Fungal Heme Oxygenases: Functional Expression and Characterization of Hmx1 from *Saccharomyces cerevisiae* and CaHmx1 from *Candida albicans*†

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ABSTRACT: Heme oxygenases convert heme to free iron, CO, and biliverdin. *Saccharomyces cerevisiae* and *Candida albicans* express putative heme oxygenases that are required for the acquisition of iron from heme, a critical process for fungal survival and virulence. The putative heme oxygenases Hmx1 and CaHmx1 from *S. cerevisiae* and *C. albicans*, respectively, minus the sequences coding for C-terminal membrane-binding domains, have been expressed in *Escherichia coli*. The C-terminal His-tagged, truncated enzymes are obtained as soluble, active proteins. Purified ferric Hmx1 and CaHmx1 have Soret absorption maxima at 404 and 410 nm, respectively. The apparent heme binding K_d values for Hmx1 and CaHmx1 are 0.34 ± 0.09 μM and 1.0 ± 0.2 μM, respectively. The resonance Raman spectra of Hmx1 reveal a heme binding pocket similar to those of the mammalian and bacterial heme oxygenases. Several reductants, including ascorbate, yeast cytochrome P450 reductase (CPR), human CPR, spinach ferredoxin/ferredoxin reductase, and putidaredoxin/putidaredoxin reductase, are able to provide electrons for biliverdin production by Hmx1 and CaHmx1. Of these, ascorbate is the most effective reducing partner. Heme oxidation by Hmx1 and CaHmx1 regiospecifically produces biliverdin IXα. Spectroscopic analysis of aerobic reactions with H_2O_2 identifies verdoheme as a reaction intermediate. Hmx1 and CaHmx1 are the first fungal heme oxygenases to be heterologously overexpressed and characterized. Their heme degradation activity is consistent with a role in iron acquisition.

Iron is an essential cofactor for many cellular processes. Fungi, including *Saccharomyces cerevisiae* and *Candida albicans*, can cope with tremendous variations in the abundance of environmental iron. Studies with the baker's yeast *S. cerevisiae* have demonstrated that under iron deprivation the cell expresses a set of genes involved in the acquisition of iron from the environment and its mobilization from intracellular stores. These genes include the Aft1p regulon (*1*−*3*). One of the Aft1p target genes is *HMX1* which exhibits low sequence identity with known heme oxygenases (HOs[1]). *C. albicans* is an opportunistic pathogen that can switch from commensal colonization to invasive infection (*4*, *5*). Hemoglobin (Hb) has been identified as a host signal to shift to the infectious state and to upregulate CaHmx1 for the metabolism of heme from Hb (*5*). CaHmx1 has been shown to allow the pathogen to utilize extracellular heme or Hb as a source of iron and to confer a nutritional advantage for growth in the mammalian host (*5*−*7*).

Heme oxygenases catalyze the oxidation of heme to biliverdin, CO, and free iron in the presence of O_2 and a suitable electron donor. Heme serves as both the prosthetic group and the substrate for this enzyme, and the overall catalytic process requires three molecules of O_2 and seven electrons (*8*). The oxidation of heme to biliverdin by the known mammalian heme oxygenases proceeds through three distinct steps: oxidation of heme to α-*meso*-hydroxyheme, conversion of α-*meso*-hydroxyheme to verdoheme, and transformation of verdoheme to biliverdin (Scheme 1).

Heme oxygenases are found in various organisms including bacteria, plants, and mammals. Three heme oxygenase isoforms have been reported in mammals, but only two, heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2), appear to play important roles in heme catabolism (*8*). Recently, several bacterial heme oxygenases have been reported including HemO, HmuO, IsdG/I, BphO, and PigA (*9*−*16*). Most of these bacterial heme oxygenases convert heme to iron, biliverdin IXα, and CO, but PigA from *Pseudomonas* is distinct in that its reaction product is biliverdin IXγ rather than IXα (*15*). All these bacterial heme oxygenases share structural and primary amino acid sequence similarity to the mammalian heme oxygenases. However, a recent X-ray structure and sequence analysis of ChuS, a heme oxygenase from a pathogenic *Escherichia coli*, showed unique features (*16*).

Fungal heme oxygenases have not been characterized at the protein level, and their identities and properties remain

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[1] Abbreviations: Hb, hemoglobin; HO, heme oxygenase; PCR, polymerase chain reaction; TB, terrific broth; CPR, cytochrome P450 reductase; Fd, ferredoxin; FdR, ferredoxin reductase; Pd, putidaredoxin; PdR, putidaredoxin reductase; SOD, superoxide dismutase; RR, resonance Raman.

