Effects of Oxygen Concentration on the Expression of Cytochrome c and Cytochrome c Oxidase Genes in Yeast*

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Oxygen is an important environmental regulator for the transcription of several genes in Saccharomyces cerevisiae, but it is not yet clear how this yeast or other eukaryotes actually sense oxygen. To begin to address this we have examined the effects of oxygen concentration on the expression of several nuclear genes (CYC1, CYC7, COX4, COX5a, COX5b, COX6, COX7, COX8, and COX9) for proteins of the terminal portion of the respiratory chain. COX5b and CYC7 are hypoxic genes; the rest are aerobic genes. We have found that the level of expression of these genes is determined by oxygen concentration per se and not merely the presence or absence of oxygen and that each of these genes has a low oxygen threshold (0.5–1 μM O2) for expression. For some aerobic genes (COX4, COX5a, COX7, COX8, and COX9) there is a gradual decline in expression between 200 μM O2 (air) and their oxygen threshold. Below this threshold expression drops precipitously. For others (COX5a and CYC1) the level of expression is nearly constant between 200 μM O2 and their threshold and then drops off. The hypoxic genes COX5b and CYC7 are not expressed until the oxygen concentration is below 0.5 μM O2. These studies have also revealed that COX5a and CYC1, the genes for the aerobic isoforms of cytochrome c oxidase subunit V and cytochrome c, and COX5b and CYC7, the genes for the hypoxic isoforms of cytochrome c oxidase subunit V and cytochrome c, are coexpressed at a variety of oxygen concentrations and switch on or off at extremely low oxygen concentrations. By shifting cells from one oxygen concentration to another we have found that aerobic genes are induced faster than hypoxic genes and that transcripts from both types of gene are turned over quickly. These findings have important implications for cytochrome c oxidase function and biogenesis and for models of oxygen sensing in yeast.

The intracellular levels and activities of a large number of proteins in Saccharomyces cerevisiae are affected by oxygen tension (1–3). Many of these proteins are involved in metabolic pathways or processes that use oxygen or reactive oxygen species as substrates. They include: cytochromes of the respiratory chain; enzymes involved in the synthesis of heme, sterols, or unsaturated fatty acids; and enzymes that function in the oxidative stress response (1, 3). The effects of oxygen on the intracellular levels of many of these proteins have been shown to be exerted through the transcription of their genes. Most of these oxygen-regulated genes can be placed into one of two groups: aerobic genes, which are transcribed optimally in the presence of air; and hypoxic genes, which are transcribed optimally under anoxic or microaerophilic conditions.

It is not yet clear how oxygen is sensed in yeast and most other eukaryotes (3). Also unclear is how an “oxygen sensor” transmits its signal to a signal transduction pathway for the activation or repression of oxygen-responsive genes and whether there is more than one signal transduction pathway involved in oxygen-regulated transcription. Most previous studies on oxygen-regulated gene expression in yeast have been done with cells grown either aerobically or anaerobically or with hem1 mutant cells grown either in the presence or absence δ-aminolevulinate. These studies have revealed that some genes are down-regulated in the absence of oxygen (or δ-aminolevulinate), and others are up-regulated (1). Although these studies have been useful in identifying some of the molecular components that are involved in oxygen-regulated gene expression they have not provided insight concerning how cells actually sense different oxygen concentrations. One obvious question raised by these studies is whether oxygen-regulated genes respond in an “all-or-none” fashion to the presence of oxygen or whether they respond in a graded fashion to different oxygen concentrations.

The dose-response effects of oxygen on the expression of the genes for cytochrome c oxidase are especially interesting because as a major consumer of oxygen (4) this enzyme plays an important role in determining the rate of energy production in yeast and other eukaryotes (5, 6). Yeast cytochrome c oxidase contains polypeptide subunits encoded by both nuclear and mitochondrial genes (6, 7). The three largest subunits (I, II, and III) are encoded by mitochondrial genes (COX1, COX2, and COX3); these perform the catalytic functions of the holoenzyme (5–8). The other subunit polypeptides are encoded by nuclear genes; some of these modulate catalysis, whereas others function in the assembly or stability of the holoenzyme (6, 7). Active preparations of yeast cytochrome c oxidase contain at least six subunits (IV, Va or Vb, VI, VII, VIIa, and VIII) encoded by the nuclear COX genes (COX4, COX5a or COX5b, COX6, COX7, COX9, and COX8, respectively) (6, 7). COX5a and COX5b encode interchangeable isoforms, Va and Vb, of subunit V (5, 9–11). The other subunits are specified by single copy genes. All of these genes, except COX5b, are aerobic genes (2, 3). Because COX5a is an aerobic gene and COX5b is a hypoxic gene, the expression of the subunit V isoforms, Va and Vb, is inversely regulated by oxygen tension (5, 12). The differential effects of oxygen on the expression of Va and Vb are especially interesting because these isoforms have differential effects on holoenzyme activity (13, 14).

