We have studied the function and expression of the flavohemoglobin (YHb) in the yeast *Saccharomyces cerevisiae*. This protein is a member of a family of flavohemoproteins, which contain both heme and flavin binding domains and which are capable of transferring electrons from NADPH to heme iron. Normally, actively respiring yeast cells have very low levels of the flavohemoglobin. However, its intracellular levels are greatly increased in cells in which the mitochondrial electron transport chain has been compromised by either mutation or inhibitors of respiration. The expression of the flavohemoglobin gene, *YHB1*, of *S. cerevisiae* is sensitive to oxygen. Expression is optimal in hypoxic conditions or in air and is reduced under hypoxic and anaerobic conditions. The expression of *YHB1* in aerobic cells is enhanced in the presence of antimycin A, in thiol oxidants, or in strains that lack superoxide dismutase. All three conditions lead to the accumulation of reactive oxygen species and promote oxidative stress. To study the function of flavohemoglobin in vivo, we created a null mutation in the chromosomal copy of *YHB1*. The deletion of the flavohemoglobin gene in these cells does not affect growth in either rho+ or rho− genetic backgrounds. In addition, a rho+ strain carrying a *yhb1* deletion has normal levels of both cyanide-sensitive and cyanide-insensitive respiration, indicating that the flavohemoglobin does not function as a terminal oxidase and is not required for the function or expression of the alternative oxidase system in *S. cerevisiae*. Cells that carry a *yhb1* deletion are sensitive to conditions that promote oxidative stress. This finding is consistent with the observation that conditions that promote oxidative stress also enhance expression of *YHB1*. Together, these findings suggest that YHb plays a role in the oxidative stress response in yeast.

Hemoglobins, defined as hemoproteins that bind oxygen reversibly, have been detected in a wide range of organisms including vertebrates, invertebrates, higher plants, fungi, and bacteria (1, 2). Although all of these hemoglobins have a conserved heme binding domain, the “myoglobin fold,” they are otherwise divergent in both structure and complexity (2). Whereas most vertebrate hemoglobins are composed of two types of polypeptide subunits, α and β, which have single heme domains and form αβ2 tetramers, invertebrate and microbial hemoglobins are more varied. For example, the bacterial and fungal hemoglobins that have been characterized to date fall into two general categories: dimeric hemoproteins composed of two single heme domain polypeptides and monomeric flavohemoproteins composed of a single polypeptide containing a single heme binding domain and a single flavin binding domain. The first type of hemoglobin (VGB) is present in the bacterium *Vitreoscilla* (3). This protein has partial primary sequence similarity to plant leghemoglobin (3). The second type of hemoglobin has been found in the bacteria *Escherichia coli* (4–6) and *Alcaligenes eutrophus* (7) and in the fungi *Candida norvegica* (8) and *Saccharomyces cerevisiae* (9, 10). The N-terminal regions of these proteins bind heme and have primary sequence homology to VGB and plant leghemoglobin (2, 10). The C-terminal region has an FAD-binding domain and is related to proteins in the ferredoxin-NADP+ reductase (FNR) family (11). A third type of hemoglobin is found in *protozoa* (*Paramecium caudatum*, *Tetrahymena pyriformis*, and *Tetrahymena thermophila*) and the cyanobacterium, *Nostoc commune* (12). This is a single heme domain polypeptide that is considerably smaller than the other two types of microbial hemoglobins discussed above. Although the hemoglobins from *protozoa* and *Nostoc* have primary sequence similarity with one another, they show no significant sequence similarities with other hemoglobins. Like microbial hemoglobins, the hemoglobins of invertebrates are also extremely diverse (2). They fall into four categories: 1) single-heme domain single subunit hemoglobins (~16 kDa); 2) two-heme domain subunits that assemble into multi-subunit complexes (250–800 kDa); 3) multi-heme domain subunits that assemble into multi-subunit complexes (240–8,000 kDa); and 4) single-heme domain multi-subunit complexes in which some of the polypeptide chains are disulfide linked (2).

