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Effects of a transition from normoxia to anoxia on yeast cytochrome *c* oxidase and the mitochondrial respiratory chain Implications for hypoxic gene induction

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Abstract

Previous studies have demonstrated that the mitochondrial respiratory chain and cytochrome *c* oxidase participate in oxygen sensing and the induction of some hypoxic nuclear genes in eukaryotes. In addition, it has been proposed that mitochondrially-generated reactive oxygen and nitrogen species function as signals in a signaling pathway for the induction of hypoxic genes. To gain insight concerning this pathway, we have looked at changes in the functionality of the yeast respiratory chain as cells experience a shift from normoxia to anoxia. These studies have revealed that yeast cells retain the ability to respire at normoxic levels for up to 4 h after a shift and that the mitochondrial cytochrome levels drop rapidly to 30–50% of their normoxic levels and the turnover rate of cytochrome *c* oxidase (COX) increases during this shift. The increase in COX turnover rate cannot be explained by replacing the aerobic isoform, Va, of cytochrome *c* oxidase subunit V with the more active hypoxic isoform, Vb. We have also found that mitochondria retain the ability to respire, albeit at reduced levels, in anoxic cells, indicating that yeast cells maintain a functional mitochondrial respiratory chain in the absence of oxygen. This raises the intriguing possibility that the mitochondrial respiratory chain has a previously unexplored role in anoxic cells and may function with an alternative electron acceptor when oxygen is unavailable.

Keywords: Mitochondria; Hypoxia; Cytochrome oxidase isozyme; Oxygen sensing

1. Introduction

The transcription of many prokaryotic and eukaryotic genes is regulated by the availability of oxygen [1–8]. These oxygen-regulated genes can be placed into one of two categories; *aerobic* genes, which are transcribed optimally under normoxic conditions and *hypoxic* genes which are transcribed optimally under hypoxic and anoxic conditions. In recent years, considerable progress has been made in our understanding of the molecular pathways involved in regulating transcription of these oxygen-responsive genes [9–11]. In particular, many oxygen-responsive transcription factors have been identified and characterized in mammals, yeast, and bacteria. Despite a great deal of progress in understanding how oxygen-regulated transcription factors function, the nature of the proximal events involved in oxygen sensing has remained elusive.

Two fundamentally different types of models for how cells sense oxygen and regulate oxygen-sensitive genes have been proposed. In the first model, oxygen itself has a direct effect on transcription, either by acting on a transcription factor itself or by affecting a component that interacts with a transcription factor. In a second model, oxygen is sensed more distally by a proximal oxygen sensor, a signal is produced, and the signal initiates a signal transduction cascade that affects distal transcription factors. Support for the first type of model was initially provided by studies on the *E. coli* FNR protein [12], iron-sulfur clusters in central neurons [13], mammalian iron responsive elements [14] and more recently, from studies on the HIF-

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1α protein in the nematode *C. elegans* [10]. Evidence for the second type of model has come from an analysis of a two-component oxygen sensing system in the nitrogen fixing bacterium *Rhizobium meliloti*. Here, the hemokinase FixL functions as a proximal oxygen sensor, which starts a phosphorylation cascade that ultimately leads to the up-regulation of genes involved in nitrogen fixation and hydrogen utilization [2,15]. Evidence for the involvement of hemoprotein oxygen sensors has also come from studies with other bacteria, yeast, and mammalian cells (for review, see [2,16]). While some hemoprotein oxygen sensors contain a heme-PAS domain [9,16], others do not. Many PAS domain proteins are part of two-component signaling pathway in which oxygen binding to the PAS domain affects an enzymatic activity of an associated domain in the protein itself.

An example of a non-PAS domain hemoprotein that has been implicated in oxygen sensing in eukaryotic cells is cytochrome *c* oxidase (COX). Previously, we have shown that yeast COX and the mitochondrial respiratory chain are required for the induction of some hypoxic nuclear genes as yeast cells undergo a transition from normoxic to anoxic growth [17] and that exposure to low oxygen concentrations leads to transient oxidative stress both in the mitochondrion and cytosol [18]. Similar results have been obtained with mammalian cells in culture [19]. These findings suggest that COX and the mitochondrial respiratory chain function in oxygen sensing, and raise the possibility that reactive oxygen species (ROS) function as signaling molecules in a signal transduction pathway to the nucleus.

The transient increase in intracellular levels of ROS as yeast cells experience low oxygen concentrations implies that one or more enzymes of the mitochondrial respiratory chain, the major producer of ROS [18], experiences functional changes during a transition from normoxic to anoxic growth. Of particular interest in this regard is COX. Active preparations of yeast COX contain a total of nine different polypeptide subunits as well as four redox-active metal centers (heme a, heme a_3, Cu_A and Cu_B) [20]. All nine subunit polypeptides are required for a fully active holo cytochrome *c* oxidase. Subunits I, II and III are products of mitochondrial genes (*COX1*, *COX2* and *COX3*, respectively) and make up the catalytic core of the enzyme [21,22]. Subunits IV, V, Va or Vb, VI, VII, and VIIa are encoded by nuclear *COX* genes (*COX4*, *COX5a* or *5b*, *COX6*, *COX7*, *COX8* and *COX9*, respectively). Some of them function to modulate catalysis [23,24] while others function to affect assembly or stability of the holoenzyme [25]. In addition to these nine subunits, some yeast COX preparations contain two additional polypeptides (subunits VIa and VIb) [26,27]. These two polypeptides are not required for catalysis [24] but may regulate its rate [28]. COX subunit V in yeast has both *aerobic* and *hypoxic* isoforms [24]. *COX5a* encodes the aerobic isoform, Va, while *COX5b* encodes the hypoxic isoform, Vb. Either one of these two isoforms is essential for active COX [29]. Extensive analysis of the yeast subunit V isoforms have revealed that the genes for these proteins are switched on or off at very low oxygen concentrations (0.5–1 μM O_2) [30] and that they affect the catalytic functions of the holoenzyme differentially [23]. By altering an internal step in electron transfer between heme a and the binuclear reaction center the hypoxic isoform, Vb, enhances the maximum turnover rate of the holoenzyme [23,24] threefold to fourfold relative to the aerobic isoform.

Given that the presence of oxygen-regulated isoforms of COX subunit V have differential effects on the catalytic activity of holo cytochrome *c* oxidase it was of interest to ask what role, if any, they play in hypoxic gene induction. It was also of interest to examine the level and functionality of the mitochondrial respiratory chain as a whole, as cells experience a decrease in oxygen tension. These questions are addressed in this study.