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Oxygen Regulation of Cytochrome Genes

RESULTS

Development of Culture Conditions—To examine the effects of oxygen on the expression of the CYC and nuclear COX genes in *Saccharomyces cerevisiae* it is necessary to use culture conditions where oxygen is the limiting nutrient and where the effects observed are due to oxygen concentration and not some other variable such as carbon source or growth rate.

To assure that oxygen was the limiting nutrient in these studies, cells were grown in batch fermenter cultures sparged with feed gas mixtures that contained different fixed concentrations of oxygen, and growth was terminated when oxygen demand exceeded supply. This was signaled by a decrease in the dissolved oxygen concentration in the culture. To be able to use the same criterion for high and low oxygen concentrations, where the total current to the oxygen probe was small, we harvested cells when the dissolved oxygen concentration in the culture fell to 80% of the oxygen concentration in the feed gas mixture. This occurred in early to mid-log phase depending upon the oxygen concentration in the feed gas. Cell densities reached 80% of the initial dissolved oxygen decreased as the oxygen concentration in the feed gas decreased (Table I). To ensure that cells were in steady-state growth we inoculated the batch fermenter cultures with a 10x dilution of an exponentially growing culture and adjusted the initial cell densities to allow for at least six mass doublings before harvest.

To minimize the effects of carbon source on the expression of the CYC and nuclear COX genes cells were grown on the nonrepressing carbon source, galactose. Because cells were harvested at low cell densities under our experimental conditions galactose concentration stayed nearly constant to the

### Table I

| Oxygen concentration in feed gas | Cell density at harvest* |
|---------------------------------|--------------------------|
| %                               | g wet weight/liter        |
| 21 (air)                        | 5.1                      |
| 5                               | 5.6                      |
| 1                               | 2.0                      |
| 0.5                             | 1.0                      |
| 0.05                            | 0.3                      |
| 0.01                            | 0.1                      |
| 0 (O2-free N2 + 2.5% CO2)       | 5.1                      |

*Cells were harvested when the dissolved oxygen in the culture reached 80% of the oxygen concentration in the feed gas.

Approximately 30 μg of total RNA was loaded per lane; loading was adjusted to give equal signals for hybridization to the ACT1 gene. DNA probes were prepared by random primer labeling of double-stranded DNA fragments using [α-32P]dATP or [α-32P]dCTP (DuPont NEN). Probes were an 800-bp *Xba*I fragment for *COX4*, a 500-bp *Pst*I fragment for *COX5a*, a 370-bp *Acc*I/*Bgl*II fragment for *COX5b*, a 500-bp *Stu*I/*Bgl*II fragment for *COX6*, an 800-bp *Eco*RI fragment for *COX7*, a 370-bp *Xba*I/*Sty*I fragment for *COX8*, a 240-bp *Clai*/*Nhe*I fragment for *COX9*, a 600-bp *Eco*RI/*Hind*III fragment for *CYC1*, a 400-bp *Rsa*I/*Xho*I fragment for *CYC7*, and a 520-bp *Sst*I fragment for *ACT1*. Hybridization and stringency washes were performed as described previously (11). Signal intensity was quantitated with an AMBIS Radioanalytic Imaging system. For quantitation of transcripts, signals were normalized to that for *ACT1* mRNA.