Although the function of vertebrate hemoglobins and myoglobin in oxygen binding and diffusion is well established, the function(s) of hemoglobins in other groups of organisms is unclear. Several possible functions have been proposed. For example, *Vitreoscilla* hemoglobin has been proposed to function in oxygen storage, diffusion, or delivery in cells grown at low oxygen partial pressures (3, 13, 14). In addition, it can serve as a terminal oxidase under some conditions (15). The flavohemoglobin FHP of *A. eutrophus* has been proposed to function, either directly or indirectly, in nitrate respiration (16). And the
flavohemoglobin HMP of *E. coli* has been proposed to be an oxygen sensor (17, 18).

In this study we have addressed the physiological role of the flavohemoglobin (YHb) of *S. cerevisiae*. This protein binds oxygen reversibly only when NADPH is present, indicating that it has an NAD(P)/H reductase activity for the heme domain (19, 20). Respiring *S. cerevisiae* cells normally have very low levels of YHb. However, its intracellular levels are greatly increased in cells in which the mitochondrial electron transport chain has been compromised by either mutation (i.e. the deletion of the mitochondrial genome) (21) or respiration inhibitors (e.g. antimycin A) (10, 22). Its level also increases in cells engineered to express the hypoxic isofrm, Vb, of cytochrome c oxidase subunit V under aerobic conditions (21). Here, by combining studies on the expression of YHB1, the structural gene for YHb, with studies on mutant yeast strains that lack YHb we have obtained evidence for a role for YHb in the oxidative stress response in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeasts, Strains, Plasmids, and Growth Conditions**—The following strains of *S. cerevisiae* were used: JM43 (MATa his4–580 trp1–289 leu2–3, 112 ura3–52 rho–1) (23); JM43ρ, a respiratory-deficient derivative of JM43 that lacks a functional mitochondrial genome (21); DR11, a derivative of JM43 containing a truncated YHB1 gene; DR10, a derivative of JM43ρ containing a URA3 disrupted YHb1 gene; EG103 (MATa leu2–3, 112 his3 trp1–289 tricho1–52); and EG113, a derivative of EG103 containing a URA3 disrupted SOD1 gene and a TRP1 disrupted SOD2 gene. EG103 and EG113 were kindly provided by Dr. E. Gralla (24). The original plasmid, pYHB4, containing the YHb1 gene was kindly supplied by Drs. H. Zhu and A. Riggs (10). Strains DR11 and DR10 were constructed using the one-step gene disruption technique (25) as follows. A 4-kb BamHI genomic fragment from plasmid pYHB4, which contains the YHb1 gene and flanking sequences, was cloned into the unique *BamHI* restriction fragment containing the *YHB1* gene (Fig. 1A). The vector pFL46s (29). The following plasmids were selected and subjected to Southern blot analysis to confirm the disruption and the probe fragment used to confirm the disruption are shown in Figure 1 (21). Both the DNA fragment used for transformation and the probe fragment used to achieve anaerobiosis. An aliquot (1 ml) of the reduced cell suspension was then transferred into a spectrophotometer cuvette. The cuvette was sealed with a rubber stopper and parafilm. The sample was incubated in the cuvette for 5 min at room temperature to ensure that any oxygen that may have diffused into the sample during transfer was reduced. The CO-ligated sample was prepared by adding antifoam (Dow Corning FG-10, 100 ppm) to the reduced cell suspension and bubbling very slowly with CO gas (99.5%) for 5 min. The oxidized sample was prepared by adding 25 μl of 30% H2O2 to 1 ml of the reduced sample in the cuvette and incubating the sample at room temperature for 2 min. All spectra were recorded using a SLM AMINCO DW2000 dual wavelength scanning UV-visible spectrophotometer (SLM Instruments, Inc.) with the reference monochromator set to a wavelength of 577 nm.

**Determination of Drug Resistance**—Yeasts were grown in liquid YPgal to log phase, plated onto YPD containing various concentrations of drugs, and grown at 28 °C for 3 (H2O2 and paraquat) or 5 days (diethylmaleate and diamide). In another experiment, the yeast strains were grown in YPD, plated with various concentrations of paraquat, and cultured under hypoxic conditions (95% O2 and 5% N2) for 3 days at room temperature.