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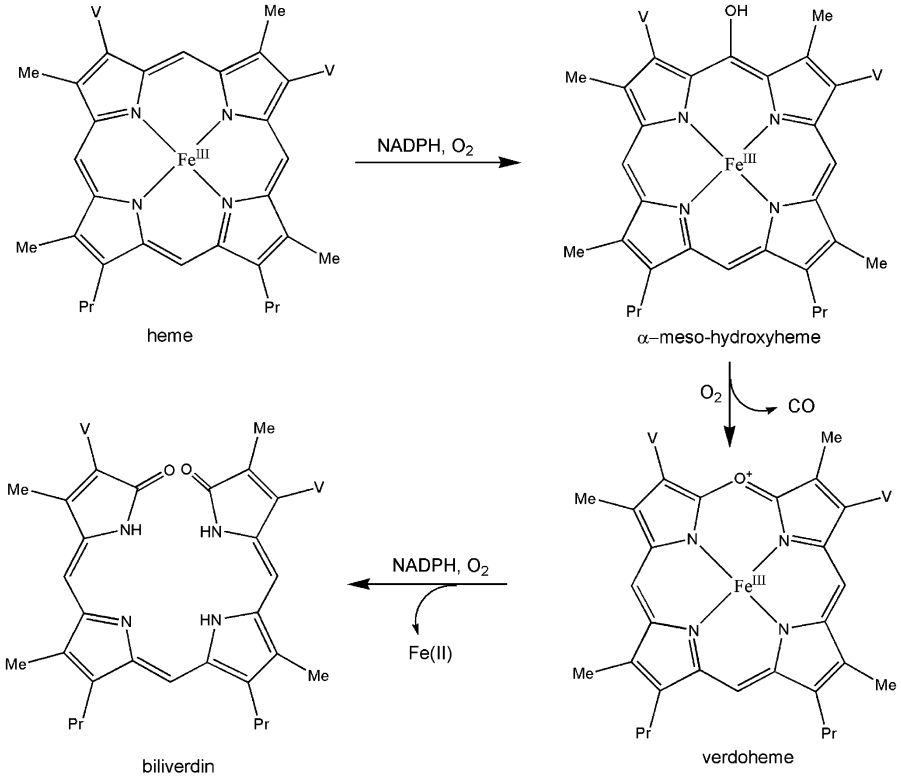
Scheme 1: The Heme Oxygenase-Catalyzed Conversion of Heme to Biliverdin[a]



[a] The substituents on the porphyrin are vinyl (V), propionate (Pr), and methyl (Me).

obscure. *S. cerevisiae* contains the gene *HMX1* exhibiting some sequence similarity with human HO-1 (21%) and bacterial HmuO (19%). However, a previous attempt to clone and express a heme oxygenase from *S. cerevisiae* suggested that the Hmx1 protein had no heme oxygenase activity (*17*). The initial sequence of *Hmx1* used for this study was later corrected in the *Saccharomyces* genome database: 44 amino acid residues at the N-terminus were missing in the sequence used in the previous study. This deletion could account for the fact that no heme oxygenase activity was observed. Deletion of the Hmx1 gene caused a decrease in heme degradation activity and suggested that Hmx1 is important for heme iron reutilization and also for the homeostasis of regulatory pools of iron and heme (*1*).

The heme oxygenase gene (*CaHmx1*) from *C. albicans* exhibits 25% sequence identity and 38% sequence similarity with human HO-1. In addition, the structural signatures for heme oxygenase as well as the proximal region of the heme-binding pocket, including His25, Ala28, Glu29, and Tyr58 in human HO-1, are all present (*5*, *18*). Previous studies of the *CaHmx1* gene have shown that it is required for iron assimilation and is regulated by heme and Hb (*5*−*7*, *19*). The regulation of CaHmx1 expression and its functions *in vivo* have been carefully examined, but the nature of the enzymatic activity remains unclear.

In this study, we have overexpressed and purified for the first time two yeast heme oxygenase proteins, Hmx1 from *S. cerevisiae* and CaHmx1 from *C. albicans*, and have characterized these proteins in terms of their structural, spectroscopic, and catalytic properties.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Hemin, sodium ascorbate, sodium dithionite, and desferrioxamine mesylate were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were of the highest grade commercially available. The spinach ferredoxin (Fd) and ferredoxin reductase (FdR) were purchased from Sigma. The human cytochrome P450 reductase, putidaredoxin (Pd), and putidaredoxin reductase (PdR) were expressed in *E. coli* and purified to apparent homogeneity as described elsewhere (*20*, *21*). The cytochrome P450 reductase from *S. cerevisiae* was kindly provided by Larissa M. Podust of Vanderbilt University.