Miscellaneous—Galactose concentration in yeast culture filtrates was determined colorimetrically with *o*-toluidine. Toluidine reagent (6% (v/v) *o*-toluidine in glacial acetic acid with thiourea as stabilizer) was obtained from Sigma Chemicals. Five ml of toluidine reagent was mixed with 0.1 ml of cell culture filtrate, heated to boiling for 10 min, cooled quickly, and read at A435 against a water blank. Assays were done in triplicate, and galactose concentrations were calculated using a 0.1% galactose standard curve. The following Matheson gases or gas mixtures were obtained from U.S. Welding (Denver): O2-free nitrogen, 1% CO2 in O2-free nitrogen, 2.5% CO2 in O2-free nitrogen, 5% O2 in nitrogen, 10% O2 in nitrogen, and certified standards containing 0.05, 0.1, 0.5, and 1.0% O2 in O2-free nitrogen.

### MATERIALS AND METHODS

**Strains and Media**—The yeast strain JM43 (*MATα leu-3, 112 his4–, thr2–, ura3–, ade2–, trp1–, can1–) was isolated by homologous recombination and used in this study (9). Strains used in this study have been described previously (9). The *MATα* strain was chosen because it has a high level of expression of *coxy* cytochrome content (11, 13), and expression of nuclear genes. We ask here if expression depends linearly on oxygen concentration or if instead expression changes at particular oxygen thresholds. We also examine the expression of the gene pairs *CYC1/COX5a* and *CYC7/COX5b* to determine if they are coexpressed at a variety of different oxygen concentrations. Finally, we examine the kinetics of appearance and disappearance of transcripts after cells are shifted from one oxygen concentration to another.

**Growth Conditions**—Cells were grown in a semisynthetic medium, SSG (per liter: 3 g Bacto yeast extract, 10 g of galactose, 0.8 g of potassium gluconate, 0.1 g of MgSO4·7H2O, 0.5 g of NaCl, 0.7 g of MgSO4·H2O, 0.5 g of FeCl3, 0.4 g of CaCl2) supplemented with 40 μg/ml leucine, histidine, tryptophan, and uracil; 0.1% Tween 80 (v/v); 20 μg/ml ergosterol; and 350 ppm Dow Corning FG-10 Silicone antifoam.

**Cell density at time of harvest for cells grown at various oxygen tensions**

1. The abbreviations used are: TN, turnover number; MOPS, 4-morpholinopropanesulfonic acid; bp, base pair.
time of harvesting. For cells grown aerobically (air) or anaerobically (2.5% CO2 in O2-free nitrogen) the galactose concentration declined only slightly, from a starting concentration of 1% (w/v) to a concentration of 0.7–0.8% (w/v), at the time of harvest. For cells grown at low to intermediate oxygen concentrations (i.e. 0.5–100 μM O2) galactose concentration declined even less because cells were harvested at lower densities. These slight changes in galactose concentration do not affect the expression of the genes studied here as judged from two observations. First, we observed no difference in the level of expression of COX5a in cells harvested at earlier times (i.e. lower cell densities) when the galactose concentration was higher. Second, the expression of COX5a and CYC1 did not change in cells grown aerobically to higher cell densities, when the galactose concentration had declined to nearly 0.4–0.5% (w/v). These findings rule out galactose concentration as a variable that affects expression of the CYC or nuclear COX genes under our experimental conditions.

The growth rate (expressed as mass doubling time) of strain JM43 in air is 2.7–2.8 h. Oxygen concentrations between 0.05 and 21% O2 (air) in the feed gas support equivalent rates of cell growth (data not shown). Surprisingly, we observed very slow growth rates (greater than an 8-h mass doubling time) with cells grown in O2-free nitrogen (i.e. anaerobic conditions) (Table II). We also observed that the growth rate in O2-free nitrogen was inversely proportional to the gas flow rate. This was surprising because it has been reported that nonsparged anaerobic cultures of S. cerevisiae grow at the same rate as aerobic cultures (21). To explain these observations we considered the possibility that carbon dioxide (CO2) was limiting in the nitrogen-sparged cultures. Although CO2 is not present initially in nonsparged cultures or in cultures that are sparged with O2-free nitrogen, it is produced metabolically by both types of culture. A feature that distinguishes nonsparged from sparged cultures is that CO2 is allowed to build up in nonsparged cultures but is blown off in sparged cultures. This could explain the slow growth observed in cultures sparged with O2-free nitrogen because sparging could result in a stripping of CO2 from the culture medium, preventing heterotrophic carbon dioxide fixation into the citric acid cycle, as observed previously for Escherichia coli (22). To examine this possibility we added CO2 to the sparge gas and asked if the growth rate was increased. From Table II it is clear that a gas mixture containing 1% CO2 in O2-free nitrogen supports a growth rate twice that supported by O2-free nitrogen and that a gas mixture containing 2.5% CO2 in O2-free nitrogen supports a growth rate that is close to that supported by air. Therefore, for studies comparing expression of CYC and the nuclear COX genes under aerobic and anaerobic conditions cells were grown anaerobically in a gas mixture containing 2.5% CO2 in O2-free nitrogen.