2. Materials and methods

2.1. Yeast strains and media

Saccharomyces cerevisiae JM43 (MATα *his4-580 trp1-289 leu2-3*, *112 ura3-52* [ρ+]) [31] and HC5b (MATα *his4-580 trp1-289 leu2-3*, *112 ura3-52 cox5b::LEU2 cox5a-Δ::URA3* [ρ+] plasmid Yep13-511) [32] strains were used in this study. Yeast cultures were grown in SSG-TEA, a semi-synthetic media supplemented with Tween 80, ergosterol, silicon antifoam and the amino acids leucine, histidine, tryptophan and uracil as needed [33].

2.2. Culture conditions for shifts between normoxia and anoxia

Shifts between normoxic and anoxic growth conditions were performed in a 5-l volume of SSG-TEA in a New Brunswich BioFlow 3000 fermentor, as described [34]. The temperature (28 °C), stirring speed (250 rpm), gas sparge rate (5 l/min) and pH (5.0) were controlled automatically by the fermentor system software. The fermentor was inoculated at a low cell concentration with aerobically grown, mid-exponential precultures and grown sparged with filtered house air. The cell density was measured turbidometrically using a Klett–Summerson photoelectric colorimeter fitted with a No. 54 green filter. Once a cell density of 0.8 g wet weight/ml was attained, the sparge gas was changed from filtered house air to 2.5% CO_2/97.5% N_2 [30]. The pH was maintained at 5.0 for the duration of the culture growth by addition of 1 M KOH as needed. The sparge gas flow rate was controlled manually. To ensure that the culture was strictly anoxic, the anoxic sparge gas mixture is certified 99.999% oxygen free and was passed through an Oxiclear O_2 absorber (Lab-Clear, Oakland, CA). The dissolved oxygen concentration of the media was measured by a Mettler–Toledo oxygen electrode fitted into the fermentor

vessel. The culture was grown anoxically for a maximum of 24 h or to a cell density of 5.5 g wet weight/ml. Anoxic cultures are protected from the light by covering the fermentor flask with aluminum foil [35]. Cells were harvested from the fermentor via a harvesting coil which was submerged in a salted ice water bath to quick chill the cells. Cells were centrifuged at 5000 rpm for 10 min, 4 °C, resuspended in cold water and centrifuged again. Anoxic cells were harvested and washed in the presence of 4 g/ml erythromycin and 1 μM chloramphenicol. Cell pellets were stored under an overlay of water containing erythromycin and chloramphenicol at 4 °C until use. For RNA isolation, centrifuge bottles and tubes were rinsed with RNAse Away (Molecular BioProducts, San Diego, CA, USA) and the harvested cells were flash frozen in liquid nitrogen and stored at −70 °C. Steady state anoxic cultures were grown as stated above with the exception that they were grown for six generations under anoxia.

2.3. Measurement of cell respiration

Whole cells (0.2 g/ml in 40 mM $NaPO_4$, pH 7.4) were added to 3 ml of aerated, 30 °C, 40 mM $NaPO_4$, pH 7.4, plus 0.67% (w/v) glucose to start the reaction. The reaction was conducted at 30 °C with stirring for a minimum of 3 min. To determine the cyanide sensitive rate 1 mM KCN was added to the assay solution either at the end of the assay or before the addition of cells to separate assays. The assays were conducted in a StrathKelvin oxygen electrode system and data were collected and manipulated using the accompanying software.

2.4. Spectrophotometric methods

Room temperature spectra were taken of whole cells using an Aminco DW2000 spectrophotometer upgraded to a Windows based software system (OLIS Globalworks v.2.0.167) by OLIS (Bogart, GA, USA). Cell suspensions of 0.1 g/ml in 40 mM KPO_4, pH 7.4 were sequentially reduced and then oxidized with 1% (v/v) glucose, solid sodium dithionite and 0.2% (v/v) H_20_2. Reduced minus oxidized difference spectra were used to calculate the cytochrome concentration. Extinction coefficients used to determine the cytochrome concentration were as follows: 24.6 mM^{-1} cm^{-1} (Δ550–540 nm) for cytochrome $c+c_1$, 25.6 mM^{-1} cm^{-1} (Δ562–577 nm) for cytochrome b, 16.5 mM^{-1} cm^{-1} (Δ601–630 nm) for cytochrome $a+a_3$ [7].

2.5. Isolation of mitochondria

Mitochondria were isolated from harvested cells using zymolyase as indicated in [34] with modifications to allow for more complete disruption of the cell wall for cells harvested 12 to 24 h post anoxia following the protocol of [36]. The isolated mitochondria were stored at 4 °C under an overlay of 150 μl of resuspension buffer (10 mM $NaPO_4$, pH 7.4) and resuspended the next morning. The mitochondria were stored at 4 °C for up to 4 days. Erythromycin (4 g/ml) and chloramphenicol (1 μM) were included in all steps of the mitochondrial isolation to prevent adaptation to aerobic conditions.

2.6. SDS-PAGE electrophoresis and Western blotting

SDS-PAGE was conducted on a Hoeffer SE 400 vertical slab gel unit following the method of [37]. Isolated mitochondria were lysed in loading dye containing 4% SDS and 2.5% β-mercaptoethanol and incubated at 30 °C for 30 min. The SDS/protein ratio was maintained at greater than 1.4:1. Hence supplemental SDS was added to the sample before incubation as required. Electrophoresis was conducted at 70 V for 2.5 h followed by 140 V for 15 h. The gel was blotted and visualized as previously described [7]. The primary polyclonal antibody recognized both COX subunits Va and Vb.

2.7. RNA isolation and Northern blotting

Total RNA isolation, electrophoresis and Northern Blotting were carried out as described in [7] with the following modifications; the random primer extension kit used to radiolabel the DNA probes was supplied by Promega, (Madison, WI, USA), the Northern blots were prehybridized, and hybridized at 55 °C, subjected to the first wash at 55 °C, with the second wash occurring at 65 °C. The RNA transcript level was normalized to the total RNA loaded per lane (1 μg) which was electrophoresed in 1% TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.0) on a 1.5% TBE-agarose gel, visualized by ethidium bromide staining (0.5 mg/ml) [38] and quantitated using the same software used to quantitate the Northern blots (Image Quant version 5.2).

2.8. Miscellaneous methods

Protein concentration was determined by the method of Lowry [39]. Heme concentrations were determined as indicated in [40].