**Induction of YHB1**—To study the transcriptional induction of YHB1 by oxidative stress inducing drugs, JM43 was grown in YPgal to log phase and pelleted. The yeast cells were then resuspended in the same volume of fresh YPgal, aliquoted, and incubated for 1 h with the appropriate drug. Total RNA was prepared as described previously (30).

**Northern Blotting**—For Northern analysis, RNA samples (30 μg) were separated on 1.5% agarose gels containing 0.22M formaldehyde (31), transferred to a Schleicher & Schuell Nytran membrane, and hybridized according to the manufacturer’s suggestions. DNA probes were prepared by random-primer labeling of double stranded DNA fragments using [α-32P]dCTP (32). Probes were a 600-bp SspI fragment for YHB1, a 500-bp StyI fragment for ACT1 (the gene encoding actin), a 500-bp Psfl fragment for COX5a (the gene encoding the Va isofrom of subunit Vc of cytochrome c oxidase), and a 370-bp AccI/BglII fragment for COX5b (the gene encoding the Vb isofrom of subunit Vb). Because YHB1 and ACT1 mRNAs migrate to a similar position in the gel, the Nytran membranes were hybridized with the YHB1 probe and then stripped and hybridized with the ACT1 probe. Blots were quantitated with an AMBIS Radioanalytic Imaging System.

**Miscellaneous**—Whole cell respiration was measured with a YSI oxygen electrode (33). Southern blots of total DNA (33) were done as described previously. Paraquat, diethylmaleate, and diamide were obtained from Sigma; 2.5% CO2 in O2-free N2 and 95% O2 plus 5% N2 were obtained from U.S. Welding (Denver, CO); and 99.5% CO was obtained from General Air Service and Supply (Denver, CO).

**RESULTS**

YHb Is Not Essential for Cell Growth—To determine if YHb is essential for cell growth, we constructed strains carrying a null mutation in its structural gene, YHB1 (10). This gene is located in the right arm of chromosome VII, distal to the Ade3 locus (34). As described under “Experimental Procedures,” the YHB1 gene was disrupted in two isochromosomal strains, JM43 and JM43ρ, that are rho- and rho+, respectively. The yhb1 derivatives of these strains are designated DR11 and DR10, respectively. Confirmation that the genomic copy of YHB1 was disrupted in these strains was obtained by comparing genomic Southern blots of JM43 with DR11 (Fig. 1) and JM43ρ with DR10 (not shown). Digestion of genomic DNA from either JM43 or JM43ρ with BamHI, which cuts outside of the YHB1 gene (Fig. 1A), yields a single band of about 4.6 kb (Fig. 1B). Digestion of genomic DNA from either DR11 or DR10 with BamHI yields a single band that is about 5.4 kb (Fig. 1B). This is the expected result for a YHB1 gene carrying a 1.1-kb insertion (containing the URA3 gene) and a 0.3-kb deletion.

To determine the effects of the YHB1 null mutation on cell growth, cells were grown on media containing repressing (e.g., dextrose) or nonrepressing (e.g., galactose) carbon sources. From Table I it is clear that the growth rates of the ρ0*yhb1* and ρ0*yhb1* strains (DR11 and DR10, respectively) were unaffected relative to their ρ+ counterparts (JM43 and JM43ρ, respectively), on either carbon source. This confirms the results of a recent report that a yhb1 ρ0* strain is phenotypically similar to a YHB1 ρ0*. | 1 | The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); GSH, glutathione; ROS, reactive oxygen species. |
A Role of Yeast Hemoglobin in the Oxidative Stress Response

Fig. 1. Gene disruption of the chromosomal copy of YHB1. A gene disruption of YHB1 was constructed as described under "Experimental Procedures." A, schematic diagram of the chromosomal locus of the parent strain JM43 and the location of the URA3 gene insertion in the yhb1:URA3 gene disruption strain DR11. The restriction sites, BamHI (B), HindIII (H), and BgIII (Bg), are shown. The 300-bp cross-hatched HindIII fragment was removed and replaced by a 1.1-kb HindIII fragment containing the URA3 gene. The shaded region represents the YHB1 coding region, and the direction of transcription is shown by the arrow. B, Southern blot analysis of genomic DNA from JM43 and DR11. Genomic DNA was digested with BamHI and probed with the BgIII-HindIII probe shown in A.

