown cells were 470 and 170 nmol O/min/mg protein, respectively, when NADH was the substrate. Respiratory rates using NADH, succinate or malate/pyruvate as substrates were generally in the proportion of 2:1:1. Mitochondria from control cells showed significantly higher ACR values with malate/pyruvate than with NADH or succinate. Also, ADP/O values were significantly higher for malate/pyruvate (2.2±0.1) than for NADH (1.4±0.2) or succinate (1.4 ± 0.2). Cyanide-insensitive mitochondria did not exhibit respiratory control (ACR ~ 1) when either NADH or succinate was the substrate. In contrast, with malate/pyruvate, ACR and RCR values were 1.6±0.2 and 1.3±0.1, respectively, and the ADP/O value was 0.8 (Table 1), showing that phosphorylation occurs under these conditions.

3.4. Complex I activity in C. albicans mitochondria

The ADP/O ratios represented in Table 1 indicate that in both cyanide-sensitive and cyanide-resistant *C. albicans* mitochondria oxidation of malate/pyruvate forms more ATP than oxidation of external NADH, suggestive of Complex I proton pumping. This was supported by the observation that malate/pyruvate respiration was almost completely inhibited by rotenone, whereas respiration on external NADH or succinate was insensitive to rotenone (Table 2). Similarly, mitochondria from cells grown in the presence of antimycin A were rotenone sensitive (data not shown). To assess whether Complex I activity was growth phase dependent, as is the case in the related species *C. utilis* [38,39], mitochondria were also isolated from stationary phase *C. albicans* cells grown in the absence of antimycin A. While mitochondria isolated from stationary phase *C. albicans*, like intact stationary phase blastoconidia, had gained cyanide-resistance, they remained sensitive to rotenone (Table 2).

NADH-Q oxidoreductase activity in log phase *C. albicans* mitochondria was assessed directly in an enzyme assay, using disrupted mitochondria, thus exposing both internal and external NADH-Q oxidoreductases to NADH. This showed that NADH oxidation could be partly inhibited by 10 μg/ml rotenone (Fig. 4), a concentration that is sufficient to completely inhibit NADH oxidation in beef heart mitochondria [40]. Increase in the rotenone concentration did not further inhibit *C. albicans* NADH-Q oxidoreductase activity (data not shown). NADH oxidation by disrupted *S. cerevisiae* mitochondria, which lack Complex I [9–11], was insensitive to rotenone (Fig. 4). These observations, together with the data from Tables 1 and 2, are further support for a matrix-facing NADH-Q oxidoreductase in log phase *C. albicans* that is a rotenone sensitive proton pump similar to Complex 1. Rotenone inhibition of disrupted mitochondria from *C. albicans* was 70±10%, suggesting that when all NADH-Q reductases are equally

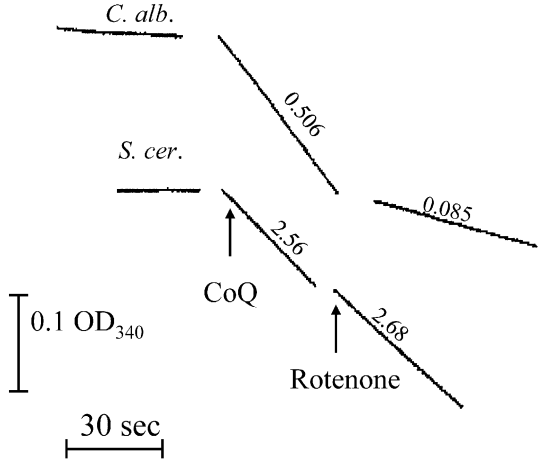

Fig. 4. NADH-Q oxidoreductase activity in disrupted mitochondria of *C. albicans* (*C. alb.*, upper graphs) and *S. cerevisiae* (*S. cer.*, lower graphs). Mitochondrial membranes were suspended to 77 and 11 μg/ml, respectively, in enzyme buffer containing 0.1 mM NADH at 30 °C. Oxidation of NADH was initiated by addition of coenzyme Q_1 (CoQ, 0.15 mM) and followed spectrophotometrically at 340 nm. Rotenone (10 μg/ml) was added were indicated. Consumption of NADH is expressed as Δμmol NADH/min/mg protein.

accessible to the same concentration of NADH, most oxidation occurs via the rotenone-sensitive pathway.