3. Results

3.1. Respiration capacity decreases when cells are shifted to anoxia

To determine the effects of exposure to anoxia on the mitochondrial respiratory chain we first examined the levels of cyanide-sensitive respiration in strain JM43, after shifting cells from growth under normoxic conditions to growth under anoxic conditions. These shifts are performed in a fermentor in which oxygen levels drop rapidly reaching anoxic levels within 10 min after

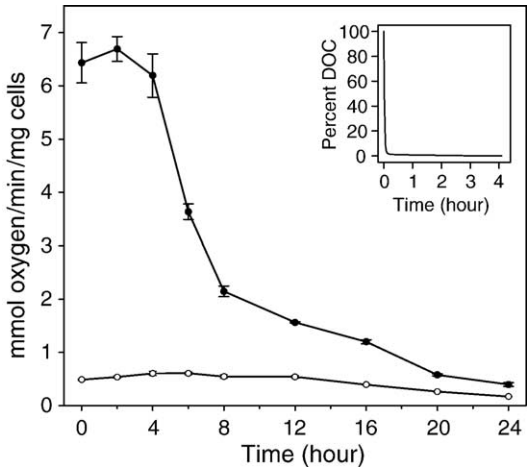

Fig. 1. Respiration capacity of *S. cerevisiae* JM43 cells during adaptation to anoxia. *S. cerevisiae* cells were harvested during a 24 h shift culture from normoxic to anoxic conditions. Respiration was measured under aerobic conditions. KCN-sensitive respiration (●) was obtained by subtracting the KCN-insensitive oxygen consumption (○) from the total oxygen consumption. Each data point represents an average of 3–5 independent assays. The cells at the 0-h time point were harvested under normoxic conditions. The inset shows the dissolved oxygen concentration in the culture media after the sparge gas was changed from air to 97.5%N_2/2.5%CO_2.

the shift (Fig. 1 insert). Hence, cells are essentially anoxic during the entire duration of the experiment. To assess the capacity of cells to respire after being deprived of oxygen, we harvested cells at different times after the shift and measured the level of cyanide-sensitive whole cell respiration in the presence of air. Respiration capacity assessed in this way is a measure of both the functionality and level the mitochondrial respiratory chain as a whole. From Fig. 1, it is clear that the level of cyanide-sensitive oxygen respiration in whole JM43 cells remains nearly constant for the first 4 h after a shift to anoxia and then declines dramatically to a level, at 24 h after the shift, which is 10–15% of that found in normoxic cells.

In contrast, cyanide-insensitive oxygen consumption declines only slightly during this period. While cyanide-sensitive respiration is a measure of the energy-producing mitochondrial respiratory chain, cyanide-insensitive oxygen consumption results from many different oxygen-dependent reactions in several different cellular compartments, including the mitochondrion [41]. From Fig. 1, it can be seen that cyanide-insensitive oxygen consumption accounts for 8% of the total respiration in aerobic cells and nearly 33% of respiration in cells exposed to anoxia for 24 h (Fig. 1).

It is noteworthy that cells retain the ability to respire after 24 h of incubation in the complete absence of oxygen, the electron acceptor used by both cyanide-sensitive respiration and cyanide-insensitive oxygen consumption. This indicates that an intact cyanide-sensitive respiratory chain as well as the enzymes involved in cyanide-insensitive oxygen consumption are retained for several hours after exposure to anoxia.

3.2. Cytochrome levels decrease in S. cerevisiae during a shift to anoxic growth

JM43 cells harvested at different times after a shift from normoxia to anoxia were monitored by difference spectroscopy to assess the effects of exposure to anoxia on cytochrome levels. Low temperature spectra done at liquid nitrogen temperatures were used to obtain a qualitative evaluation of the cytochromes that were present. However, because low temperature spectra are not quantitative, we used room temperature difference spectra to quantitate cytochrome levels. Room temperature difference spectra of intact cells reveal that exposure to anoxia has both qualitative and quantitative effects on mitochondrial cytochromes (Fig. 2). Pigments with spectral signatures diagnostic of cytochromes $c+c_1$ (maximum absorbance at 550 nm), b (maximum absorbance at 562 nm), and cytochromes $a+a_3$ (maximum absorbance at 603 nm) are easily observed in cells at the beginning of the shift. From low temperature spectra of these cells we have found that cytochrome c contributes about 60% of the absorbance at 550 nm while cytochrome c_1 contributes the rest [23]. Because cytochromes a and a_3 have overlapping absorption bands in both low temperature and room temperature spectra, it is difficult to quantitate them separately. From Fig. 2, it is clear that both cytochromes $c+c_1$ and cytochrome

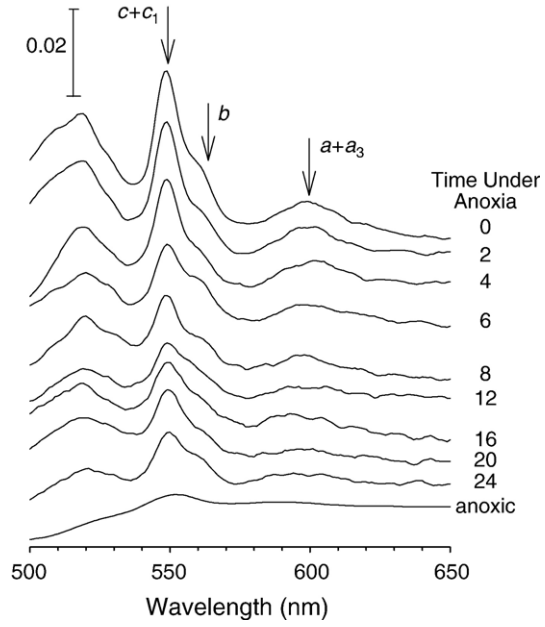

Fig. 2. Reduced minus oxidized difference spectra of *S. cerevisiae* JM43 during adaptation to anoxia. Suspensions of whole cells (0.1 g/ml) were reduced sequentially with glucose and sodium dithionite prior to being oxidized with hydrogen peroxide. The sample was measured spectrophotometrically after the addition of each reductant or oxidant. Difference spectra were constructed by subtracting the absolute oxidized spectra from the absolute reduced spectra. The absolute spectra were smoothed, using the data acquisition software, as required. The peaks corresponding to the respiratory cytochromes are indicated by the arrows; cytochrome $c+c_1$, 550 nm, cytochrome b, 560 m, and cytochrome $a+a_3$, 601 nm. The time after the shift is indicated on the right.

