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Induction of Alternative Oxidase Activity in *Candida albicans* by Oxidising Conditions

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Abstract*—Candida albicans* ATCC 10231 had low endogenous activity of the alternative oxidase compared with that of *C. albicans* ATCC 10261. In *C. albicans* ATCC 10231 the endogenous activity declined as the cultures aged. Alternative oxidase activity could be induced in *C. albicans* ATCC 10231 by treatment with cyanide, but the induction of this activity required the presence of oxygen which could be replaced, at least in part, with high concentrations of potassium ferricyanide. We infer from this that the expression of the gene encoding the alternative oxidase is under the control of a redox-sensitive transcription factor.

***Keywords*—**alternative oxidase, *Candida albicans*, enzyme induction; oxygen; redox potential.

I. Introduction

CYANIDE-RESISTANT respiration has been reported in many organisms, especially in higher plants and fungi. The alternative oxidase (AOX) responsible for this activity is a quinol:oxygen oxidoreductase which is insensitive to cyanide, unlike most other terminal oxidases. Like the other enzymes of the respiratory electron transfer chain, the AOX is associated with the inner mitochondrial membrane [1], but, unlike several of those enzymes, it is not coupled to proton translocation.

Alternative oxidase activity is inhibited by compounds such as SHAM [2], disulfiram and *n*-alkyl gallates [3, 4], none of which inhibit cytochrome oxidase. Conversely, cyanide inhibits cytochrome oxidase, but does not inhibit AOX. These two inhibitors can be used to distinguish between O_2 uptake catalysed by AOX and cytochrome oxidase. The disruption of electron transfer by cyanide and SHAM would affect ATP synthesis and the activity of metabolic pathways, either of which would inhibit growth. It has been suggested that AOX is a viable target in the treatment of trypanosomes [5] and that it plays an important role in the stress response in various fungi [6] and in plants [7]. Morover, hydroxamic acids (such as SHAM) inhibit the growth of some bacteria, some of which also have an AOX homologue [1], and other fungi [8, 9]. While does SHAM inhibit the growth of various *Candida* spp., including *C. albicans*, this is not due to the inhibition of AOX [10, 11].

While the AOX of higher plants appears to be present constitutively in many species, this is not necessarily the case in fungi. For example, *Hansenula anomala* and *Magnaporthe grisea*) appear to lack detectable levels of the enzyme in normal circumstances [12, 13], whereas others (such as *Aspergillus niger* and *Neurospora crassa*) generally have only low levels of AOX [14, 15]. In each of these species AOX activity can be induced or enhanced by a variety of treatments. For example, treatment with respiratory inhibitors such as antimycin A or cyanide, which inhibit the cytochrome bc_1 complex and cytochrome oxidase, respectively, results in the appearance of AOX activity after a lag of as much as several hours, depending on the experimental conditions and the species. *Candida albicans* ATCC 10231, like some other *Candida* spp., has a constitutive AOX and an inducible AOX encoded by different genes [16, 17].

The AOX of *C. albicans* has been reported to be sensitive to azide as well as SHAM [18], unlike that from other fungi, including *H. anomala* [19], and the induction of AOX activity is relatively rapid [18]. Here we show that the induction of the AOX activity in *C. albicans* is dependent on the strain, the growth phase, on oxygenation and on the redox state of the medium.

II. Materials and Methods

Candida albicans ATCC 10231 and *C. albicans* ATCC 10261 were grown in an orbital incubator at 30ºC in liquid YPD (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) bacteriological peptone, 2% (*w*/*v*) glucose). Routinely, cultures (50 mL YPD in a 250 mL conical flask) were inoculated with sufficient cells from an overnight culture to get an initial A_{600} of 0.4 and grown for 15 h, unless otherwise specified, at 30°C in a shaking incubator (at 200 min^{-1}).

Cells were harvested by centrifugation at 1300 × *g* for 15 min at 4°C, washed with 50 mM KH_2PO_4-KOH pH 5.6 and then resuspended to 5 mL in the same buffer. The O_2 uptake by cell suspensions (2 mg mL^{-1} DW in 50 mM KH_2PO_4-KOH pH 5.6) was measured polarographically with a YSI 5300 O_2 electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, USA) at (30 ± 1)°C. The O_2 concentration in the cuvette was digitised each second (each datum represented the average of 1000 determinations) using hardware and software developed in-house [20]. Where necessary, cell suspensions

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were oxygenated using a standard fish tank pump and the O_2 concentration was monitored using the oxygen electrode.