3.5. Influence of the mode of cellular respiration on susceptibility to antifungal agents

As *C. albicans* is an important opportunistic infective agent in humans [19], we investigated whether expression of the alternative respiratory pathway influenced its sensitivity to clinically employed antimycotics. Cells were grown to log phase in broth with or without antimycin A. The sensitivity of each culture to 5-fluorocytosine, amphotericin B, fluconazole, ketoconazole, itraconazole and miconazole was assessed with a commercial assay (Fungitest®, Bio-Rad). The assay is based on a microdilution method in RMPI 1640 medium supplemented with a redox indicator. Reduction of the indicator by growing cells, which turns the medium from purple to pink, was observed with all growth controls. Comparison of the results revealed no differences in sensitivity between *C. albicans* respiring through the conventional or the alternative respiratory pathways. Both cultures, each tested in triplicate, were inhibited by all six antifungal agents included in the assay, as indicated by the absence of a color change of the growth-indicator (data not shown).

Since *C. albicans* is a common resident of the human oral cavity [35,36], it was also of interest to investigate whether expression of the alternative respiratory pathway influenced its sensitivity to naturally occurring salivary antifungal proteins. Histatin 5 is a small cationic peptide present in human saliva that exerts strong fungicidal activity against *C. albicans* and other fungal species [35,41]. Results obtained after exposure of cells expressing the cyanide-sensitive or the cyanide-resistant pathway to histatin 5 indicated that there are no marked differences in sensitivity between both cultures for this peptide (Fig. 5).

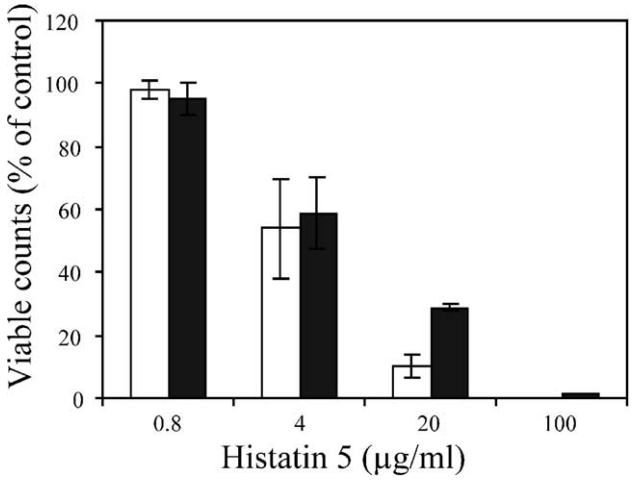

Fig. 5. Susceptibility of cells respiring through the conventional and alternative pathway to histatin 5. *C. albicans* cells were grown to log phase in the absence and presence of antimycin A (10 μM), suspended in 1 mM potassium phosphate pH 7.0 (OD_{620}=0.4) and exposed for 1.5 h to various concentrations of histatin 5. Viability was determined by colony counting 48 h after plating of the cells on agar. White bars, cyanide-sensitive cells, grey bars, cyanide-resistant cells. Represented are the means±S.D. of two experiments performed in duplicate.

4. Discussion

The present study was undertaken to elucidate the constitution of the respiratory chains in the human pathogen *C. albicans* using the classical method of ADP/O determination and selective respiratory chain inhibitors, together with enzyme inhibition assays. The data obtained, together with earlier studies on partly uncoupled *C. albicans* mitochondria [24,27] and on whole *C. albicans* cells [23], allowed us to construct a model for the various respiratory chains in *C. albicans* mitochondria (Fig. 6). Our data support the presence of a rotenone-sensitive and proton-pumping NADH-Q oxidoreductase similar to Complex I, as well as an alternative, external rotenone-insensitive NADH-Q oxidoreductase.