Construction of Expression Plasmids. The expression vectors for the yeast heme oxygenases were constructed in the pCW(Ori$^+$) plasmid (*22*). The genomic DNAs from *S. cerevisiae* and *C. albicans* were kindly provided by the laboratories of Erin O'Shea and Alexander Johnson at UCSF, respectively. Amino acid residue analysis and alignment with human HO-1 suggests that the C-termini of the proteins contain a lipophilic membrane-binding domain (Figure 1). The C-terminal truncated genes lacking 23 residues for Hmx1 and 20 residues for CaHmx1, and an added 6 × His-C-terminal tag, were amplified using PCR. The amplified PCR fragments were purified by agarose gel electrophoresis and cloned into the pCW(Ori$^+$) vector using the *Nde*I and *Xba*I restriction sites. The cloned vectors were verified by nucleotide sequencing and restriction digestion.

C. albicans translates CTG as Ser instead of Leu, the amino acid for which CTG is a universal codon (*23*, *24*). The open reading frame of *CaHmx1* contains a CTG

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                                                    *
HO-1      .........M ERPQPDSMPQ D.....LSEA LKEATKEVHT QAENAEFMRN
Hmx1      .........M EDSSNTIIPS PTDVGALANR INFQTRDAHN KINTF...MG
CaHmx1    MQYKSSGATS KLSQVEIIPA KTDVGALANR INLETRSLHD RADKT...VT

HO-1      FQKGQVTRDG F..KLVMASL YHIYVALEEE IERNKESPV. ........FA
Hmx1      IKMAIAMRHG FIYRQGILAY YYVFDAIEQE IDRLLNDPVT EEELQTSTIL
CaHmx1    LKFALALRNY KVYRQGLQAF YHVFASIEKA LYRQLE.... .KKDEWSEML

HO-1      PVYFPEELHR KAALEQDLAF WYGP...... RWQEVI.PYT ..PAMQRYVK
Hmx1      KQFWLEDFRR STQIYKDLKL LYSNTFKSTE SLNEFLATFQ KPPLLQQFIN
CaHmx1    EQVWKPEIAR AGKAEQDLLF FYDD...... NKEKFIKPIM ..PAQIEFCK

HO-1      RLHEVGRTEP ELLVAHAYTR YLGDLSGGQV LKKIAQKALD LPSSGEGLA.
Hmx1      NIHENIHKEP CTILSYCHVL YLALFAGGKL IRSNLYRRLG LFPNFEKLSQ
CaHmx1    HILEVTEEKP YLLFAYLHVM YLALFAGGRI MRSSVLKATG MYPQRDGLSH

HO-1      .........F FTFPNIASA. .TKFKQLYRS RMN...SLEM TPAVRQRVIE
Hmx1      KELVKKGTNF FTFSDLGPTE ETRLKWEYKK NYELATRTEL TEAQKLQIIS
CaHmx1    DDVVRMGTNF FTF.DV..PD EDLLRLTYKR DYELVTRNGL TEEQKLEIIE

HO-1      EAKTAFLLNI QLFEELQELL THDTKDQSPS RAPGLRQRAS NKVQDSAPVE
Hmx1      VAEGIFDWNF NIVAEIGELN RRELMGKFSF KCITYLYEEW MFNKDSATRR
CaHmx1    ESKYIFEHDV KCVAELEKHN MDKLSGTWTY FLVTRGY... ..........

HO-1      TPRGKPPLNT RSQAPLIRWV LTLSFLVATV AVGLYAM
Hmx1      ALHTVMLLVL SIIAIWVLYF LVKSFLSIV. .......
CaHmx1    ...YAALVLF SLLALIYLRR VVNKLT.... .......
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FIGURE 1: Sequence alignment of Hmx1 and CaHmx1 with human HO-1. The amino acid sequences were aligned using the software T-Coffee from the Swiss Institute of Bioinformatics (http://www.ch.embnet.org/software/TCoffee.html). The regions of high sequence homology are underlined, and the proximal histidine ligand is marked with an asterisk (*). The residues believed to constitute the lipophilic portion are shown in bold.

encoding a Ser that must be corrected by site-directed mutagenesis to enable expression of the correct recombinant protein in *E. coli*. Site-directed mutagenesis to change Leu5 to a Ser was carried out using Quick-Change mutagenesis (Stratagene, La Jolla, CA). The change in nucleotides was confirmed by nucleotide sequencing.

The conserved histidine residues in Hmx1 and CaHmx1 that correspond to the proximal heme ligand in human HO-1 (His25) were proposed to be His30 for Hmx1 and His39 for CaHmx1 based on sequence alignments with human HO-1 (Figure 1). The putative proximal histidine ligand mutants Hmx1 H30A and CaHmx1 H39A were constructed from the wild-type pCW(Ori$^+$) vectors using site-directed mutagenesis.