**Expression of CYC and Nuclear COX Genes under Aerobic and Anaerobic Conditions**—As a first step toward understanding how oxygen concentration affects the expression of oxygen-regulated genes we first determined the overall level of change in expression of the aerobic genes COX4, COX5a, COX6, COX7, COX8, COX9, CYC1, and the hypoxic genes COX5b and CYC7 in cells grown to steady state under aerobic conditions (i.e. air saturation, which corresponds to an oxygen concentration of 200 μM O2 at 28 °C and at the 1,350-m elevation of our laboratory in Boulder, CO) or anaerobic conditions (2.5% CO2 in O2-free nitrogen). RNA isolated from JM43 cells was subjected to Northern blot analysis using probes specific for each of the above mentioned genes as well as ACT1 and ACT2 as an internal control. From Fig. 1 (lanes 1 and 5) it is clear that mRNA levels from all of these aerobic genes are reduced in anaerobic cells. Some of these aerobic genes (COX4, COX5a, COX6, COX7, COX8, and CYC1) exhibit very little expression under anaerobic conditions, whereas others (COX5a and COX9) have moderate levels of expression. When normalized to ACT1 the level of expression of these genes varies from 3% (for CYC1) to 40% (for COX9) of their level in aerobically grown cells (Table III). It is also clear that the hypoxic genes COX5b and CYC7 are more tightly regulated by oxygen than the aerobic genes (Fig. 1, lanes 1 and 5) (Table III). They are expressed under anaerobic conditions but are not detected in air.

**Effects of Oxygen Concentration on Expression of CYC and Nuclear COX Genes**—To examine how oxygen concentration per se affects the expression of CYC and nuclear COX genes we first examined the level of gene expression in cells grown at oxygen concentrations, between 200 and 10 μM O2. From Figs. 1 and 2 it is clear that transcript levels for the aerobic genes COX4, COX5a, COX6, COX7, COX8, and CYC1 decrease relative to their aerobic levels in cells grown at 100, 50, and 10 μM O2. When normalized to ACT1 mRNA the levels of

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**TABLE II**

| Gas          | Growth rate* (h) |
|--------------|------------------|
| O2-free N2   | 8.0–9.0          |
| O2-free N2 + 1% CO2 | 4.2–4.4        |
| O2-free N2 + 2.5% CO2 | 3.0–3.2       |
| Air          | 2.7–2.8          |

* Mass doubling time.

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**TABLE III**

| Gene      | Ratio of gene transcript to ACT1 transcript* |
|-----------|--------------------------------------------|
| **Aerobic genes** |                                             |
| COX4      | 1                                           |
| COX5a     | 1                                           |
| COX6      | 0.8                                         |
| COX7      | 0.6                                         |
| COX8      | 0.4                                         |
| CYC1      | 0.3                                         |
| **Hypoxic genes** |                                             |
| COX5b     | ND*                                         |
| CYC7      | 1                                           |

* The ratio for aerobic genes was normalized to a value of 1 for expression under aerobic conditions; the ratio for hypoxic genes was normalized to a value of 1 for expression under anaerobic conditions.

* ND, not detected.
mRNA from all of the aerobic genes, except COX5a and CYC1, declined to about 40–70% of their aerobic levels at 100 μM and stayed nearly constant at oxygen concentrations between 100 and 10 μM O₂ (Fig. 2A). In contrast, the mRNA levels from COX5a and CYC1 are nearly the same in cells grown in air, 100, 50, and 10 μM O₂. These mRNAs are present at 85–95% of their aerobic levels in cells grown in 10 μM O₂ (Fig. 2B). From Figs. 1 and 2B it is also clear that no transcripts from the hypoxic genes, COX5b and CYC7, are observed in cells grown at oxygen concentrations between 200 and 10 μM O₂.