Table I

| Yeast strains | Mass doubling times |
|--------------|---------------------|
|              | in YPD              | in YPGal             |
| JM43         | 1.5                 | 2.2                  |
| DR11         | 1.5                 | 2.2                  |
| JM43p<sup>o</sup> | 2.0              | 3.8                  |
| DR10         | 1.8                 | 3.4                  |

strain with respect to growth on fermentable carbon sources (34). In addition, we have found that the anaerobic growth rates and cell yields for the rho<sup>-</sup> yhb1<sup>-</sup> strain (DR11) and the rho<sup>-</sup> YHB1 strain (JM43) are exactly the same.

The finding that the growth characteristics of YHB1 and yhb1<sup>-</sup> strains are the same may be interpreted in one of two ways. Either YHB1 is the only gene encoding a flavohemoglobin in S. cerevisiae and hence its protein product, YHb, is not essential for cell growth, or flavohemoglobins are encoded by more than one gene and when YHB1 is rendered nonfunctional a flavohemoglobin isoform of YHb, encoded by another gene, is produced and is a functional substitute for YHb. The most direct way to distinguish between these two possibilities is to examine a yhb1<sup>-</sup> strain for the presence of flavohemoglobin pigment. Insofar as the level of flavohemoglobin is extremely low in respiration-proficient strains and is elevated in respiration-deficient strains (21), the flavohemoglobin pigment is most easily observed in rho<sup>-</sup> strains (21). When subjected to difference spectroscopy (reduced minus oxidized), JM43p<sup>o</sup> cells reveal an absorption maximum at 561 nm, a shoulder at 551 nm, and an absorption minimum at 582 nm (Fig. 2, trace 2). The shoulder at 551 nm corresponds to reduced cytochrome c. The maximum at 561 nm and minimum at 582 nm, correspond to reduced YHb. They are similar to those for purified human hemoglobin (Fig. 2, trace 1). It is clear that DR10, the yhb1<sup>-</sup> derivative of JM43p<sup>o</sup>, lacks these maxima and minima but retains an absorption maximum at 551 nm (Fig. 2, trace 3). This is expected for a strain that retains cytochrome c but lacks YHb. A more sensitive assay for the presence of YHb is difference spectroscopy in the presence of CO. CO difference spectroscopy of JM43p<sup>o</sup> reveals the characteristic flavohemoglobin maximum at 440 nm as well as a minimum at 422 nm (Fig. 3, trace 1) reported earlier (21, 35). In contrast, CO difference spectroscopy of DR10 reveals the absence of a CO-binding pigment with these absorption characteristics (Fig. 3, trace 2).

Together, these findings demonstrate that DR10 lacks any flavohemoglobin pigment and rule out the possibility that a YHb isoform, which could substitute for YHb, is present in DR10. This conclusion is supported by a BLAST search of the entire yeast genome; no other genes with significant similarity to YHB1 were found. Because JM43p<sup>o</sup> and DR10 have similar growth rates on YPD and YPGal, we conclude that YHb, which is present in JM43p<sup>o</sup> but absent in DR10, is not essential and does not affect growth rates or yields, at least under our standard laboratory conditions.