III. Results

A. Comparison of C. albicans strains

As isolated from various exponential phase cultures, *C. albicans* strains ATCC 10231 and ATCC 10261 consumed O_2 at similar rates, but the addition of 3 mM KCN to a suspension of *C. albicans* ATCC 10231 rapidly reduced the activity by about 85%, but had no significant effect on the rate of O_2 uptake by *C. albicans* ATCC 10261 (Fig 1). In either case, the residual rate of O_2 uptake in the presence of cyanide was insensitive to any further cyanide addition, but was partly inhibited by 3 mM SHAM, whereas SHAM in the absence of KCN had no significant effect on the rate of O_2 uptake by either strain (Fig 1).

While 3 mM KCN rapidly inhibited the rate of O_2 uptake by *C. albicans* ATCC 10231, after a period of sustained inhibition (Fig 1A), lasting as much as 20 minutes, the rate of O_2 uptake gradually increased, approaching the uninhibited rate (3 nmol O_2 min^{-1} mg^{-1} DW, Fig 2A), a phenomenon we have not observed with *C. albicans* ATCC 10261 in which the rate of O_2 uptake was not significantly affected by KCN (Figs 1 and 2B). The residual rate observed immediately after KCN addition to *C. albicans* ATCC 10231 could not be inhibited by SHAM, but the subsequent stimulated rate was SHAM-sensitive (Fig 2A). In the case of *C. albicans* ATCC 10261, the O_2 uptake activity observed in the presence of KCN (Fig 2B) was also SHAM-sensitive (Figs 1 and 2B). The rate of O_2 uptake by *C. albicans* ATCC 10261 was not significantly affected by SHAM in the absence of KCN (Fig 1).

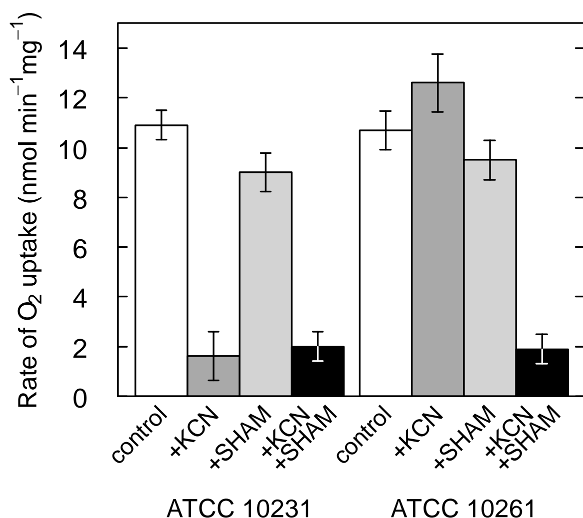

Fig. 1. Endogenous rates of O_2 uptake by *C. albicans* ATCC 10231 (left) and ATCC 10261 (right) and the effect of 3 mM KCN, 3 mM SHAM or both KCN and SHAM. The rate of O_2 uptake was determined as soon as practicable after KCN or SHAM addition. Oxygen uptake was followed using 2 mg mL^{-1} DW in the cuvette in 50 mM KH_2PO_4-KOH pH 5.6. The errors represent ± SEM for at least 6 exponential phase cultures.

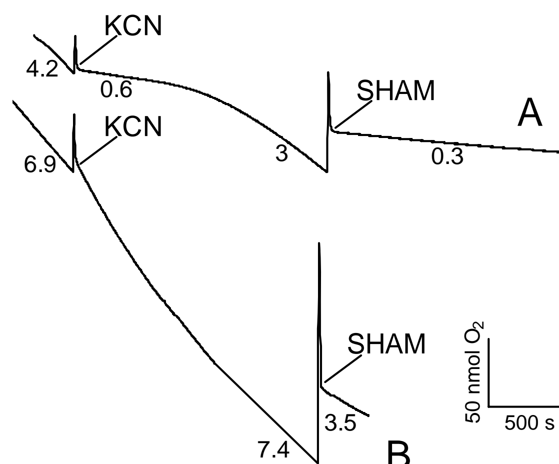

Fig. 2. Oxygen electrode traces of suspensions of *C. albicans* ATCC 10231 (A) and ATCC 10261 (B) illustrating the induction of cyanide-insensitive O_2 uptake in the former (A) but not the latter (B). At the times indicated 3 mM KCN or 3 mM SHAM (A) or 0.5 mM SHAM (B) were added (the sharp spikes are addition artefacts). As SHAM was dissolved in 2-methoxyethanol, in which O_2 is more soluble than it is in water, the addition of SHAM was necessarily associated with an addition of O_2 resulting in an increase in O_2 concentration. The numbers on each O_2 electrode trace represent the rate of O_2 uptake (nmol O_2 min^{-1} mg^{-1} DW).

