Protection from nitrosative stress by yeast flavohemoglobin

Limin Liu*, Ming Zeng†, Alfred Hausladen†, Joseph Heitman*‡, and Jonathan S. Stamler*§

*Howard Hughes Medical Institute, and Departments of †Medicine, Pulmonary and Cardiology Divisions, ‡Biochemistry, and §Genetics, Duke University Medical Center, Durham, NC 27710

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Yeast hemoglobin was discovered close to half a century ago, but its function has remained unknown. Herein, we report that this flavohemoglobin protects Saccharomyces cerevisiae from nitrosative stress. Deletion of the flavohemoglobin gene (YHB1) abolished the nitric oxide (NO)-consuming activity of yeast cells. Levels of protein nitrosylation were more than 10-fold higher in yhb1 mutant yeast than in isogenic wild-type cells after incubation with NO donors. Growth of mutant cells was inhibited by a nitrosative challenge that had little effect on wild-type cells, whereas the resistance of mutant cells to oxidative stress was unimpaired. Protection conferred by yeast flavohemoglobin against NO and S-nitrosothiols was seen under both anaerobic and aerobic conditions, consistent with a primary function in NO detoxification. A phylogenetic analysis indicated that protection from nitrosative stress is likely to be a conserved function among microorganismal flavohemoglobins. Flavohemoglobin is therefore a potential target for antimicrobial therapy.

Hemoglobin is expressed throughout most, if not all, phyla. The common perception that it is involved solely in transport of O2 and CO2 has been challenged by a series of recent discoveries on the nitric oxide (NO)-related functions of hemoglobins of mammalian, invertebrate, and bacterial origin. Mammalian hemoglobin functions in erythrocytes to conserve and dispense NO (1–4). NO bound at the heme is transferred oxidatively to a highly conserved cysteine within the β-subunit where it is in position to exert bioactivity. Transport of the NO group to target effectors regulates blood flow and hence O2 delivery. Chemical transfer and transport of the NO group is linked to the allosteric state of hemoglobin (1–4). The β-subunit of hemoglobin may subserve a related function in mouse macrophages where its expression is induced by stimuli that generate NO (5).

By contrast, the more ancient hemoglobins of nematodes and bacteria variously metabolize NO and O2. The periercetic hemoglobin of Ascaris lumbricoides is an NO-primed deoxygenase, employing NO to detoxify O2 (6). In this case, NO is transferred oxidatively from the hemoglobin to a critical cysteine within the heme pocket where it is poised to react with O2. NADPH then supports the transformation of NO/O2 into innocuous nitrate (NO3−). The function of Escherichia coli flavohemoglobin (HMP) is to protect from NO and NO-donating S-nitrosothiols (SNOs; refs. 7–9). It has two NADH-dependent activities that consume NO: an NO oxygenase activity (9) that generates mainly nitrate (8, 9), and an NO reductase activity that generates nitrous oxide (N2O; refs. 9–11). NO and O2 are cosubstrates in the first reaction (9), whereas the second operates under anaerobic conditions (9, 11).

Expression of the E. coli hemoglobin gene (hmp) is induced by nitrite, NO, and and SNO (8, 9, 12, 13) and confers protection from each of them. Mutants deficient in HMP show compromised ability to metabolize NO and SNO to nitrate. The flavohemoglobin of Salmonella typhimurium shares 94% amino acid sequence identity with that of E. coli (14) and also protects against acidified nitrite, NO, and SNO (14, 15). The more distantly related flavohemoglobin in Ralstonia eutropha (formerly Alcaligenes eutrophus) is implicated in production of N2O during denitrification obligate in anaerobic growth; however, disruption of the gene does not affect anaerobic growth on nitrite or nitrate, because there is an independent route of nitrate reduction (16). Thus, although the functions of hemoglobins have clearly evolved around interactions with NO, they are diverse in nature, and it is unclear to what degree function is conserved throughout phylogeny.