YHB Does Not Function in Respiration—To examine the possibility that YHb functions as a terminal oxidase, we measured the rates of whole cell respiration in the yhb1<sup>-</sup> mutants, DR10 and DR11 (Table II). Cyanide-sensitive and cyanide-insensitive rates were determined. In the presence and the absence of cyanide the yhb1<sup>-</sup> mutation has little if any effect on the respiration rates in the rho<sup>-</sup> strain. This finding indicates that the YHb does not contribute to either the mitochondrial respiratory chain (i.e. cyanide-sensitive) or to cyanide-insensitive respiration by an alternative respiratory chain (36). Similar results were obtained with the rho<sup>-</sup> strains JM43p<sup>o</sup> and DR10. As expected, respiration in the absence of cyanide is greatly reduced because these strains lack functional mitochondrial respiratory chains. Considered together, these results clearly show that YHb is not required for respiration in vivo.
have examined the expression of its gene, to gain insight concerning possible functions of the YHb, we examined the expression of its gene, ACT1 and YHB1, in hyperoxic conditions. The dissolved oxygen concentration increased rapidly during the first 10 min after the shift and then decreased more slowly throughout the remainder of the experiment (Fig. 5). The mRNA levels from both YHB1 and COX5a increased slightly during the first 10 min after the shift and then decreased to about 30% of their aerobic levels. COX5b, the gene for the hypoxic isoform (Vb) of cytochrome c oxidase subunit V (40), was expressed transiently at low levels between 15 and 20 min after the shift. It is expressed at higher levels in steady state anaerobic cultures (Fig. 5, lane 11). From these findings it is clear that YHB1 is optimally expressed in the presence of air and that its expression declines as cells become hypoxic. It is also clear that YHB1 mRNA levels parallel those of the aerobic gene COX5a and not the hypoxic gene COX5b.

In a second experiment cells were shifted from air to 95% oxygen. The dissolved oxygen concentration increased rapidly during the first 5 min after the shift and then more slowly throughout the remainder of the 120-min incubation period (Fig. 6). The mRNA levels for both YHB1 and COX5a declined initially and then rose gradually to their steady state levels. When normalized to the ACT1 transcript the level of expression of YHB1 mRNA was somewhat higher (a 20%) than the level of expression of COX5a during the majority of time the cells were exposed to hyperoxia. So, although YHB1 is expressed like an aerobic gene at oxygen concentrations between atmospheric and anaerobic, it is hyperexpressed relative to an aerobic gene under hyperoxic conditions.

Cells That Carry a yhb1 Deletion Mutation Are Sensitive to Oxidative Stress.—The finding that YHB1 is hyperexpressed relative to the aerobic gene, COX5a, in hyperoxic conditions suggested a possible role for YHb in the oxidative stress response. To examine this possibility we compared the sensitivity of YHB1- and yhb1- cells with a variety of conditions that can induce oxidative stress. Four types of conditions were used to promote oxidative stress in intact yeast cells: exposure to thiol oxidants that affect glutathione (GSH) levels in the cell, exposure to hydroperoxides, exposure to reduct recycling compounds, and exposure to hyperoxia (41–44). Two thiol oxidants, diamide and diethylmaleate, were used. These modify the GSH

Effects of Oxygen Tension on YHB1 Transcription—In order to gain insight concerning possible functions of the YHb, we have examined the expression of its gene, YHB1. Similar studies on the expression of the Vitreoscilla hemoglobin revealed that it was expressed optimally under hypoxic conditions; these findings were among the first to suggest that this protein functions as an oxygen scavenger (3, 13, 14). In S. cerevisiae there are two classes of yeast genes: “aerobic” genes, which are expressed optimally in the presence of air, and “hypoxic” genes, which are expressed optimally at low oxygen concentrations (37–39). To examine the effects of oxygen on YHB1 expression and determine if it is an aerobic or hypoxic gene, we compared the expression of YHB1 with that of ACT1, the actin gene. The ACT1 transcript the level of expression in air.

To determine if YHB1 is optimally expressed at some oxygen concentration other than those used for the experiment shown in Fig. 4, we performed shift experiments. First, we shifted cells from air to nitrogen. For this experiment JM43 cells were grown up to mid-log phase in a fermentor in the presence of air, and then the sparge gas was shifted from air to nitrogen. Upon shifting cells from air to nitrogen 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. 5). The mRNA levels from both YHB1 and COX5a increased slightly during the first 10 min after the shift and then decreased to about 30% of their aerobic levels. COX5b, the gene for the hypoxic isoform (Vb) of cytochrome c oxidase subunit V (40), was expressed transiently at low levels between 15 and 20 min after the shift. It is expressed at higher levels in steady state anaerobic cultures (Fig. 5, lane 11). From these findings it is clear that YHB1 is optimally expressed in the presence of air and that its expression declines as cells become hypoxic. It is also clear that YHB1 mRNA levels parallel those of the aerobic gene COX5a and not the hypoxic gene COX5b.