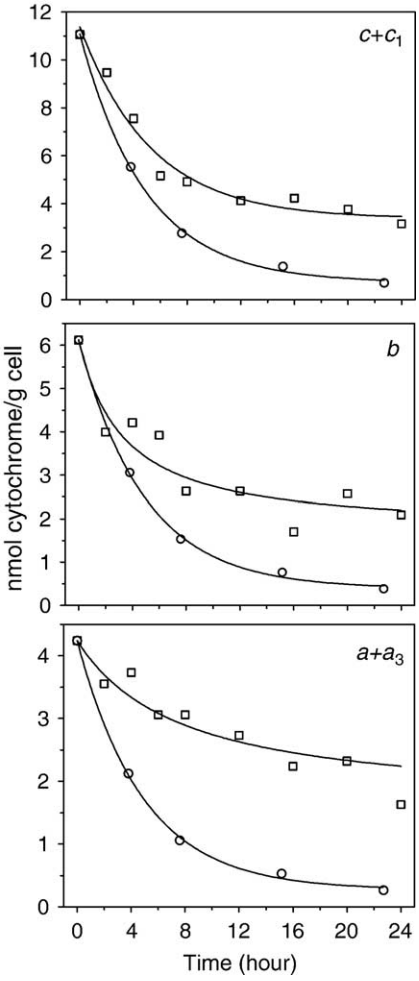

Fig. 3. Actual vs. theoretical cytochrome concentration in *S. cerevisiae* JM43 cells adapting to anoxia. The measured cytochrome concentration (□) was calculated from the reduced minus oxidized difference spectra shown in Fig. 2. The slope through the measured concentration is a best fit exponential decay with three to five variables. The theoretical cytochrome concentration was calculated from the measured 0 h concentration divided by the measured culture doubling time (○).

b are readily observable out to 24 h after a shift. In contrast, the spectral signatures for cytochromes $a+a_3$ are strong for the first 6 h after a shift and then become more diffuse. Interestingly, beginning 6 or so hours after a shift to anoxia the difference spectra in this region experience a blue shift with maximal absorbances that range from 595 to 600 nm. A similar finding has been reported when rat carotid bodies or HepG2 cells are exposed to low oxygen concentrations [42]. By quantitating cytochrome levels, we have determined that cytochromes $c+c_1$, cytochrome *b*, and cytochromes $a+a_3$ levels decline rapidly after a shift and reach levels that are 27%, 33% and 35%, respectively, of their aerobic levels by 24 h after the shift.

3.3. New cytochromes are produced in anoxic cells

There are at least three possible explanations for why the levels of respiratory cytochromes decline upon a shift from normoxic to anoxic growth (Figs. 2 and 3). First, the decline in cytochrome concentration could be due to anoxia-induced degradation of the cytochromes per se. Second, the cytochromes could be stable but in the absence of oxygen no new cytochromes would be synthesized. In this model, cytochrome levels would decline by simple dilution and would be reduced by a factor of two with every cell doubling. Third, cytochromes could be synthesized under anoxic conditions but at a lower level than in normoxic cells. If the first mechanism were operative, we would expect cytochrome levels to decline rapidly upon cellular exposure to anoxia, at a rate that exceeds the rate predicted by simple dilution due to cell doubling. If the second mechanism were operative, the rate of decline in cytochrome levels should be that expected by simple dilution due to cell doubling. And if the third mechanism were operative cytochrome levels should decline at a rate that is slower than that expected by simple dilution due to cell doubling. Fig. 3 shows our measured cytochrome levels compared to cytochrome levels predicted from simple dilution due to cell mass doubling. The levels predicted (open circles) are based on the initial measured concentration and actual culture doubling time. The measured cytochrome concentrations decline at differing rates, but all decline at a rate that is slower than predicted by simple dilution. This result is consistent with the third mechanism and indicates that each cytochrome is being synthesized and assembled by the dividing cells during the shift to anoxic growth.

The production of cytochromes under anoxic conditions requires that both the protein and heme prosthetic groups be synthesized in the absence of oxygen. The best understood cytochromes in this regard are cytochrome *c* and COX. Previous work has shown that the genes for iso-2-cytochrome *c* as well as many COX subunits are transcribed in the absence of oxygen [30], and all of the polypeptide subunits of COX are present, at reduced levels, in anoxic cells [33]. In addition, the genes coding for many of the proteins required for the assembly of COX are expressed in the absence of oxygen [4,6]. In order to determine how oxygen availability affects intracellular heme levels we extracted total, non-covalently bound heme (i.e., hemes A and B), from cells that had been grown normoxically or anoxically (Table 1). It is clear from these data that both types of heme are present in cells that had been grown in the complete absence of oxygen for several generations. To ask if these and other hemes form functional cytochromes in anoxic cells we grew cells to steady state in the complete absence of oxygen and then assayed for their ability to

Table 1
Heme concentration of *S. cerevisiae* JM43 cells

	Normoxic[a]	Anoxic
Heme A	6.5	1.8
Heme B	90	38

[a] Heme concentration expressed in pmol/mg protein as described in [40].

Table 2
Respiratory capacity of *S. cerevisiae* JM43 cells

	mmol/min/mg cells	%
Normoxic	9.1±1.7	100
Anoxic	1.0±0.3	11

Respiration capacity of *S. cerevisiae* JM43 grown under normoxic and steady state anoxic conditions.

respire when given oxygen. From Table 2, it is clear that these cells have cyanide-sensitive respiration, albeit at a reduced level, compared to that seen in normoxic cells.

By demonstrating that yeast cells control the level of their respiratory cytochromes in an oxygen-regulated manner, the above findings substantiate several previous studies ([43] and references therein). In addition, our findings indicate that yeast cells have the ability to produce a functional respiratory chain in the complete absence of oxygen, which usually functions as its physiological electron acceptor.