Saccharomyces cerevisiae was discovered 47 years ago (17), but its function has remained unknown. It is a flavohemoglobin sharing only 38% amino acid sequence identity with E. coli HMP (18, 19). The expression of the yeast flavohemoglobin gene YHB1 is elevated under aerobic conditions (20, 21), whereas E. coli hmp expression is not affected by O2 (12). YHB1 is not required for respiration, fermentation, or growth under any O2 tension (20–22). It has been suggested that YHB1 may protect against oxidative stress (20), but this function has been questioned (21, 22). Herein, we report that yeast YHB1 is required to metabolize NO and thereby protects against nitrosylation of cellular targets and inhibition of cell growth under both aerobic and anaerobic conditions. That is, the primary function of YHB1 is to protect against a nitrosative stress.

Materials and Methods

Construction of yhb1 Mutants. The entire ORF of the YHB1 gene (GenBank accession no. Z73019) in the haploid yeast strain Y190 (CLONTECH) was deleted by using either KanMX2 (23) or hphMX (24) cassettes. Primers HMPKOse (TTTACCATTTTACAAACACACAAAGACCTTTATTCATGATACAGCTTGCCCTCGTC) and HMPKOas (AATCGTAATATAAATGTGAAGTTTCCAGGTACATCTGACA-CTGGATGGCGCGG) were used to amplify the cassettes and add YHB1 sequences to both ends by PCR. Cells stably transformed with recombinant KanMX2 and hphMX were selected by their resistance to G418 (200 mg/ml) and hygromycin (200 μg/ml), respectively. Replacement of the YHB1 gene by KanMX2 or hphMX in the genome was confirmed by detection of YHB1–KanMX2 or YHB1–hphMX fusion fragments, respectively, when PCR was performed with primers HMP1512se (CAGTTTCCGT-GTTGTGGTCAAG) and Kanas (GTCGACACTGGATGCGGCG). Primers HMP1512se and HMP1899as (CAATGTTCTGTATCTAATGA) were used to amplify the wild-type YHB1 fragment. Replacement of the YHB1 gene by KanMX2 and hphMX was also carried out in the diploid yeast strain JK935 (25). After the cassette positively targeted one of the two alleles, diploid cells resistant to either G418 or hygromycin were induced to

Abbreviations: DETA NONOate, 2,2′-dihydroxytrosohydroxanolobis-ethanamine; GSNO, S-nitroso-glutathione; SNO, S-nitrosothiol; X-NO, nitrosylated peptides and proteins; YPD, yeast extract/petitone/dextrose.

*To whom reprint requests should be addressed. E-mail: STAML001@mc.duke.edu.

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sporulate. Haploid clones of YHBI wild-type and yhb1 mutant cells were then obtained by tetrad dissection.

The YHBI gene was amplified from genomic DNA of yeast strain Y190 by PCR with primers hmp5'-XbaI (CTAGGTCAGTGCACGTATCTATCTAGTTGCT) and hmp3'-EcoRI (CCGGAAATTCCTCTGTTAGACACTGTCG-GCTTATT). It was then cloned into the XbaI–EcoRI sites of plasmid YEp4912 (26). This plasmid was transformed into YhBI mutant strains to restore protein (Yhb1) activity.

**NO Metabolism.** NO consumption by whole-cell lysates in the presence of NADH (250 μM) or by intact cells (OD600 = 0.5) was measured in 2 ml of PBS with 0.1 mM diethylenetriamine pentaacetic acid by using an NO electrode as described (5). Alternatively, NO-dependent NADH consumption was measured by following the decrease in absorbance at 340 nm. Anaerobic assays were performed in Thunberg tubes (sealed cuvettes). Enzyme preparations were incubated with up to 200 μM NO in an anaerobic chamber. Reactions were initiated by adding 100–200 μM NADH from a sidearm.