Together, these findings indicate that there is a gradual decline in the mRNA levels from the aerobic genes in cells grown at oxygen concentrations below 10 μM O₂, and that expression of all aerobic genes declined gradually in cells grown at oxygen concentrations between 10 and 1 μM O₂; mRNA levels at 1 μM O₂ are about 40–66% of their mRNA levels in air (200 μM O₂) (Fig. 3). At oxygen concentrations below 1 μM O₂ the levels of expression of COX4, COX6, COX7, COX8, and COX9 show a precipitous decline (Fig. 3A). These findings suggest that 1 μM O₂ is a threshold for expression of COX4, COX6, COX7, COX8, and COX9. In contrast to COX4, COX6, COX7, COX8, and COX9, the transcript levels from COX5a and CYC1 show a sharp decline below 0.5 μM O₂, instead of 1 μM O₂ (Fig. 3B). Hence, it appears that these two genes have a lower oxygen threshold than the other aerobic genes.

The dose-response curves for the hypoxic genes COX5b and CYC7 are somewhat simpler than the curves for the aerobic genes. These genes are expressed anaerobically but are not expressed in cells grown at oxygen concentrations of 0.5 μM O₂ or above (Fig. 3B). Because 0.5 μM O₂ is the lowest steady-state oxygen concentration for which we can harvest sufficient cells for RNA analysis we were unable to determine the expression behavior of these genes in steady-state cells grown at oxygen concentrations between 0 and 0.5 μM O₂. Consequently, we...
were unable to determine the oxygen threshold for expression of these genes.

Kinetics of Induction of Aerobic and Hypoxic Genes—As a second way of examining the effects of oxygen on transcript levels we performed shift experiments and followed the kinetics of induction of aerobic or hypoxic genes. Two kinds of shift experiments were done. Cells were shifted from anaerobic to aerobic conditions to follow the induction of aerobic genes and from aerobic to anaerobic conditions to follow the induction of hypoxic genes. In the first experiment cells were grown under anaerobic conditions (2.5% CO₂ in O₂-free nitrogen) for 6.4 generations, and then the sparge gas was changed to air. Upon shifting the cells from anaerobic to aerobic conditions the dissolved oxygen concentration in the fermentor increased rapidly, approaching a plateau within 5 min after the shift (Fig. 4). The mRNA levels for all of the aerobic genes (COX4, COX5a, COX6, COX7, COX8, COX9, and CYC1) increased immediately after the shift but exhibited different kinetics of induction (Fig. 4). Some of these genes were fully induced (i.e. to their levels in steady-state aerobic cells) quickly, and others were induced more slowly. Those genes that were induced quickly included CYC1 and COX8. The CYC1 gene was induced fully within 15 min after the shift, was induced to a level that was about 50% higher than its aerobic level after 30 min, and then declined to its aerobic level. COX8 was induced fully by 90 min after the shift. Those genes that were induced more slowly include COX5a, COX7, COX4, COX6, and COX9; these genes were only partially induced by 120 min, the last time point of the experiment. Transcripts from these genes reached between 80 and 25% (for COX9) of their levels in steady-state aerobic cells, 120 min after the shift.

To examine the kinetics of induction of hypoxic genes COX5b and CYC7, cells were grown in air until the dissolved oxygen reached 80% of its aerobic value, and then the sparge gas was changed from air to 2.5% CO₂ in O₂-free nitrogen. Upon shifting cells from air to nitrogen the dissolved oxygen concentration in the fermentor fell rapidly during the first 10 min and then decreased more slowly throughout the remainder of the experiment (Fig. 5). COX5b was up-regulated slightly within 30 min after the shift, and its transcript level continued to increase (to about 20% of its aerobic level) throughout the 120-min period of observation (Fig. 5). In contrast, CYC7 mRNA was expressed transiently between 15 and 30 min and not expressed for the remainder of the 120-min period of observation (Fig. 5). These findings indicate that the kinetics of induction of COX5b and CYC7 are different and that neither gene is fully induced by 120 min after a shift to anaerobiosis. This is in agreement with the recent finding that it typically takes 12 h for COX5b to be induced fully and more than 18 h for CYC7 to be induced fully following a shift from aerobic to anaerobic conditions. By comparing these findings with those for the aerobic genes CYC1 and COX5a it is obvious that the genes for the aerobic isoforms of cytochrome c and cytochrome c oxidase subunit V are induced much more quickly than the genes for the hypoxic isoforms. It is also obvious that whereas the aerobic isoform gene pairs COX5a/CYC1 have similar kinetics of induction the hypoxic isoform gene pairs COX5b/CYC7 are induced with different kinetics.