3.4. The turnover rate of cytochrome c oxidase exhibits a transient rise followed by a rapid decline when cell are shifted to anoxia

It is clear from Fig. 4 that there is an immediate but transient increase in the in vivo turnover rate of cytochrome *c* oxidase when yeast cells are shifted from normoxia to anoxia. The turnover rate increases during the first 2 h after the shift, reaching a maximum that is 20% higher than its normoxic level before declining to a minimum at 24 h that is about 15% of that in normoxic cells. These findings have interesting implications concerning the effects of oxygen on the functionality of COX in vivo. First, the transient increase in turnover rate observed immediately after a shift just precedes the transient increase in oxidative stress observed when yeast cells experience anoxia [18], suggesting that this change in turnover rate may contribute to cellular oxidative stress. Second, the reduced turnover rate observed several hours after the shift may indicate that the COX that is present in anoxic cells is catalytically different

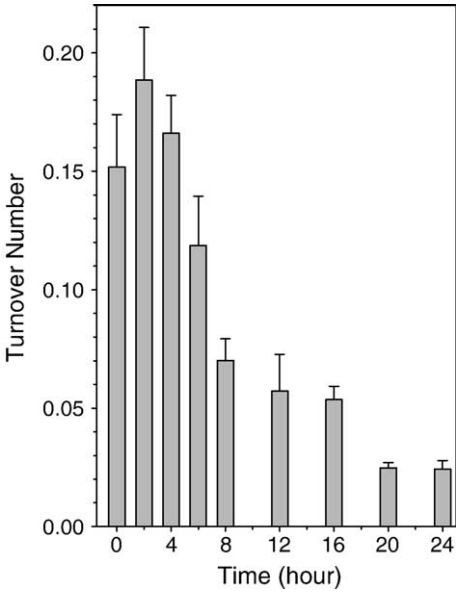

Fig. 4. Turnover rate of COX in *S. cerevisiae* JM43 cells adapting to anoxia. The turnover rate for COX in *S. cerevisiae* JM43 cells was calculated using the measured respiration rate (Fig. 1) and cytochrome *c* oxidase concentration (Fig. 3) determined at each time point harvested. Units of turnover rate are expressed as 1 mole oxygen consumed/min/mole cytochromes $a+a_3$.

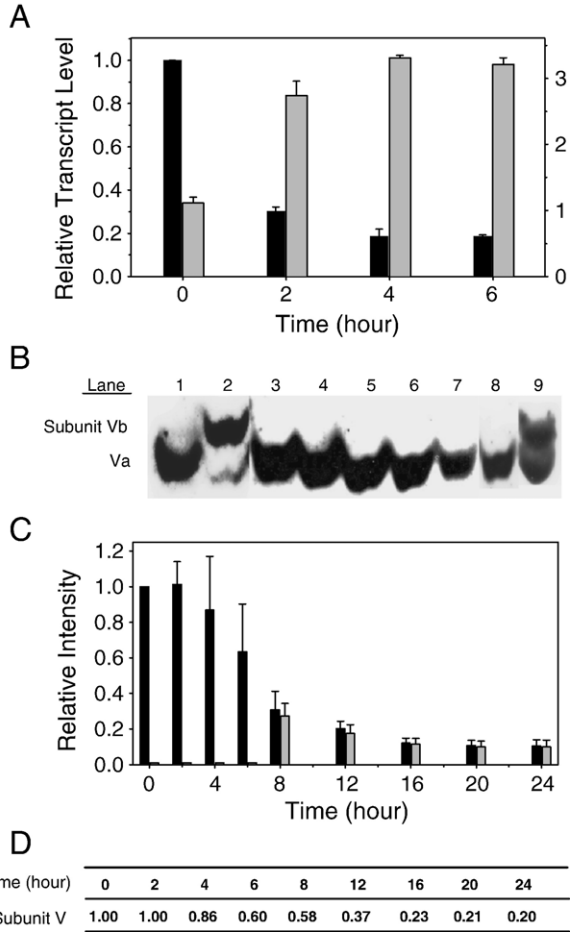

Fig. 5. Effects of a shift from normoxia to anoxia on expression of COX5a and COX5b and subunit V isoform levels. (A) Relative transcript level of *COX5a* and *COX5b.* Total RNA isolated from cells adapting to anoxia was electrophoresed and blotted. The RNA transcript levels were normalized to the RNA loaded per lane as described in Materials and methods. Transcript levels for *COX5a* are expressed relative to the level at 0 h and at 24 h for *COX5b* then set to 1.0. *COX5a*; black bars and left axis, *COX5b*; grey bars and right axis. (B) Western Immunoblot of isolated mitochondria. Mitochondria were isolated from cells harvested during adaptation to anoxia (see Materials and methods). Subunit Va and Vb were separated by SDS-PAGE and visualized by Western Immunoblotting. Lanes 1 to 9 show steady state aerobic, steady state anaerobic, and 0 h, 1 h, 2 h, 3 h, 4 h, 5 h and 20 h post anoxia, respectively. (C) Concentration of COX subunits Va and Vb in isolated mitochondria adapting to anoxia. The amount of subunit Va and Vb was quantitated as signal intensity/μg protein loaded per lane. Data points are an average of 5–7 immunoblots from three experiments. Black bars are subunit Va and grey bars are subunit Vb. (D) Total Subunit V present during adaptation to anoxia as a percentage of the 0-h level.

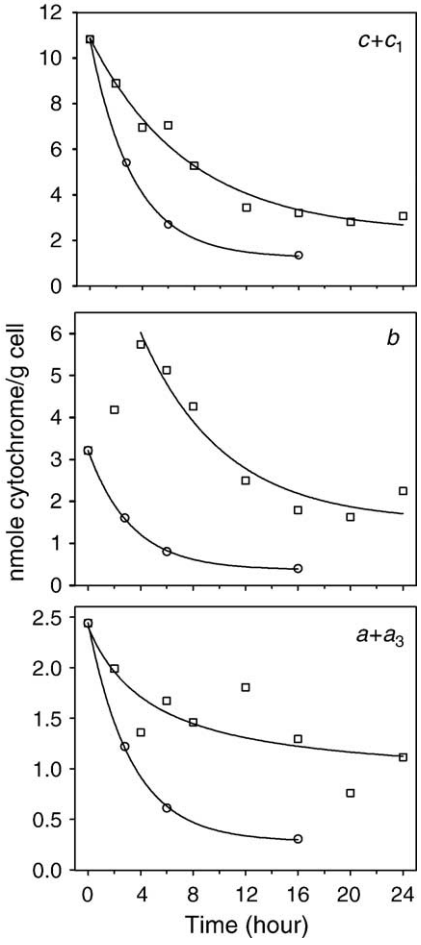

Fig. 6. Actual vs. theoretical cytochrome concentration in *S. cerevisiae* HC5b cells adapting to anoxia. The measured cytochrome concentration (□) was calculated from reduced minus oxidized difference spectra of whole cells. The slope through the measured concentration is a best fit exponential decay using 3–5 variables. The theoretical cytochrome concentration (○) was calculated from the 0 h concentration divided by the measured culture doubling time. For cytochrome *b*, the best fit exponential decay was determined from the 4 to 24 h data.

than the COX that is present in normoxic cells. Finally, these data suggest that the maximal turnover rate of yeast COX is not achieved in cells grown in the presence of oxygen, its physiological substrate, but rather is achieved in cells that have been deprived of oxygen for short periods of time.