B. Induction of AOX activity in relation to growth phase

The endogenous rate of O_2 uptake of *C. albicans* ATCC 10231 declined approximately exponentially with culture age (Fig 3). The rate of O_2 uptake observed following the addition of KCN is a measure of the activity of the AOX, which also declined as the culture aged (Fig 3). Cells taken from exponential phase cultures (10-45 mg mL^{-1} DW) were more sensitive to KCN (exhibiting only 20% of the endogenous rate of O_2 uptake after KCN addition) than those taken from stationary phase cultures (>45 mg mL^{-1} DW), which were relatively insensitive to the addition of KCN (exhibiting >50% of the endogenous rate of O_2 uptake after KCN addition).

The induction of SHAM-sensitive AOX activity also varied with the growth phase of the culture (Fig 4). Cells from exponential phase cultures developed realtively high rates of SHAM-sensitive O_2 uptake within about 1000 s (Fig 2A) and so the stimulation of the KCN-inhibited rate 1000 s after KCN addition was arbitrarily used to monitor the induction of AOX activity after the addition of KCN (Fig 4). Cells from stationary phase cultures failed to exhibit any further induction of AOX activity within 1000 s (Fig 4) and *C. albicans* ATCC 10261 showed no evidence of any induction of AOX activity within 1000 s (Fig 2A).

The kinetics of the appearance of SHAM-sensitive O_2 uptake after the addition of KCN also depended on the growth phase of the culture, but this will be described elsewhere.

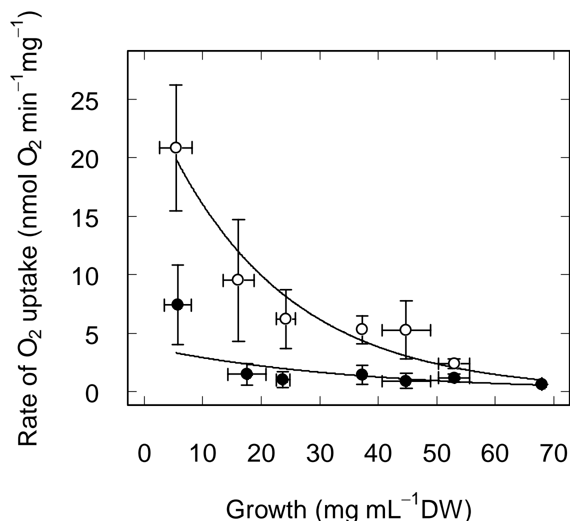

Fig. 3. The endogenous rate of O_2 uptake (○) and the residual activity immediately after the addition of 3 mM KCN (●) during the growth of batch cultures of *C. albicans* ATCC 10231. The data represent the mean (± 95% confidence intervals) and the exponential curves are intended only as a guide. Data were obtained from at least 4 independent cultures.

gradual SHAM-sensitive increase in the rate of O_2 uptake, consistent with the induction of AOX activity (Fig 5). In these experiments, the lag before the appearance of AOX activity following the transfer of the anaerobically-incubated cells to aerobic conditions in the O_2 electrode (Fig 5) was consistently shorter than that observed in the standard induction assay (an example of which is shown in Fig 2A).

The O_2 required in the induction of AOX activity could be at least partially replaced with potassium ferricyanide (FeCN). The presence of 50 mM FeCN in the incubation medium had no significant effect on the rate of O_2 uptake by cells incubated aerobically with KCN (Fig 6). However, cells incubated anaerobically with KCN, but without FeCN (or with 50mM potassium ferrocyanide) exhibited a rate of oxygen consumption (about 3 nmol O_2 min^{-1} mg^{-1} DW) which was only 22% of that of cells incubated anaerobically with 50 mM FeCN. Since the FeCN was slowly reduced during the incubation, the conditions gradually became less oxidising. In contrast, FeCN had little effect on the rate of O_2 uptake of oxygenated suspensions incubated with KCN (Fig 6).