**Nitrosylation (X-NO).** Amounts of X-NO in the total lysate, in the fraction of the lysate that passed a Bio-Gel P-6 column from Bio-Rad (high-mass X-NO), and in a fraction filtered through a 5-kDa cut-off ultrafiltration membrane (low-mass X-NO) were measured by photolysis chemiluminescence (27). The data were normalized against protein content of the total lysate. High-mass X-NO was also obtained indirectly by subtracting the low-mass X-NO from the total in the lysates.

**Growth Inhibition.** Mid-log phase (OD600 0.4–0.6) cells were diluted to an OD600 of about 0.05 and cultured aerobically in yeast extract/peptone/dextrose (YPD) supplemented with varying concentrations of 2,2′-(hydroxynitrosodihydrone)bis-ethanamine (DETA NONOate, Cayman Chemicals, Ann Arbor, MI) or H2O2. Cell growth was monitored by OD600 measurements on either undiluted or diluted culture. Readings were made only in a linear OD600 cell-concentration range that had been determined experimentally.

**Anaerobic Study.** Yeast colonies grown on YPD plates under room air were transferred and incubated for a minimum of 24 h in a glove box (Coy Laboratory Products, Grass lake, MI) where the O2 concentration was kept below 1 ppm. After culture in liquid medium for another 24 h, both YHBI and yhb1 cells were assayed for their growth in the presence of various concentrations of DETA NONOate and S-nitrosoglutathione (GSNO). Cell lysates were prepared from the anaerobic culture, and their NO- and NADH-consuming activities were measured under various conditions.

**Results**

**Saccharomyces cerevisiae** readily consumes NO under aerobic conditions (vide infra). Spectral analysis of the partially purified NO-metabolizing protein indicated that it is a flavohemoprotein (data not shown). To determine whether yeast flavohemoglobin is critical for NO metabolism, the entire coding region of YHBI was deleted by a PCR-mediated method (23–25) from both Y190 and JK93d parental strains (Fig. 1; and data not shown). YHBI deletion is not lethal, in accord with previous reports (20–22). NO-consuming enzyme activities were not detected in either yhb1 extracts (Fig. 2A) or intact cells (Fig. 2B), regardless of genetic backgrounds (Y190 or JK93d) or the mutagenic cassettes used (KanMX2 or hghMX; Figs. 1 and 2; data not shown). Transformation of the YHBI gene into yhb1 mutants restored the NO-metabolizing activity (data not shown). Thus, the flavohemoglobin is essential for NO metabolism in *S. cerevisiae.*
To understand better the metabolic function of yeast flavohemoglobin in protecting against NO, both YHB1 wild-type and yhb1 mutant cells were treated aerobically with the NO donor DETA NONOate and then analyzed for NO content. Low levels of nitrosylation of high molecular mass molecules were detected in YHB1 cells after exposure to NO (Fig. 3). In contrast, the levels of nitrosylation were more than 10-fold higher in isogenic yhb1 mutants. These NO complexes (X-NO) included a protein class that was sensitive as well as a class that was resistant to HgCl2 treatment, behavior identified with SNO and metal-NO complexes, respectively (ref. 28; data not shown). Thus, flavohemoglobin in yeast cells prevents X-NO of multiple targets, including protein thiols, by metabolizing NO.

yhb1 mutants were hypersensitive to the cytostatic effects of NO in room air. For example, yhb1 cells grew much slower than wild-type cells in the presence of 3 mM DETA NONOate (Fig. 4A and C), whereas growth rates of yhb1 mutants and YHB1 wild-type cells were comparable in the absence of drug (Fig. 4B). The mutant cells were also hypersensitive to the cytostatic effect of GSNO (data not shown). In contrast, they were not more sensitive than wild-type cells to the toxic effects of H2O2 (Fig. 4B). When YHB1 deletion was performed in diploid JK93d cells, tetrad analysis revealed cosegregation of antibiotic resistances (G418r and hygR) both with loss of NO-consuming activity and with NO hypersensitivity; a 2:2 ratio was seen in all four complete tetrads studied (Fig. 4C; data not shown). NO resistance was restored in yhb1 cells transformed with a plasmid expressing wild-type YHB1 but not by vector alone (data not shown). Taken together, these data show that YHB1 is required to protect cells from nitrosative stress.