3.5. The switching of subunit V isoforms is not responsible for changes in turnover rates when cells are exposed to anoxia

At the time of the shift from normoxia to anoxia yeast cells contain COX subunit Va, the aerobic isoform of subunit V. Because the hypoxic Vb isoform supports a higher turnover rate than Va and is up-regulated when cells experience hypoxia, it was of interest to ask if the change in turnover rate could be explained by subunit V isoform switching. Three types of experiments were performed to address this question. First, we examined the level of transcripts from *COX5a* and *COX5b* after a shift from normoxia to anoxia. As can be seen in Fig. 5A, there is a rapid decline in *COX5a* mRNA levels and a slow but steady increase in *COX5b* mRNA levels immediately after a shift to anoxia. Second, we assessed the level of subunits Va and Vb in mitochondria from JM43 cells at different times after a shift to anoxia by immunoblot analysis, using an antibody made to a synthetic peptide constructed to correspond to the 20 amino acids at the carboxyl terminus that is common to both Va and Vb [23]. It is clear from Fig. 5B that subunit Vb is not detectable for up to 5 h after the shift and that the only subunit V isoform present during this period is Va. Immunoblot analysis of mitochondria isolated from cells out to 24 h reveals that subunit Vb is not detectable until after 6 h, and is present in levels that approximate those of Va out to 24 h (Fig. 5C). The total subunit V content, both Va and Vb, shows a continual decline immediately after a shift to anoxia. It stabilizes at about 20% of the aerobic level between 16 and 24 h (Fig. 5D).

The above data make it unlikely that the increase in COX turnover rate between during the first 2 h after a shift can be explained by subunit V isoform switching. To confirm this we did a third experiment in which we looked at the behavior of the respiratory chain and COX in a strain, HC5b, constructed to carry just subunit Vb. Because the Vb promoter is functional, albeit at low levels in normoxic cells, it is possible to increase cellular levels of Vb merely by expressing it from a high copy plasmid under normoxic conditions [29]. Indeed, the level of respiration in HC5b is comparable to that in JM43 ([29] and data not shown). Cytochrome levels in HC5b decline after a shift from

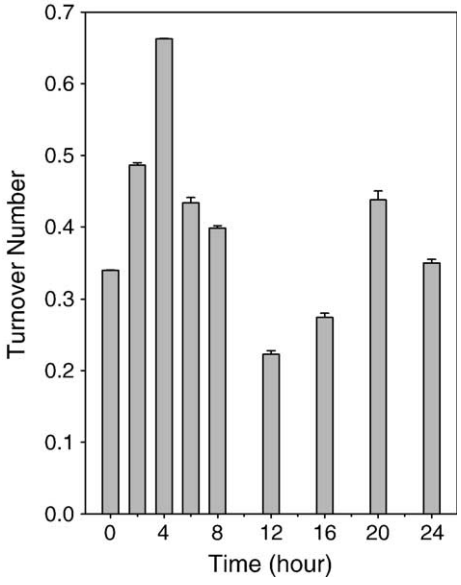

Fig. 7. Turnover rate of COX in *S. cerevisiae* HC5b whole cells adapting to anoxia. The turnover rate of COX was calculated from the respiration capacity measured in whole cells (data not shown) and the cytochrome concentration shown in Fig. 6. Units of turnover rate are expressed as 1 mole oxygen consumed/min/mole cytochromes $a+a_3$.

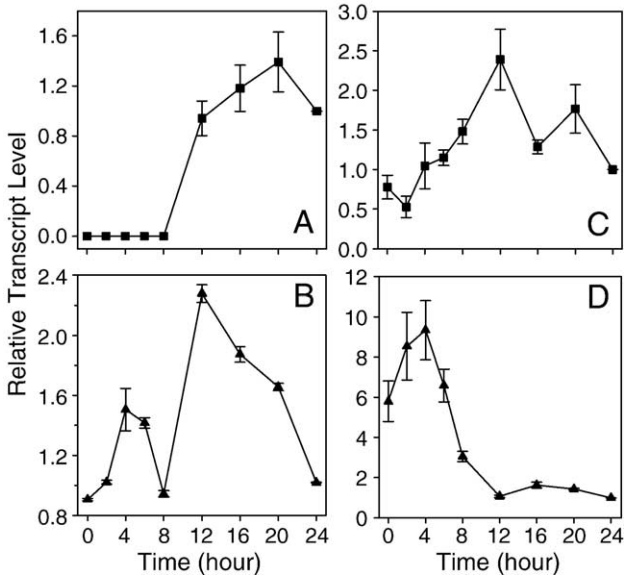

Fig. 8. Expression of CYC7 and OLE1 in *S. cerevisiae* strains JM43 and HC5b during adaptation to anoxia. Total RNA was isolated from JM43 and HC5b cells at different times during a shift to anoxia. RNA was electrophoresis, blotted and normalized as described (Materials and methods). The transcript level is expressed relative to the 24-h value and set to 1.0. (A) *CYC7* from strain JM43; (B) *CYC7* from strain HC5b; (C) *OLE1* from strain JM43; (D) *OLE1* from strain HC5b.

normoxia to anoxia (Fig. 6). Interestingly, the turnover rate of COX in HC5b also increases during the shift from normoxia to anoxia. However, in contrast to JM43, the turnover rate of COX in HC5b increases by nearly 90% and is maintained at nearly its normoxic level for up to 24 h after the shift from normoxia to anoxia (Fig. 7). These findings clearly demonstrate that COX isozymes containing either Va or Vb show an increase in turnover rate after cells are exposed to anoxia and, together with the results presented above, clearly rule out the possibility that this increase is due to isoform switching. They also reveal, however, that cells containing the Vb isozyme alone maintain a higher turnover rate at 24 h after a shift than cells containing a mixture of Va and Vb isozymes.