Expression and Purification. Expression and purification of the truncated yeast heme oxygenases were carried out as previously described with some modifications (*25*). The *E. coli* strains transformed with pCW(Ori$^+$) vectors were inoculated into TB medium containing 100 μg/mL ampicillin and 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expression cultures were grown at 37 °C for 3 h and then at 28 °C with shaking at 200 rpm for 24 h in 1 L Fernbach flasks. The soluble proteins were separated by ultracentrifugation, and purified using a Ni^{2+}-nitrilotriacetic acid agarose column eventually eluted with imidazole (400 mM). The eluted fractions were concentrated and dialyzed. Reconstitution with heme was performed as previously described (*25*) so that hemin was added to the purified proteins to give a final 1:1 heme:protein ratio. The samples were applied to a hydroxyapatite Bio-Gel HTP column (Bio-Rad Laboratories, Hercules, CA) preequilibrated with 10 mM potassium phosphate buffer (pH 7.4), and the proteins were eluted with 150 mM potassium phosphate buffer (pH 7.4) after first washing with 10 mM potassium phosphate buffer (pH 7.4).

Spectroscopic Characterization. Sodium dithionite was added to reduce the purified ferric heme oxygenases. The CO−ferrous heme oxygenase complexes were generated by passing CO gas through solutions of the ferrous heme oxygenases. UV−visible spectra were collected at room temperature on a Cary Varian UV/visible spectrometer in 100 mM potassium phosphate buffer (pH 7.4) using 1 cm path length quartz cuvette.

Resonance Raman (RR) spectra were obtained on a McPherson 2061/207 spectrograph equipped with a Princeton instrument liquid-N_2-cooled (LN-1100PB) CCD detector. Kaiser Optical supernotch filters were used to attenuate Rayleigh scattering. Excitation sources consisted of an Innova 302 krypton laser (413 nm) and a Liconix 4240NB He/Cd laser (442 nm). Spectra were recorded in a 90°-scattering geometry on samples at room temperature. Frequencies were calibrated relative to indene and CCl_4 standards and are accurate to ± 1 cm^{-1}. CCl_4 was also used to check the polarization conditions. Optical absorption spectra of the Raman samples were obtained on a Cary 50 Varian spectrophotometer to monitor the sample (fully oxidized, fully reduced, CO complex) both before and after laser illumination.

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter. The samples were at a concentration of 1 μM in 10 mM potassium phosphate buffer, pH 7.4. The spectra were an average of 2 scans recorded at 25 °C with a bandwidth of 1 nm, a 0.1 nm step size, and a 1 s time constant and were baseline corrected.

HPLC Analysis of Biliverdin Isomers. Biliverdin formation reactions included 2 μM heme oxygenase, 30 μM hemin, 50 mM sodium ascorbate, and 2 mM desferrioxamine mesylate in 0.50 mL of 100 mM potassium phosphate buffer (pH 7.4) with 150 units each of catalase and SOD. Incuba-

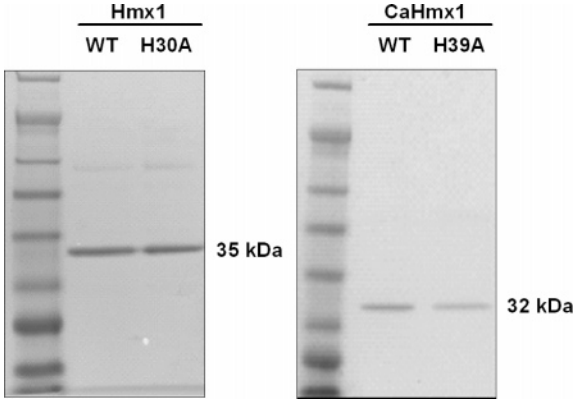

FIGURE 2: SDS−PAGE of the purified wild-type and mutant Hmx1 and CaHmx1 proteins.

tions were done for 4 h at room temperature. The extraction of the biliverdin products and their conversion to the dimethyl esters were done as previously described (*26*). The biliverdin dimethyl esters were analyzed by HPLC on a YMC ODS-AQ column (S-5, 120 Å, 4.6 × 250 nm) at a flow rate of 1.0 mL/min using a distilled water as solvent A, and CH_3-OH as solvent B. The percentage of solvent B was held for 5 min to 30% and increased linearly to 75% over 5 min; the percentage of solvent B was then increased to 95% over 35 min and held for 5 min.