Rotenone-sensitivity was independent of the growth phase of the cells and appeared to be the only active internal NADH-Q oxidoreductase in this species. If cells respire via the alternative, cyanide-insensitive respiratory pathway, Complex I is the only site where proton pumping can occur, since the activity of AOX is not associated with proton translocation. Flow of electrons through an alternative NADH-Q oxidoreductase and AOX would therefore theoretically lead to no proton pumping and thus to no mitochondrial ATP synthesis. Although there is evidence that respiration without proton pumping may serve an important biological purpose [42], no fungi are known in which Complex I is absent and AOX is present [3]. Therefore, the observed Complex I activity in *C. albicans* mitochondria expressing AOX (from antimycin A grown cells and from stationary phase cells) was expected. In contrast to *C. utilis* [38,39], we found that Complex I was also active in log phase *C. albicans* cells, and, thus, that it is expressed in conjunction with either AOX or with cytochromes bc_1 and aa_3.

In addition to Complex I, *C. albicans* cells express external NADH-Q oxidoreductase activity. *C. albicans* may contain only one external alternative NADH-Q oxidoreductase, like the yeast *Yarrowia lipolytica* [4], but we cannot exclude the possibility that several external alternative NADH-Q oxidoreductases are present, as is the case in *S. cerevisiae* [3,4]. Complex I activity in *C. albicans* shows that the genes for Complex I in this species (Ref. [43] and *C. albicans* genome sequence data available to date (http://www-sequence.stanford.edu/group/candida/index.html) are expressed and functional. The *C. albicans* genome furthermore contains at least three genes, *YMR145*, *YMX6* and *NDI1*, which show a high degree of similarity to the conserved nucleotide binding domains in *S. cerevisiae* alternative NADH-Q oxidoreductase genes *SCNDI1*, *SCNDE1* and *SCNDE2*. Future studies should elucidate

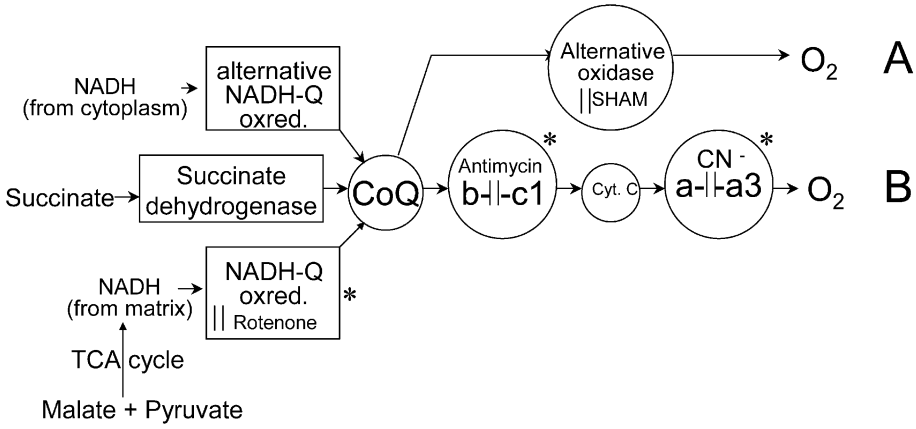

Fig. 6. Diagram of the alternative (A) and conventional (B) respiratory chain in *C. albicans* mitochondria. The matrix-facing NADH-Q oxidoreductases contains Complex I type characteristics, such as rotenone sensitivity, and proton pumping. Evidence for an internal, rotenone-insensitive NADH-Q oxidoreductase could not be deduced from the data obtained. *C. albicans* contains at least one external alternative NADH dehydrogenase, but the presence of additional external NADH dehydrogenases cannot be excluded. The alternative oxidase (AOX) branches off from the conventional respiratory chain at the Coenzyme Q level. Indicated are the sites of inhibition of rotenone, antimycin A, cyanide and SHAM, the sites of entry of NADH and succinate and the sites of proton pumping (*).

the localization and functions in *C. albicans* of the *YMR145*, *YMX6* and *NDI1* gene products in more detail.