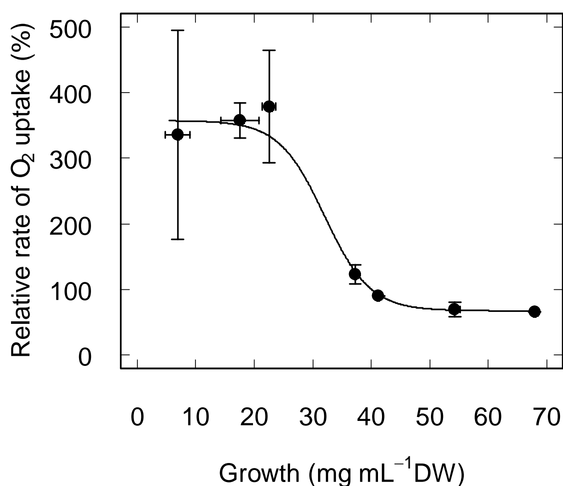

Fig. 4. The relative rate of O_2 uptake 1000 s after the addition of 3 mM KCN during the growth of batch cultures of *C. albicans* ATCC 10231. The data represent the mean of at least 4 independent cultures (± 95% confidence intervals) and the smooth curve is intended only as a guide. The values are expressed relative to the rate observed immediately after the addition of KCN (Fig 3).

C. *Effect of redox state on the induction of AOX activity*

The induction of the AOX activity in *C. albicans* ATCC 10231 generally required oxygen (Fig 5). Cells incubated anaerobically in the presence of cyanide for 1 hour exhibited a slow rate of oxygen consumption (2.9 nmol O_2 min^{-1} mg^{-1} DW) compared with cells which had been incubated aerobically in the presence of cyanide (9.8 nmol O_2 min^{-1} mg^{-1} DW). Furthermore, aerating cells which had been incubated anaerobically for 1 hour in the presence of cyanide caused a

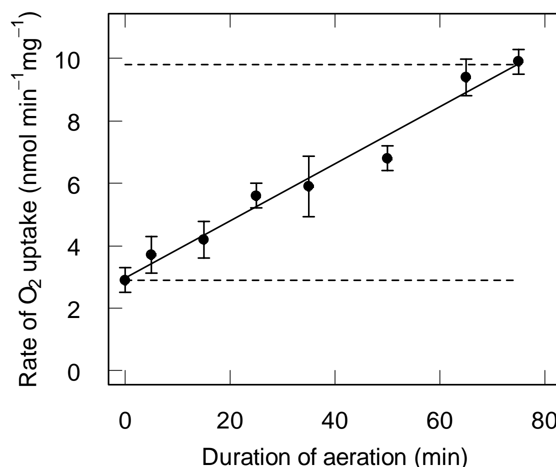

Fig. 5. The effect of oxygenation on the induction of KCN-insensitive O_2 uptake by *C. albicans* ATCC 10231. Five millilitres of cell suspension (15 mg mL^{-1} DW) were placed in each of two vials for 10 minutes to ensure that the suspension was anaerobic before 2.4 mM KCN was added to each, after which one was aerated (using a fish tank pump) and the other was kept tightly sealed. After one hour, samples were taken from each vial and the rate of O_2 uptake was determined (indicated by the upper and lower dashed lines). At this time aeration of the anaerobic vial was started and samples were taken intermittently after the start of bubbling and the rate of O_2 uptake was measured using 1.5 mg mL^{-1} DW in the cuvette in 50 mM KH_2PO_4-KOH pH 5.6. The data shown are representative at least 4 experiments (± 95% confidence intervals). The straight line is the least squares fit to the data (r = 0.988, $p < 0.001$) and is intended only to guide the eye.

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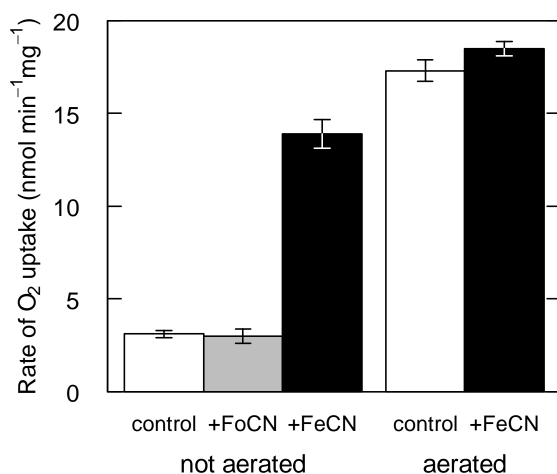