3.6. Effect of cytochrome c oxidase subunit V isozymes on hypoxic gene induction

To ask if the COX subunit V isozymes affect the induction of hypoxic nuclear genes we compared the kinetics of appearance of transcripts from two hypoxic genes, *CYC7* and *OLE1*, in JM43 and HC5b. Both genes have been shown previously to require the mitochondrial respiratory chain for their increased expression when cells are exposed to anoxia. The data in Fig. 8 reveals that there are two significant differences between JM43 and HC5b in the level of expression and kinetics of induction of both genes. First, transcript levels for both genes are much higher under normoxic conditions (0 h) in strain HC5b than in JM43 (Fig. 8). Second, in HC5b, both *CYC7* and *OLE1* are induced sooner after the shift to anoxia than they are in strain JM43. Interestingly, the *CYC7* transcript from *S. cerevisiae* HC5b shows two phases of induction, suggesting two levels of regulation of this transcript. *CYC7* is present at a high level aerobically and is strongly induced with the onset of anoxia (Fig. 8B), whereas in *S. cerevisiae* JM43, the *CYC7* transcript is not present at detectable levels until 12 h post anoxia (Fig. 8A). These findings reveal that the type of subunit V isozyme present in normoxic cells at the time of a shift from normoxia to anoxia has a significant affect on both the level of expression and the kinetics of appearance of at least two hypoxic genes that require the mitochondrial respiratory chain for their induction. Previously, we have shown that the respiratory chain is not required for the down-regulation of aerobic genes in cells exposed to anoxia [33]. To confirm that the differences we observe between JM43 and HC5b are attributable to the effects the subunit V isoforms have on respiration, we looked at the down-regulation of *CYC1* in both strains after a shift to anoxia. As expected, the down-regulation of *CYC1* after a shift is identical in both strains (Fig. 9).

Considered together, our findings that the isoforms of subunit V can affect the expression pattern of at least two hypoxic genes provides additional support for the conclusion that COX and crosstalk from the mitochondrial respiratory chain is important for the expression of some oxygen-regulated nuclear genes.

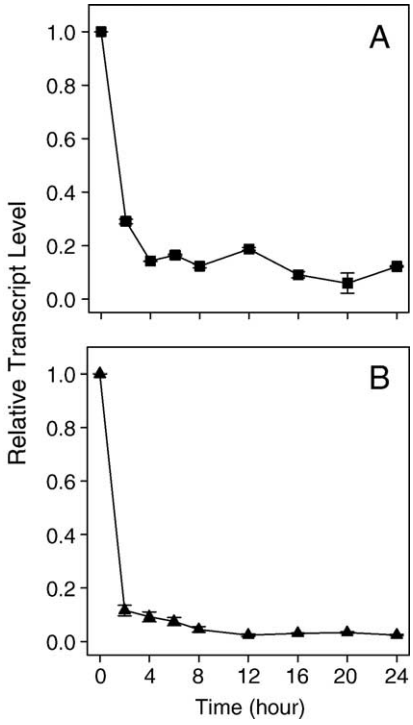

Fig. 9. Expression of CYC1 in *S. cerevisiae* JM43 and HC5b during adaptation to anoxia. Total RNA was isolated at different times during the shift anoxia. RNA was electrophoresed, blotted and normalized as described (Materials and methods). Transcripts levels are expressed relative to the 0 h time and set to 1.0. (A) JM43; (B) HC5b.

4. Discussion

In our continuing effort to understand the involvement of the mitochondrial respiratory chain in the induction of hypoxic nuclear genes in eukaryotes, we have been examining the effects of cellular exposure to anoxia on the level and functionality of the mitochondrial respiratory chain in yeast. The results reported here provide important new insight concerning the effects of cellular exposure to reduced oxygen concentrations on COX and its involvement in hypoxic gene induction. First, they reveal that although cytochrome levels decline starting immediately after cellular exposure to anoxia mitochondrial respiration capacity does not decline until 4 h. Second, the turnover rate of COX exhibits two changes. It increases transiently after cellular exposure to anoxia and then declines. The increase in turnover rate corresponds with the timing of the transient increase in oxidative stress we observed previously and cannot be explained by subunit V isoform switching. Third, the normoxic and hypoxic isoforms of COX subunit V have differential effects on the induction of hypoxic nuclear genes, providing further support for a role of COX as a cellular oxygen sensor. And fourth, a functional mitochondrial respiratory chain is present, albeit at greatly reduced levels, in anoxic cells.

4.1. Effects of reduced oxygen levels on the mitochondrial respiratory chain and cytochrome c oxidase

By using an experimental strategy in which growing cells are shifted rapidly from normoxic to anoxic growth and then monitored for 24 h in a fermentor, it has been possible to analyze both short term and long term effects of reduced oxygen levels on the respiratory chain in general and COX in particular. For convenience, we consider short term effects to be changes that occur during the first mass doubling (i.e. 4 h) after the shift and long term effects to be changes that occur between 4 and 24 h after the shift. In the short term, we have found that the capacity of mitochondria to respire is maintained at normoxic levels even though the levels of cytochromes $c+c_1$, b and $a+a_3$ drop by 20 to 30%. These findings are intriguing because they indicate that cells maintain their ability to respire at normoxic levels for at least one cell doubling in the absence of oxygen, their physiological electron acceptor.

We also find that the turnover rate of COX, with oxygen as an electron acceptor, increases above its normoxic rate during the first 4 h after a shift. This finding implies that cellular exposure to anoxia results in an immediate alteration of COX per se. Although it is well established that the hypoxic isoform, Vb, of COX subunit V enhances the turnover rate of COX relative to its aerobic counterpart, Va [24] we have been able to determine that the increase in turnover rate we observe during the first 4 h is not attributable to subunit V isoform switching. Indeed, the only subunit V isoform that is present during this period is Va, the isoform that was present in cells at the beginning of the shift. The Vb isoform does not appear until 8 h after the shift. Additional evidence that the increase in turnover rate is not subunit V isoform-dependent comes from our finding that the turnover rate of COX in a strain constructed to carry just Vb also exhibits an increase during the first 4 h after a shift from normoxia to anoxia.

Given that isoform switching is not involved in the alteration in turnover rate of COX in the short term, it is likely that the increase in turnover rate we observe is due to allosteric regulation of the holoenzyme. It has been known for some time that ATP and the intramitochondrial ATP/ADP ratios have marked effects on the kinetic properties of COX from a variety of eukaryotes including yeast [28,44–46]. During cellular exposure to low oxygen, ATP generation by oxidative phosphorylation drops in yeast and other organisms [47,48]. This leads to a decrease in ATP/ADP ratio. In yeast cells, this decrease in ATP/ADP ratio is responsible for a drop in cytosolic pH due to decreased activity of the plasma membrane and vacuolar H^+/ATPases, and an influx of protons across the plasma membrane via H^+ symport with phosphate [48]. The net result of shifting yeast cells from normoxic to anoxic growth is a dramatic short term increase in the ratio ([cytoplasmic PO_4][ADP]/[ATP]) [48]. The increase in this ratio would be expected to enhance the activity of COX and may account for the increased turnover rate we observe during the first 4 h after a shift.