When *C. albicans* cells were grown in the presence of antimycin A, cyanide-insensitive AOX activity was induced. In accordance with previous studies on AOX from a variety of species [12], AOX in *C. albicans* did not contribute to ATP synthesis, since oxidation of external NADH or succinate, which donate electrons downstream from Complex I, was not coupled to ATP formation. The observed ADP/O ratios were 2.2 for electrons passing through Complexes I, III, and IV, 1.4 for electrons passing through Complexes III and IV, and 0.8 for electrons passing through Complex I and AOX. These values are in agreement with theoretical values of an ADP/O ratio of 2.5 for the full conventional respiratory chain (10 H^+ pumped, with 4 H^+/ATP molecule), 1.5 for external NADH-Q oxidoreductase in conjunction with cytochrome oxidase, and 1.0 for Complex I alone [44].

Respiration via AOX is clearly less efficient than respiration via the conventional chain. Since only one proton pumping complex is involved (Complex I), 1 ATP is formed compared to 2.5 when the conventional respiratory pathway is used. Thus, alternative respiration operates at a considerable energy cost, and much of this is lost as heat. While in Araceae, the function of AOX in thermogenic inflorescence is possibly related to heat production [12], it seems unlikely that heat production is a major function of AOX in unicellular microorganisms. With respect to the low energy conservation, alternative respiration has been compared to other energy dissipating mechanisms in the respiratory chain, such as natural uncoupler proteins [12,32]. One potential role for uncoupled respiration in general is to facilitate glycolytic ATP production by acting as a sink for cytoplasmic NADH formed during fermentation under conditions of excess of nutrient availability [45]. However, since AOX is present in plants and also in strictly aerobic yeasts such as *C. parapsilosis* [22], it is unlikely that this is the major function of AOX. A third and attractive possible role of uncoupled respiration is to protect cells from superoxide radical formation by preventing excessive reduction of the Q-pool [42]. Indeed, in both plant and fungi, expression of AOX can be induced by inhibition of the conventional respiratory pathway, presumably leading to an increase in the Q reduction level and in oxygen radical production, but also by externally applied oxidative stress conditions [14,17,18] or by salicylate, an inhibitor of catalase [17,18]. In addition, antisense suppression of AOX expression in transgenic tobacco cells significantly increases ROS levels whereas overexpression of AOX lowers ROS levels [13]. Based on these observations, it has been postulated that the predominant and universal role of AOX in plant as well as in fungal cells might be the protection against oxygen radical formation by lowering the Q-redox poise.

Apart from the importance of the physiological role of AOX in unicellular microorganisms such as *C. albicans*, it was of interest to investigate the clinical consequence of AOX expression in this microorganism since it constitutes an important human opportunistic pathogen [19,41]. AOX expression by *C. albicans* in vivo has yet to be elucidated; however, it is anticipated that in the oral cavity where *C. albicans* is a resident, substrate limitation, growth in oral biofilm or at sites of infections may create stress conditions that induce alternative respiration. Therefore, we studied if AOX expression would influence antifungal susceptibility and germ tube formation. The latter is considered to be related to adhesion and invasive growth and therefore an important virulence factor [46]. A commercial antifungal susceptibility test was chosen with three possible outcomes (susceptible, intermediate, or resistant) to identify clinically

important differences rather than to identify small differences in susceptibility that would have little clinical relevance. The tests indicated no differences in susceptibility, and thus no differences in response to clinical treatment are to be expected if cells change their expression levels of AOX. Likewise, AOX expression did not alter the susceptibility to the natural salivary peptide histatin 5, nor did it enhance germ tube formation (data not shown), indicating that there are no marked differences in virulence between cells expressing the conventional or the alternative respiratory pathway.

This study shows that *C. albicans* contains a variety of respiratory systems whose expression depends on growth conditions. Future study should elucidate in more detail the factors that regulate the expression of the individual complexes and the partitioning of electrons between both respiratory chains, and the contribution of this flexible adaptation in the successful establishment of *C. albicans* in complex microbial ecological systems and on its survival under conditions of oxidative stress.

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