We next addressed the question of whether YHB1 protects cells anaerobically. As shown in Fig. 5A, there was no difference in growth rates in a glove box between yhb1 mutant and isogenic YHB1 cells. Growth of yhb1 mutant cells, however, was clearly slower than...
that of wild-type \( YHB1 \) cells in the presence of DETA NONOate (Fig. 5).

Resistance to NO toxicity was restored by transformation of plasmids containing \( YHB1 \) into \( yhb1 \) mutant cells (data not shown). Thus, \( YHB1 \) also protects against NO anaerobically. By comparison with aerobic cultures, the anaerobically cultured cells were more sensitive to NO toxicity (Figs. 4 and 5A)—a finding in keeping with their level of Yhb1 expression, which, although readily detectable (Fig. 5B), was lower than under aerobic conditions (Fig. 5B). In addition, NO is more stable, and its effective concentration in the medium was greater anaerobically.

NO-dependent NADH consumption by partially purified preparations of yeast hemoglobin was detected under both anaerobic and aerobic conditions (not shown). Extremely rapid consumption of NADH in aerobic preparations was slowed approximately 350-fold by removal of oxygen. Such anaerobic NO-mediated NADH consumption activity was stable over several days. Spectroscopic studies performed on the purified \( E. \) coli enzyme while NADH was being consumed anaerobically showed the visible spectrum of Fe(II)NO. Moreover, Fe(III)NO complexes generated anaerobically (in the presence of NO excess) were transformed by NADH addition into Fe(II)NO. Taken with previous studies (2, 9, 11), these data are consistent with a reaction mechanism in which NO is reduced to NO\(_2\) at the ferrous heme \[ \text{Fe(II)NO} \rightarrow \text{Fe(III)NO}_2 \], ultimately yielding N\(_2\)O (2NO\(_2\)+2H\(^+\) → N\(_2\)O + H\(_2\)O). The role that is envisioned for NADH is to reduce the oxidized heme iron generated in production of NO\(_2\); a new round of catalysis would then be initiated by NO binding to the ferrous heme.

**Discussion**

We have shown that the yeast flavohemoglobin Yhb1 is required for metabolism of NO and consequently protects yeast from NO-related toxicity under both aerobic and anaerobic conditions. By metabolizing NO, Yhb1 prevents nitrosylated protein concentrations from reaching what are presumably hazardous levels (that is, Yhb1 protects against a nitrosative stress; Fig. 3). Yhb1 might also facilitate denitrosylation of thiol- or transition metal-containing peptides or proteins by scavenging NO that has been liberated from these complexes, either enzymatically or nonenzymatically (refs. 9 and 29–31; in a process perhaps even facilitated by Yhb1 itself), thereby shifting the equilibrium toward the denitrosylated state. Irrespective of mechanism, Yhb1 evidently protects against NO and GSNO by shielding vital targets from nitrosative stress.

Yhb1 is protective against NO under strictly anaerobic conditions (Fig. 5). Anaerobic protection against NO by flavohemoglobin has been noted in \( S. \) typhimurium (14) and \( E. \) coli (8). However, the high affinity of flavohemoglobin for O\(_2\) (32) had led some to worry that trace O\(_2\) may not have been excluded adequately in these studies. These concerns may have been reinforced by our previous finding that NO bound to heme in HMP under anaerobic conditions was readily replaced by O\(_2\), and that NADH-dependent transformation of NO was much slower in the anaerobic than aerobic condition (9). Indeed, we also find that NO-stimulated NADH consumption by Yhb1 is slower under strictly anaerobic conditions than in air. Notwithstanding these kinetic differences, we now clearly show that Yhb1 is