Spectrophotometric Monitoring of Reaction Intermediates. The reactions supported by H_2O_2 were performed to identify intermediates in the oxidation of heme. The spectrum of either wild-type Hmx1 or CaHmx1 (4 μM) was first recorded after addition of 20 μM H_2O_2 under an aerobic atmosphere. Following maximum loss of the Soret band, pyridine was added to the solution to a final concentration of 20% for pyridine verdohemochrome production.

RESULTS

Expression and Purification of Hmx1 and CaHmx1. Both the native Hmx1 and CaHmx1 proteins are anchored to the membrane by C-terminal lipophilic peptides analogous to those of human HO-1 (*25*). Initial expression and purification of the native recombinant Hmx1 and CaHmx1 proteins showed that they were localized in the membrane fractions (data not shown). In order to overcome the low yields and difficulties in purifying membrane-bound proteins, the C-termini of the proteins were truncated (by 23 residues (Hmx1) and 20 residues (CaHmx1) and C-terminal 6 × His-tags were added (Figure 1 and Supporting Information, Figure S1). Purification of Hmx1 and CaHmx1 gave proteins with single bands on SDS−PAGE at 35 and 32 kDa, respectively (Figure 2). No green coloration due to the accumulation of biliverdin was observed during the expression, in contrast to what was seen with human HO-1 and some bacterial heme oxygenases (*10*, *25*, *28*). Interestingly, the absorption spectra of the purified proteins before reconstitution with hemin showed distinct Soret bands at 404 nm for Hmx1 and 410 nm for CaHmx1 (data not shown). These results suggest that the recombinant proteins acquire and retain small amounts of heme from either the cytosol or the expression medium, but cannot oxidize it, presumably because there is no optimal reduction partner for the yeast heme oxygenases in *E. coli* cells. Hmx1 and CaHmx1 may not be able to accept electrons

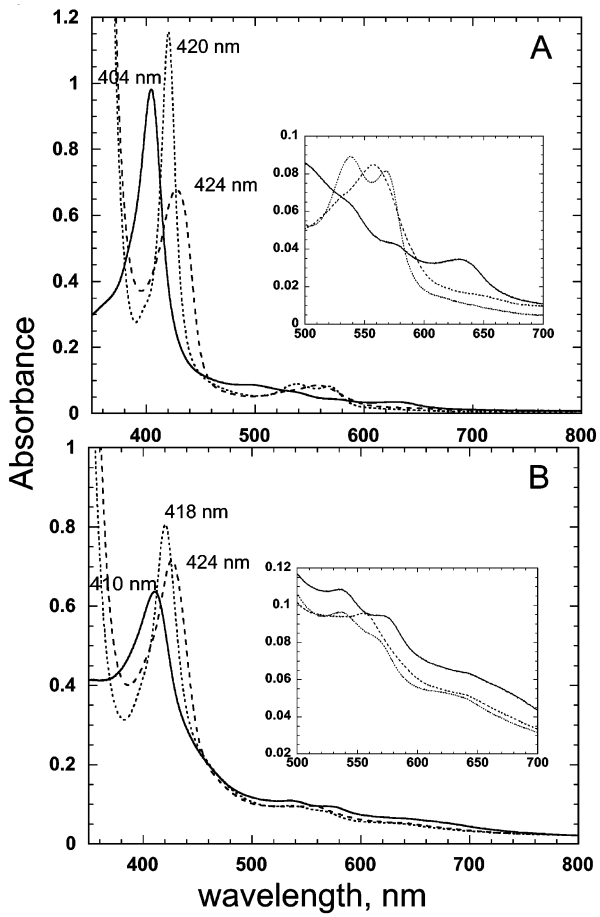

FIGURE 3: UV/visible absorption spectra of wild-type Hmx1 (A) and CaHmx1 (B) proteins. The spectra are of the ferric heme complex (—), the ferrous complex (− − −), and the ferrous carbon monoxide complex (- - -). The insets show an expanded region of the spectra.

from the *E. coli* flavodoxin and flavodoxin reductase, contrary to what was observed upon expression of human HO-1 in *E. coli*. Based on the pyridine hemochrome method, the extinction coefficients for Hmx1 and CaHmx1 are 169 and 121 mM^{-1} cm^{-1}, respectively (*29*).

Properties of the Heme−Hmx1 and Heme−CaHmx1 Complexes. After incubation with free hemin followed by removal of excess hemin on a hydroxyapatite column, the proteins Hmx1 and CaHmx1 show characteristic Soret absorptions at 404 and 410 nm, respectively (Figure 3). Reduction of the ferric Hmx1 with dithionite results in a shift of the Soret band to 424 nm. The CO complex is readily observed with a Soret maximum at 420 nm and α/β bands at 563 and 537 nm, respectively (Figure 3A). In addition, the ferric protein possesses a distinct absorption band near 630 nm characteristic of a high-spin ferric heme (Figure 3A). CaHmx1 exhibited similar Soret band shifts to 424 nm for the ferrous form and 418 nm for the CO-bound ferrous form, but had a weaker absorption at 630 nm (Figure 3B).