In the long term, we have found that respiration capacity and cytochrome levels in both JM43 and HC5b strains decline dramatically between 4 and 24 h after cells are exposed to anoxia. This is not surprising considering that most of the genes that encode subunits of respiratory proteins are aerobic genes that are down-regulated under anoxic conditions [24]. We have also found that the turnover rate of COX in JM43 declines dramatically during this period. It is not clear why the COX turnover rate in JM43 decreases during this period. JM43 has functional *COX5a* and *COX5b* genes and expresses both subunit V isoforms between 8 and 24 h after the shift. Although expression of Vb would be expected to increase the turnover rate of COX this effect is damped down by the presence of the hypoxic isoform, iso-2 cytochrome *c*, of cytochrome *c* [23] that is expressed during this period. The co-expression of the hypoxic isoforms of COX subunit V and cytochrome *c* cannot, however, explain a reduction in turnover rate. Other factors relating to adaptation to anoxia [47,48] are most likely involved. It is interesting that no such decline in COX turnover rate is observed in HC5b. This strain has been engineered to carry only the Vb isoform of subunit V. As expected, the turnover rate of COX at the time of the shift is higher than in JM43. It is noteworthy that during the first 4 h after a shift to anoxia the increase in COX turnover rate in this strain is substantially higher than that in JM43 and that the turnover rate is maintained at nearly the normoxic level between 4 and 24 h. The maintenance of the COX turnover

rate at its normoxic level in this strain is more than likely due to the continued presence of the Vb isoform throughout the duration of the shift.

4.2. The subunit V isoforms of cytochrome c oxidase affect the induction of at least two hypoxic genes

The higher COX turnover rate in HC5b relative to JM43 allowed us to examine whether the two COX subunit V isoforms affected the expression of two hypoxic genes, *CYC7* and *OLE1*, when cells are exposed to anoxia. Both genes had been shown earlier to require the respiratory chain for their induction under these conditions [17]. It is clear that both genes are induced earlier in HC5b than in JM43. Given the differences in COX turnover rates in these strains at the start of the shift and during the first 4 h after the shift and the finding that induction occurs in HC5b during this period, it is interesting to speculate that either the turnover rate of COX per se or its increase during the first 4 h after a shift affects the expression of these genes and, in fact, may be an early determinant in their induction when cells are exposed to anoxia. Although more work is required to understand the mechanistic basis for this the findings presented here clearly support our earlier conclusion that COX is involved in the induction of some hypoxic nuclear genes.

4.3. Anoxic yeast cells contain cytochromes and a functional respiratory chain

Our findings that new cytochromes are made during the 24-h period after a shift and that anoxic cells contain cytochromes extends our previous findings that several COX genes are expressed in anoxic cultures and that all of the COX subunits are present in mitochondria from anoxic cells [30,33]. Moreover, our observation that anoxic cells are capable of respiring oxygen indicates that anoxic cells have a functional respiratory chain.

Previous studies have reported that heme biosynthesis in yeast requires molecular oxygen because two of the enzymes (coproporphyrinogen III oxidase and protoporphyrinogen IX oxidase) in the heme biosynthetic pathway require oxygen as a substrate [49]. However, it has also been reported previously that cells from anoxic cultures also contain small amounts of heme [50]. The latter has been assumed to result from trace oxygen levels in these cultures [50]. It is unlikely that our finding that anoxic cells contain hemes A and B is due to the presence of trace amounts of oxygen in our cultures for several reasons. First, we have used extremely pure gases and oxygen scrubbers to remove any trace levels of oxygen that may be present. Second, we monitor oxygen concentrations throughout our experiments with extremely sensitive dissolved oxygen probes. And third, any trace amounts of oxygen in the fermentor would be rapidly metabolized leaving it oxygen free. Given these considerations, we believe that it is more likely that yeast cells do not require oxygen as an obligate electron acceptor, as proposed earlier [51]. Although it is usually assumed that two of the enzymes (coproporphyrinogen III oxidase and protoporphyrinogen IX oxidase) in the yeast heme biosynthetic pathway require oxygen as a substrate it has been reported previously that at least one of these enzymes, purified coproporphyrinogen III oxidase, can function in the absence of oxygen [52]. It has also been suggested that electron carriers other than oxygen can function during heme biosynthesis [53]. In this regard, it is interesting that *E. coli* has both an oxygen-dependent and oxygen-independent coproporphyrinogen III oxidase [54]. Recently, *hemN*, a gene coding for an oxygen-independent coproporphyrinogen III oxidase was cloned and sequenced from *E. coli* [55] and a number of other bacteria (see ref in [55]). The *E. coli* enzyme uses S-adenosyl methionine as an electron acceptor; it has been crystallized and its mechanism is under study [56]. A second gene, *hemZ*, was also shown to catalyze oxygen-independent coproporphyrinogen III oxidase activity [57]. A search of the *S. cerevisiae* ORF protein database using the conserved cysteine motif found to be common to all *hemN* proteins revealed one match to a protein of unknown function, YDR210W. A second search using the sequence common to *hemZ* found 38 hits in the yeast ORF protein database. This finding together with the fact that S-adenosyl methionine is present in yeast cells [58] opens the possibility that oxygen-independent proto- and coproporphyrinogen oxidases exist in yeast. Studies addressing this possibility are currently underway.

The finding that anoxic cells are able to respire when put in the presence of oxygen and that this respiration is cyanide-sensitive clearly indicates that a functional respiratory chain is maintained in the absence of oxygen. The maintenance of this chain under anoxic conditions implies that it functions in the absence of oxygen, most likely by using an alternative electron acceptor. The possibility that the mitochondrial respiratory chain can use an alternative electron acceptor when oxygen is unavailable is interesting in the context of the induction of hypoxic nuclear genes. Previously, we have reported that cyanide inhibits the hypoxic induction of *CYC7* and *OLE1* when applied to both aerobic cells and cells that have been deprived of oxygen for 1 h [17]. The latter finding implies that the respiratory chain is functional in the absence of oxygen. It supports the hypothesis that the chain is capable of using an electron acceptor other than oxygen and that electron transport to this alternative acceptor is instrumental in the hypoxic gene induction pathway.

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