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levels in the cell as follows. GSH is oxidized by diamide and is depleted by conjugation with diethylmaleate (45). Because GSH is an important antioxidant and helps maintain a reducing environment in the cell, its depletion or oxidation mimics some of the effects of oxidative challenge (46). These studies were done in the rho<sup>r</sup> strains JM43<sup>r</sup> and DR10 because the level of YHb in rho<sup>r</sup> cells is negligible (21). From Fig. 7 it is clear that yhb<sup>r</sup> cells are more sensitive than YHB<sup>1</sup>r<sup>-</sup> cells to diethylmaleate and, to a lesser extent, diamide under aerobic conditions. In addition, growth rates of yhb<sup>r</sup> cells are slower than YHB<sup>1</sup>r<sup>-</sup> cells when exposed to diethylmaleate and diamide. Under hyperoxic conditions both strains become more sensitive to these compounds, but the sensitivity of the yhb<sup>r</sup> strain is more greatly enhanced than the YHB<sup>1</sup>r<sup>-</sup> strain (data not shown). Paraquat, a redox-recycling drug that generates superoxide (47), had a minor effect when added to cells in air. However, sensitivity of the yhb<sup>r</sup> strain is greatly enhanced under hyperoxic conditions. Unlike the results obtained with the three drugs mentioned above, yhb<sup>r</sup> cells were no more sensitive to H<sub>2</sub>O<sub>2</sub> than YHB<sup>1</sup>r<sup>-</sup> cells when grown either in air or under hyperoxic conditions. This may be due to a high level of catalase and/or peroxidase in these yeast strains.

To confirm that the effects observed above are due to the lack of a functional YHb protein, we transformed DR10 with the pFL46s-YHB1 plasmid. From Fig. 8 it is clear that a plasmid-borne YHB1 gene is capable of partially reversing the sensitivity of the parent strain to diethylmaleate. At present, we do not know why the plasmid-borne YHB1 gene does not completely restore drug resistance to wild type levels. However, it is most likely the result of reduced expression of the plasmid-borne YHB1 gene because CO difference spectra reveal that the transformed strain contained about half of the JM43<sup>r</sup> level of YHb (data not shown). Because the YHB1 promoter has not been characterized yet, it is possible that the 460 bp of 5′-flanking sequence on the plasmid-borne YHB1 gene was not sufficient for maximal expression. Despite the reduced expression of YHb in DR10-pFL46s-YHB1 compared with JM43<sup>r</sup>, it is noteworthy that this strain was about half as resistant to diethylmaleate as JM43<sup>r</sup>. This finding, together with the fact that this strain contains about half of the JM43<sup>r</sup> level of YHb, suggests that the degree of resistance to oxidative stress parallels the intracellular level of YHb. When considered together these findings support the conclusion that YHb plays a role in the oxidative stress response in yeast.

YHB1 Transcription Is Up-regulated under Conditions of Oxidative Stress—Many genes that encode proteins that function as antioxidants or participate in oxidative stress response pathways are induced in cells that are exposed to reagents that promote oxidative stress (43, 44, 48). To determine if YHB1 expression is affected in a similar manner, RNA isolated from cells treated with diethylmaleate, diamide, paraquat, and hydrogen peroxide were subjected to Northern analysis (Fig. 9). Northern analysis was also performed on RNA isolated from cells treated with antimycin A, which enhances the production of reactive oxygen species (ROS) by inhibiting complex III of the mitochondrial electron transport chain and which has been shown previously to increase intracellular levels of YHb (10, 22). Using the ACT1 gene as a control, we have found that H<sub>2</sub>O<sub>2</sub>, diamide, and diethylmaleate all enhance expression of YHB1 by 1.5–2-fold within 1 h after exposure. The level of YHB1 expression in antimycin A-treated cells is enhanced...
nearly 3-fold. In contrast, paraquat had no effect. Because the level of superoxide produced in the presence of paraquat may be kept low by the cell’s cytosolic and mitochondrial superoxide dismutases (encoded by the \( SOD1 \) and \( SOD2 \) genes, respectively; Ref. 24), we examined \( YHB1 \) expression in an \( sod1^{−} sod2^{−} \) strain. From Fig. 10 it is clear that \( YHB1 \) expression is elevated about 3-fold in an \( sod1^{−} sod2^{−} \) strain, relative to the \( SOD1^{+} SOD2^{+} \) parent. Thus, it is likely that superoxide as well as other ROS can participate in the up-regulation of \( YHB1 \).