Spectroscopic titrations of Hmx1 and CaHmx1 with heme were used to measure the heme binding constants for the proteins (Figure 4). Heme titrations with Hmx1 and CaHmx1 gave the apparent binding constants of $K_d = 0.34 \pm 0.09$ μM and 1.0 ± 0.2 μM, respectively. The calculated K_d values indicate a similar heme affinity to that of human HO-1 or the bacterial heme oxygenases (*11*).

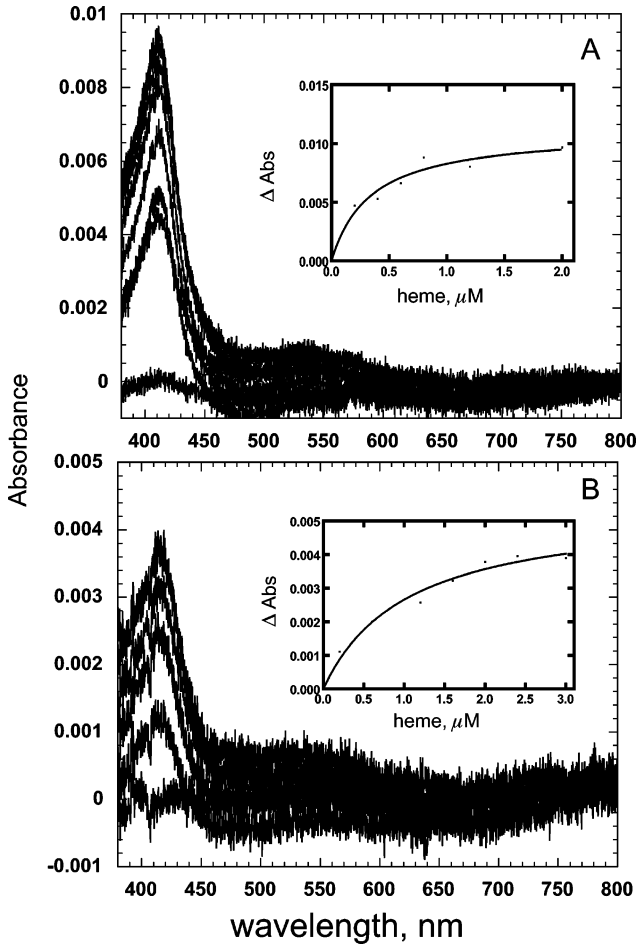

FIGURE 4: Absorption difference spectra of heme binding to wild-type Hmx1 (A) and CaHmx1 (B). Increasing concentrations of heme (0.1−3 μM) were added to both the sample (2 μM enzyme) and reference cuvettes.

Resonance Raman Spectroscopy. The high-frequency RR spectrum of the ferric protein is characteristic of a 6-coordinate high-spin (6cHS) species with ν_4, ν_3, and ν_{10} at 1371, 1482, and 1611 cm^{-1}, respectively (Figure 5A). In the reduced protein, the heme iron is in a 5cHS configuration as revealed by its ν_3 at 1469 cm^{-1} (Figure 5A). Thus, the coordination and spin states of the heme iron in ferric and ferrous wild type Hmx1 are identical to those of the mammalian and bacterial heme oxygenases and suggest the presence of a proximal histidine and a distal water ligand.

Hmx1 and CaHmx1 showed pH-dependent spectral changes similar to those of mammalian HO-1 (*25*, *30*). As the pH is raised, the Soret bands of Hmx1 shift to the red and the absorption maximum at 630 nm indicative of a high-spin species decreases (Supporting Information, Figure S2). The loss of the high-spin marker at alkaline pH suggests a transition to a low-spin conformation. The RR analysis of Hmx1 at different pH values (Figure 5B) supports this conclusion, with the alkaline pH sample exhibiting skeletal vibrations consistent with a 6cLS configuration. These results argue in favor of the axial coordination of an aqua ligand to the ferric iron. Coordination to the iron(III) lowers the pK_a value of the coordinated water molecule and facilitates its deprotonation to a hydroxyl ion above pH 8. The stronger ligand field associated with the hydroxyl group changes the iron(III) configuration from high-spin to low-spin. In the low-frequency RR spectra of the alkaline form of the ferric heme complex, a RR band observed at 554 cm^{-1} in unlabeled water downshifts to 533 cm^{-1} in $H_2{}^{18}O$ (Figure 6A). This vibrational frequency and its ^{18}O-isotope shift is typical of a ν(Fe−OH) in a 6cLS ferric−hydroxo heme complex (*31*−*33*) and is within a few cm^{-1} of those observed in mammalian and bacterial heme oxygenases (*34*, *35*).