Kinetics of Transcript Decay after a Shift—Currently, it is not known if oxygen concentration affects transcript synthesis, transcript stability, or both transcript synthesis and stability. To begin to address this we examined the RNAs from the shift experiments described above. The decay of mRNA levels from aerobic genes was followed in cells shifted from aerobic to anaerobic conditions, and the decay of transcript levels from hypoxic genes was followed in cells shifted from aerobic to anaerobic conditions. The kinetics of decay of mRNA levels from aerobic genes after a shift from aerobic to anaerobic conditions is shown in Fig. 6. As above, the dissolved oxygen concentration in the fermentor fell rapidly during the first 10 min after the shift and then decreased more slowly throughout the remainder of the experiment (Fig. 6). The mRNA levels for COX4, COX6, COX7, COX8, and COX9 change in a complex manner after the shift (Fig. 6A). They increase slightly, relative to ACT1 mRNA, during the first 10–20 min after the shift, decline

2 K. E. Kwast and R. O. Poyton, unpublished observations.
levels for levels was exponential, and the half-times for decay of mRNA occurred between 10 and 40 min after the shift. During this period of time the decay in these mRNAs had reached its anaerobic level by 120 min. Second, the major decrease after the shift; their levels continued to decrease gradually because transcripts were simply no longer synthesized and their ratios to ACT1 mRNA were decreasing as new RNA was synthesized, the expected half-time for decay would be 170 min. Because the rates of decay observed here are five to eight times faster than that, we conclude that the transcripts from these aerobic genes are degraded rapidly after the shift to anaerobiosis.

The mRNA levels from CYC1 also change in a complex manner after the shift (Fig. 6B). They increased slightly during the first 10 min after the shift and then fell rapidly between 10 and 40 min. The decay during this period was also exponential with a half-time of 11 min. Unlike these other mRNAs the mRNA from CYC1 reached its anaerobic level by 40 min after the shift. The mRNA from COX5a behaved similarly but with a slight delay before the major decrease (Fig. 6B). Its half-time for decay was 17 min. Thus, these transcripts differ from those of the other nuclear COX genes in that they decline to their anaerobic levels faster or exhibit a shorter delay before doing so.

The kinetics of decay of mRNA levels from hypoxic genes after a shift from anaerobic conditions to aerobic conditions is shown in Fig. 7. As above, the dissolved oxygen concentration increased rapidly during the first 5 min after the shift. The mRNA level from COX5b dropped rapidly and was undetectable by 20 min after the shift, whereas the mRNA level from CYC7 increased immediately after the shift, stayed high for about 30 min, and then declined. These findings are interesting for two reasons. First, they reveal that transcripts from these two hypoxic genes do not behave similarly after a shift. Second, they reveal a substantial transient increase in the levels of CYC7 mRNA after a shift from aerobic to anaerobic conditions. This increase results in mRNA levels that are 6-fold higher than those in anaerobic cells.

**DISCUSSION**

The results presented here provide several new insights concerning the oxygen-regulated transcription of the nuclear genes for cytochrome c oxidase and cytochrome c. First, they demonstrate that the level of expression of aerobic genes is determined by the concentration of oxygen and not merely by its presence or absence. Second, they reveal that hypoxic genes are regulated more tightly than aerobic genes with respect to
the oxygen concentrations over which they are expressed. Third, they show that aerobic genes are induced faster than hypoxic genes following shifts in oxygen concentration. Fourth, they reveal that transcripts from both aerobic and hypoxic genes are turned over when cells are shifted from one oxygen concentration to another. And fifth, they demonstrate that the isoform gene pairs CYC1/COX5a and CYC7/COX5b are coexpressed over a wide range of oxygen concentrations and switch on or off at extremely low oxygen concentrations. Together, these findings have important implications for the functions of cytochrome c and cytochrome c oxidase isoforms and for models of oxygen sensing in yeast.