**DISCUSSION**

The results presented here address the in vivo function of the Yhb flavohemoglobin of *S. cerevisiae*. They indicate that this protein participates in the oxidative stress response and probably does not function as an alternative oxidase or in oxygen delivery.

**Yhb Is Not Required For Respiration or Alternative Oxidase Activity in Vico**—Several previous papers have speculated that microbial hemoglobins may function as terminal or alternative oxidases (1, 2, 10, 15). The most compelling evidence for this comes from studies with *Vitreoscilla* hemoglobin. This protein is expressed optimally under microaerophilic conditions (49) and enhances cell growth under oxygen-limiting conditions when overexpressed in *E. coli* (14). Moreover, this hemoglobin is capable of rescuing terminal oxidase mutants of *E. coli* (15).

*S. cerevisiae* and other fungi have an alternative respiratory pathway, which is distinguishable from the mitochondrial respiratory pathway by its insensitivity to cyanide (36). To examine the possibility that Yhb functions as an alternative terminal oxidase in this pathway, we examined the rates of respiration in *YHB1* and *yhb1* cells. We found no difference in the rates of cyanide-sensitive or insensitive respiration in these strains, indicating that Yhb does not function in vivo.
independently of respiration during oxygen consuming reactions in the cytosol. ROS are highly unstable reactive compounds that have been shown to mutate DNA (54), oxidize proteins (55), and damage membranes (56). All aerobic organisms have evolved antioxidant defense systems to help keep the harmful effects of ROS in check. In this study, we have exposed cells to a number of conditions that generate oxidative stress and analyzed the antioxidant capacity of \( S.~cerevisiae \) cells that lack a functional \( YHB1 \) gene. Diethylmaleate and diamide treatment reduce the levels of intracellular GSH either by forming GSH conjugates via glutathione S-transferase or by oxidizing it to GSSG. The ability of yeast cells to survive exposure to these chemicals is dependent on a functional \( YHB1 \) gene. Yeast cells that lack a \( YHB1 \) gene are also more sensitive to growth in hypoxic conditions than cells that carry a functional \( YHB1 \) gene. Importantly, each of these conditions also enhance the level of expression of \( YHB1 \). Together, these results imply that YHB plays a role in the response of yeast cells to oxidative stress.

Currently, it is possible to envision at least two roles for YHB in the oxidative stress response. It may function as an antioxidant itself or it may participate in a sensing pathway that responds to oxidative stress. As a flavohemoprotein YHB may bind ROS and reduce them to water by a mechanism that is analogous to the reduction of oxygen to water by cytochrome \( c \) oxidase (57). For example, it is plausible that superoxide can bind to the \( \text{Fe}^{2+} \) form of hemoglobin, which can transfer electrons from NAD(P)H to it. Alternatively, YHB may bind to the \( \text{Fe}^{3+} \)-O form of hemoglobin and is oxidized to GSSG. The ability of yeast cells to survive autooxidation, it is plausible that either the \( \text{Fe}^{2+} \)-O form or the \( \text{Fe}^{3+} \)-O form of hemoglobin can then be reduced to water by electrons supplied from NAD(P)H. Alternatively, YHB may bind ROS and, as such, serve as a proximal sensor for oxidative stress. In this regard it is noteworthy that HMP, the \( E.~coli \) counterpart of YHB, has been proposed as an oxygen sensor (17, 18). Because the level of ROS produced is proportional to the partial oxygen pressure (58) perhaps HMP and, by extension, YHB sense oxygen indirectly via the ROS produced from it. Further study with purified YHB is required to examine these possibilities and determine the precise role of this protein in the oxidative stress response in yeast.

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