A 442 nm excitation was used to acquire the low-frequency RR spectrum of ferrous Hmx1 at neutral pH to identify the proximal ligand of Hmx1 (Figure 6B). An intense band of 218 cm^{-1} is within the characteristic range for ν(Fe-His) vibrations.

Catalytic Activities of Hmx1 and CaHmx1. Purified Hmx1 and CaHmx1 were tested for biliverdin formation using several reductants (ascorbate, yeast CPR, human CPR, Fd/FdR, and Pd/PdR). All the reductant systems supported biliverdin production by Hmx1 and CaHmx1, but ascorbate appeared to be the most potent reducing partner (Supporting Information, Figure S3). In addition, the biliverdin isomer IXγ was produced in the ascorbate coupled Hmx1 or CaHmx1-dependent oxidation as a minor product due to concurrent production of H_2O_2 and could be eliminated by the addition of SOD and catalase to the incubation mixture (Figure 7A). Hmx1 and CaHmx1 specifically produce biliverdin IXα under all conditions examined, as indicated by spectroscopic comparison (not shown) and cochromatography of the product with authentic standards of the four possible biliverdin isomers (Figure 7A). The conversions of the heme−Hmx1 and heme−CaHmx1 complexes to biliverdin in the presence of ascorbate were monitored by UV/visible spectroscopy (Figures 7B and 7C). Over a period of 1 h, the enzymatic reaction proceeded with a decrease in the Soret absorption and the α and β bands and an increase of a broad maximum at 680 nm indicative of iron-free biliverdin (Figures 7B and 7C).

Biliverdin production by the Hmx1 H30A and CaHmx1 H39A mutants was greatly decreased, or was not observable, and only trace amounts of non-regiospecific reaction products were obtained (Figure 7A). Far-UV CD spectra of the wild-type and mutant proteins indicates that the mutant proteins have the same general fold as the wild-type proteins, and thus that the loss of catalytic activity was not the result of a significant misfolding of the protein (Supporting Information, Figure S4). Titration of these mutants with imidazole did not rescue their ability to catalyze the conversion of heme to biliverdin (data not shown), in contrast to what was observed with the human HO-1 and bacterial HmuO proximal histidine mutants (*36*, *37*).

Reaction Intermediates. The conversion of heme to verdoheme by human HO-1 can be supported by H_2O_2 (*27*). Aerobic reaction of wild-type Hmx1 and CaHmx1 with H_2O_2 exhibited spectroscopic kinetic patterns analogous to those observed with human HO-1 (Figure 8) (*27*, *38*): the 660−690 nm absorption characteristic of verdoheme increased in parallel with a decrease in the Soret band. Addition of pyridine to the complex showed the characteristic pyridine verdohemochrome spectrum with a maximum at 680 nm (Supporting Information, Figure S5) (*28*). These results indicate that verdoheme is produced as an intermediate in the oxidation of heme by Hmx1 and CaHmx1 (Scheme 1).

Peroxidase Activity. Mutation of Gly139 in human HO-1 to an alanine causes a major loss of heme oxygenase activity in favor of a peroxidase activity that can be measured by

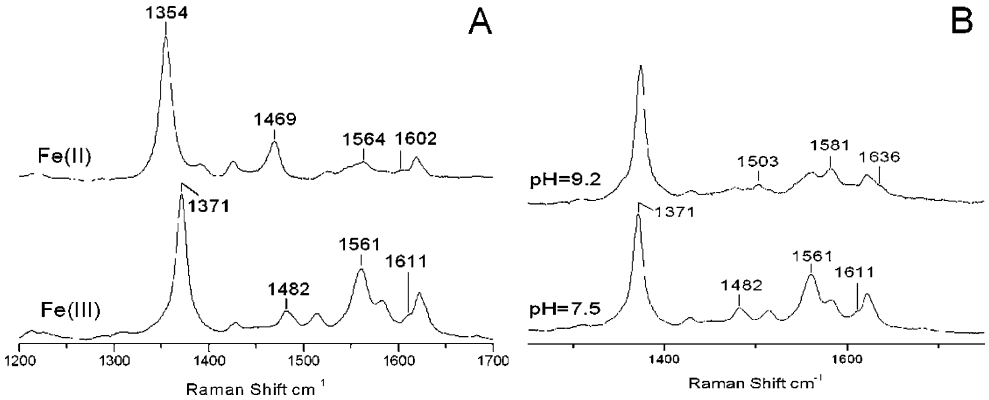

FIGURE 5: RR spectra of Hmx1 obtained with 413 nm excitation at room temperature. (A) High-frequency RR spectra of ferric and ferrous Hmx1. (B) High-frequency RR spectra of ferric Hmx1 at varying pH.