The Level of Expression of COX and CYC Genes Is Determined by Oxygen Concentration—Previously, most studies on oxygen-regulated gene expression in yeast have been done with cells grown in one of two conditions, aerobic or anaerobic. These studies have been useful in identifying molecular components (i.e. transcription factors, cis promoter elements, heme) that are involved in oxygen-regulated gene expression but have not provided insight concerning how cells actually sense oxygen. This requires an understanding of how oxygen-regulated genes respond to differences in oxygen concentration per se. Indeed, dose-response curves that relate gene expression to oxygen concentration are of fundamental importance because they can place limits on possible models for oxygen sensing and provide an experimental paradigm for examining how the molecular components of oxygen sensing pathways function. Here, we have addressed this question by examining the effects of oxygen concentration on the expression of a number of aerobic and hypoxic yeast genes. We have found that the dose-response curves relating the steady-state mRNA levels from aerobic genes to oxygen concentration are complex, exhibiting two phases. In the first phase, between oxygen concentrations of 200 and 0.5 or 1 \( \mu M \) \( O_2 \), there is a gradual decline in the transcript levels from each of these genes with decreasing oxygen concentration. In the second phase, below oxygen concentrations of 0.5 or 1 \( \mu M \) \( O_2 \), there is a rapid decline in mRNA levels as the oxygen concentration drops. Because of the biphasic nature of these dose-response curves we conclude that the oxygen concentration that occurs at the break between phase 1 and phase 2 represents an oxygen threshold for expression of each of these genes. The threshold occurs at an oxygen concentration of 1 \( \mu M \) \( O_2 \) for COX4, COX6, COX7, COX8, and COX9 and at or below 0.5 \( \mu M \) \( O_2 \) for COX5a and CYC1. Although we were unable to measure the oxygen thresholds for expression of the hypoxic genes COX5b and CYC7 it is clear that transcripts from these genes are not detectable at 0.5 \( \mu M \) \( O_2 \) but are detectable in anaerobically grown cells. This implies that oxygen thresholds for these genes are below 0.5 \( \mu M \) \( O_2 \).

Hypoxic Genes Are Regulated More Tightly Than Aerobic Genes—A striking difference between the aerobic and hypoxic genes studied here is how tightly regulated they are with respect to the presence or absence of oxygen. Although the aerobic genes are expressed optimally at 200 \( \mu M \) \( O_2 \) (air) they are also expressed under anaerobic conditions. Their level of expression under anaerobic conditions ranges from 3% (for CYC1) to 40% (for COX9) of their level of expression in air. Our dose-response curves for the expression of aerobic genes do not show the large differences in aerobic and anaerobic expression reported for some of these genes, when assayed by use of lacZ gene fusions (23, 24). The large differences noted in these latter studies may result from an effect of oxygen on some step in gene expression besides transcription. For example, because oxygen is required for heme biosynthesis (see below) and heme is required for translation (25) the large effects observed may be attributable to differences in translation or stability of \( \beta \)-galactosidase. An effect exerted through translation could explain why the effects of oxygen on gene expression, measured by quantitating Northern blots as was done here, are smaller than those measured by \( \beta \)-galactosidase activities. Whereas the latter assay requires translation of the lacZ fusion genes, the former does not.

The hypoxic genes studied here are not expressed at oxygen concentrations between 200 and 0.5 \( \mu M \); they are only detectable in aerobic cells. These findings suggest that the expression of hypoxic genes is regulated more tightly than aerobic genes vis à vis the presence or absence of oxygen. This conclusion is also supported by the kinetics of induction of aerobic and hypoxic genes. The induction of hypoxic genes is slow. Indeed, they do not reach their anaerobic levels until several hours after cells are shifted from aerobic to anaerobic conditions. In contrast, aerobic genes are induced relatively quickly. Some are completely induced by 15 min after a shift from anaerobic to aerobic conditions, whereas others take slightly longer. These differences suggest that aerobic and hypoxic genes are regulated by fundamentally different signal transduction pathways.