Fig. 6. The effect of ferri- and ferrocyanide on the induction of AOX activity. Five millilitres of culture (15 mg mL^{-1} DW) were placed in each of five vials for 10 minutes to ensure that the suspension was anaerobic, before 2.4 mM KCN was added to each. At this time, 50 mM potassium ferricyanide (FeCN) was added to two vials, 50 mM potassium ferrocyanide (FoCN) was added to one vial and an equal volume of water was added to each of the other two vials (control). Two vials (a control and one containing FeCN) were aerated (using a fish tank pump) and the others were kept tightly sealed. After one hour, samples were taken from each vial and the rate of O_2 uptake was measured using 1.5 mg mL^{-1} DW in the cuvette in 50 mM KH_2PO_4-KOH pH 5.6. The data shown are representative of at least four experiments and the errors represent ± 95% confidence intervals.

IV. Discussion

We have shown that not all strains of *C. albicans* have comparable constitutive AOX activities (Fig 1), but SHAM-sensitive O_2 uptake can be induced by the addition of cyanide in at least *C. albicans* ATCC 10231 (Fig 2A). Both the activity (Fig 3) and the induction of the activity (Fig 4) of the AOX in *C. albicans* vary with growth phase, and the induction of the activity following cyanide addition requires aerobic (Fig 5) or oxidising conditions (Fig 6).

Cells in the exponential growth phase, which are actively dividing, have the lowest proportion of cyanide-insensitive activity (about 20% of the uninhibited rate) and cells in the stationary phase have the highest relative activity of the AOX (Fig 3). This is consistent with the observations of Minagawa and Yoshimoto [12] with *H. anomala* and Kirimura *et al.* [21] with *A. niger*. Since the AOX is not coupled to proton translocation across the inner mitochondrial membrane, it does not contribute to ATP synthesis. Therefore, it is reasonable to suppose that rapidly dividing cells, with a high demand for ATP, should have the least AOX activity. Similarly, stationary phase cells, which are not dividing rapidly, have a lower requirement for ATP and are able to sustain the metabolic cost of AOX activity.

The data indicate that AOX activity in *C. albicans* ATCC 10231 is induced by oxidising conditions. While we have not shown that there is *de novo* protein synthesis, we suspect that this is the most likely explanation for at least three reasons. First, *C. albicans* lacks the cysteine in the N-terminal domain of the AOX that is associated with pyruvate activation and dimerisation of the enzyme in plant mitochondria [1, 22]. Second, if the enzyme were activated by pyruvate, it should occur essentially instantaneously, as is the case in plant mitochondria [23], but AOX activity of the enzyme remained low for up to 20 min after KCN addition (Fig 2A). Third, where the activity of existing enzyme is increased, this is mediated by reducing conditions such as a reduced quinone pool [24], elevated pyruvate concentrations [23, 25] and reduction of the enzyme [25, 26].

In various yeast, AOX activity is induced by factors which reduce the activity of the mitochondrial electron transfer chain. These include inhibitors of cytochrome oxidase or the cytochrome bc_1 complex [12, 18, 19]; copper-limited growth [27]; low glucose concentration [28]; chloramphenicol, which inhibits the synthesis of the mitochondrially-encoded subunits of the electron transfer chain enzymes [15, 29]; and various sulphur compounds [30]. Conversely, induction of the AOX activity is blocked by factors which prevent the expression of the nuclear gene(s) encoding the polypeptide. Such factors include: conditions which reduce the ATP supply, such as anaerobiosis [12] and the presence of uncouplers [14, 31]; inhibitors of extra-mitochondrial protein synthesis, such as cycloheximide [15] and antibiotics [14]; and Fe(II) chelators such as *o*-phenathroline [32], that prevent the incorporation of iron atoms that could be involved in the oxygen chemistry at the catalytic centre of the enzyme [33]. It has been argued [34] that the signal for the induction of the AOX is the accumulation of superoxide anion, however, an alternative explanation, which is consistent with our own data, is that oxidising conditions (low glucose concentrations resulting in a low intracellular [NADH]/[NAD$^+$] ratio; high O_2 concentration; high ferricyanide concentration; high concentration of oxidised glutathione [30]) promote the induction of AOX activity, whereas reducing conditions (high substrate supply, anaerobiosis) tend to block the induction.

Bunn and Poyton [35] presented a model of the oxygen regulation of transcription in yeast which could be mediated either by oxygen binding to, or through the redox state of, specific haem proteins. The data we have presented here imply that it is possible that the induction of AOX activity could be mediated by an analogous redox-sensitive process. However, such a model does not take into account the effects of inhibitors of electron transfer and protein synthesis.

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