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**Fig. 5.** Anaerobic protection against DETA NONOate by \( YHB1 \). Yeast cells were cultured in a glove box for 5 days; studies were initiated on day 3. (A) Wild-type \( Y190 \) (open) and isogenic \( yhb1 \) (filled) cells were cultured in YPD supplemented with 0 (circles), 100 \( \mu \)M (triangles), 200 \( \mu \)M (diamonds), and 400 \( \mu \)M (squares) DETA NONOate. Data are the means of two independent experiments. (B) Aerobic NO clearance by 100 \( \mu \)g of lysates of anaerobically cultured \( yhb1 \) (thin solid line) or \( YHB1 \) (thick solid line) cells or 20 \( \mu \)g of lysates of aerobically cultured \( YHB1 \) (dotted line) cells.

**Fig. 6.** Phylogenetic analysis of flavohemoglobins. The tree is based on amino acid sequence identities and was constructed by the unweighted pair-group method of averages after a Jukes-Cantor correction with the SEQWEB program (Genetics Computer Group, Madison, WI).
functionally important for NO resistance in the complete absence of O₂ (Fig. 5). It remains to be proven that conversion of NO to N₂O (refs. 9 and 11; see Results) is responsible for resistance in the anaerobic mechanism of yeast and bacterial flavohemoglobin, and the possibility of additional cofactors or NO-related substrates in vivo has not been ruled out; however, it should be noted that the flavohemoglobin in _R. eutropha_ (16) is the major source of N₂O during anaerobic growth on nitrite.

The _E. coli hmp_ gene is markedly induced by NO and SNO (8, 9, 12, 13). In contrast, flavohemoglobin expression and activity seems to be constitutive in yeast cells. No increase in Yhb1 activity was observed with DETA NONOate and/or GSNO treatments in logarithmic or stationary yeast cells under aerobic or anaerobic conditions (data not shown). Expression of the yeast _YHB1_ gene was also not significantly affected by H₂O₂, paraquat or menadione, that is, by chemicals that impose an oxidative stress (20, 22). Yhb1 expression was lower in anaerobic culture than in air (Fig. 5B), in agreement with the levels of mRNA previously reported (20, 21). This reduced expression of Yhb1 may contribute to the lower resistance afforded by _YHB1_ under anaerobic conditions (Figs. 4A and 5A). Crawford et al. (21) have reported that _YHB1_ promoter activity and mRNA levels are much higher in logarithmic phase than in stationary phase. Others noted little change in _YHB1_ mRNA levels during diauxic shifts (33). We observed that NO-consuming activity and presumably Yhb1 protein level are slightly higher in stationary cells than in logarithmic growth (data not shown). The reasons for the disparity among studies may include the relatively long half-life of Yhb1, the different culture mediums used, and/or the differences in genetic backgrounds. That Yhb1 is ultimately the enzyme activity—to be distinguished from the protective function—is most proficient in air. It is not inconceivable that the ancient flavohemoglobin (~2 billion years ago) originated in the anaerobic mode (or evolved the anaerobic mechanism) to detoxify NO, which was abundant in the earth’s primordial atmosphere. Only minor adaptations in the flavohemoglobin would have been required to accommodate the evolution of O₂ as a cosubstrate, thereby improving enzyme efficiency (8, 9, 11). Regardless of the evolutionary origin of the mechanism, it is probably a safe bet that as of 1 billion to 1.5 billion years ago (around the time of yeast and plant evolution) the flavohemoglobin was functioning dually as an “NO reductase-NO oxygenase” to protect against nitrosative stress under anaerobic and aerobic conditions. Modern flavohemoglobins may have specialized further in metabolizing NO (or related molecules) derived from environmental sources (39), from endogenous metabolic processes such as denitrification (16), or from hosts that produce NO and SNO for defense purposes (36, 40, 41).

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