The Isoform Gene Pairs CYC1/COX5a and CYC7/COX5b Are Coexpressed—The dose-response curves obtained in this study clearly show that the aerobic isoforms genes COX5a and CYC1 are coexpressed over a wide range of oxygen concentrations and have an oxygen threshold at 0.5 \( \mu M \) \( O_2 \). Similarly, the hypoxic isoform genes COX5b and CYC7 are not expressed at any oxygen concentration in the range between 0.5 and 200 \( \mu M \) (air) but are expressed in anaerobically grown cells. Thus, these genes have an oxygen concentration threshold that is below 0.5 \( \mu M \) \( O_2 \). These findings suggest that an oxygen concentration near 0.5 \( \mu M \) could serve as a signal to switch one set of isoforms on and the other set off. Moreover, they are relevant to the function of the isoform pairs, Va + iso-1-cytochrome c, and Vb + iso-2-cytochrome c. The isoforms of subunit V modulate cytochrome c oxidase activity. The catalytic constant \( TN_{\text{max}} \) (maximal turnover number) of the Vb isozyme is 3–4-fold higher than that of the Va isozyme when assayed with iso-1-cytochrome c as the substrate either in vitro (12) or in vitro (13). However, this difference in rate is reduced when the Vb isozyme is assayed in conjunction iso-2-cytochrome c instead of iso-1-cytochrome c (13). This finding lead to the hypothesis that Va and iso-1-cytochrome c and Vb and iso-2-cytochrome c act synergistically (13), but it was uncertain whether these pairs of isoforms are physiological partners over a range of oxygen concentrations. Our findings suggest that they are.

Implications for Cytochrome c Oxidase Biogenesis—The dose-response curves reported here imply that the levels of cytochrome c oxidase decrease with decreasing oxygen concentration and fall off rapidly at oxygen concentrations below 0.5 \( \mu M \) \( O_2 \). Moreover, they predict that the subunit isoforms Va and Vb switch at an oxygen concentration that is below 0.5 \( \mu M \) \( O_2 \). This switch would allow cells to assemble holocytochrome c oxidase molecules with higher TN values at low oxygen concentrations. These predictions are supported by previous studies (26, 27) which found that both cytochrome c oxidase activity and intracellular levels of cytochrome aa3 are undetectable in cells grown at oxygen concentrations below 0.1 \( \mu M \), increase in cells grown at oxygen concentrations between 0.1 and 1 \( \mu M \), and stay constant (or increase slightly) at oxygen concentrations between 1 and 200 \( \mu M \). Moreover, as predicted from the results presented here, the TN of cytochrome c oxidase was higher in cells grown at oxygen concentrations below 0.2 \( \mu M \). Considered together, these results suggest that the effects of oxygen on the expression of the nuclear COX genes are sufficient to account...
for the regulation of intracellular cytochrome c oxidase in response to oxygen. It should be noted that the presence or absence of oxygen also affects the expression of two the mitochondrially encoded subunits (subunit I and II) of cytochrome c oxidase (for review, see Ref. 6). These effects are exerted post-transcriptionally. In the absence of dose-response curves that relate subunit protein level to oxygen concentration it is not yet known if, and how, the levels of these subunits affect cytochrome c oxidase levels.

Implications for Oxygen Sensing—A great deal of evidence implicates heme as an intermediary in sensing oxygen levels in yeast and other organisms (3). Oxygen is required for two steps in heme biosynthesis, the formation of protoporphyrin IX by coproporphyrinogen oxidase III and the formation of porphyrin IX by protoporphyrinogen IX oxidase, and it has been argued that as a molecule whose synthesis requires molecular oxygen heme is ideally suited for a role in oxygen sensing (1). However, it is not yet clear how heme functions in this capacity.

There are at least three feasible pathways by which heme might function in oxygen-regulated transcription in yeast (2, 3). First, heme could serve as a ligand that binds to a transcription factor, as has been proposed for the heme-dependent transcription factor, as has been proposed for the heme-dependent oxygen sensor (for review, see Ref. 6). These effects are exerted post-translationally. In the absence of dose-response curves that relate subunit protein level to oxygen concentration it is not yet known if, and how, the levels of these subunits affect cytochrome c oxidase levels.

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There are at least three feasible pathways by which heme might function in oxygen-regulated transcription in yeast (2, 3). First, heme could serve as a ligand that binds to a transcription factor, as has been proposed for the heme-dependent oxygen sensor, and are any transcription factors involved in oxygen-regulated gene expression differentially phosphorylated in response to oxygen concentration, as suggested by the third model? Current work in our laboratory is focused on these questions.

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