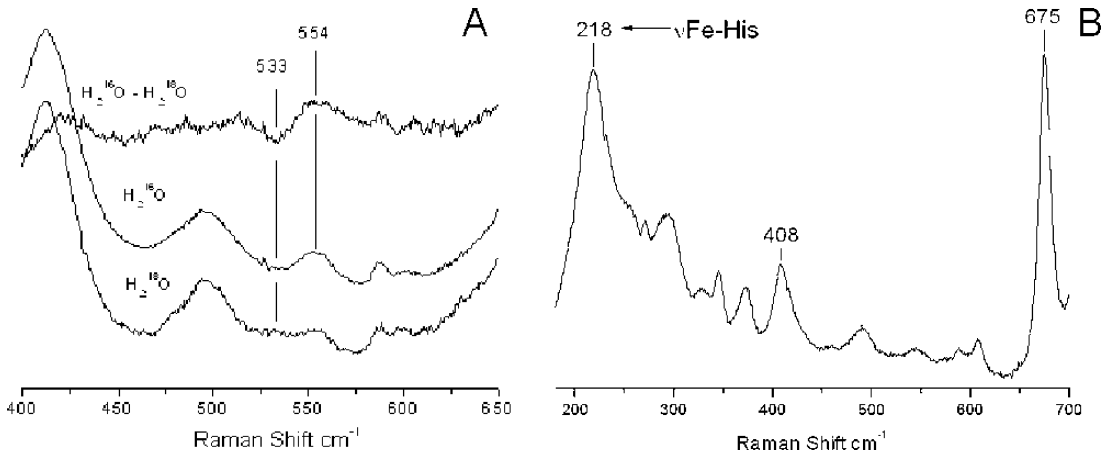

FIGURE 6: Low-frequency RR spectra of ferric Hmx1. (A) The RR spectra obtained in 25 mM CHES buffer (pH 9.5) in $H_2{}^{16}O$ and $H_2{}^{18}O$ with 413 nm excitation at room temperature. (B) The RR spectrum obtained with 442 nm excitation at room temperature.

guaiacol oxidation (*40*). Analysis of the sequences of HMx1 and CaHmx1 shows that the residue that corresponds to Gly139 is an alanine in the two fungal enzymes. We therefore investigated whether the fungal heme oxygenases have unusual peroxidase activity. The guaiacol peroxidase activity, measured as reported previously (*40*), was 0.0015 s^{-1} for Hmx1 and 0.0008 s^{-1} for CaHmx1, values very similar to the value of 0.0016 s^{-1} reported previously for wild-type hHO-1 (*40*).

DISCUSSION

Fungi require iron to perform their biological functions, including growth and pathogenic infection. Since iron availability is highly limited due to its extreme insolubility at physiological pH, iron acquisition is crucial for microorganisms (*7*, *19*, *37*, *41*). While our understanding of iron and heme metabolism in mammals has advanced greatly in recent years, iron assimilation in yeast is not well understood. Here we have investigated two of the proteins responsible for iron utilization, the heme oxygenases from the prototypical eukaryotic microorganism *S. cerevisiae* and the pathogenic fungus *C. albicans*.

Although a previous investigation reported that Hmx1 has no heme oxygenase activity (*17*), the membrane fraction from *S. cerevisiae* was shown to catalyze heme degradation in the presence of ascorbate. Furthermore, it was shown that this ascorbate-dependent activity was lost when the *Hmx1* gene was deleted (*1*). Protchenko et al. also observed that disruption of intracellular iron and heme homeostasis in *Hmx1*Δ was corrected by expression of the bacterial heme oxygenase, HmuO (*1*). The absence of heme oxygenase activity in the previous attempt to characterize Hmx1 may be due to the absence of 44 amino-terminal residues in the originally posted sequence of the gene. Bacterial heme oxygenases are soluble cytosolic proteins, whereas mammalian heme oxygenases are integral membrane-bound proteins (*42*−*44*). By immunofluorescence analysis, Hmx1 showed a peripheral and perinuclear distribution, which corresponds to the endoplasmic reticulum of yeast (*1*). We confirmed by ultracentrifugation that recombinant Hmx1 and CaHmx1 without truncation of C-terminal regions were localized to membrane fractions. The construction of soluble Hmx1 and CaHmx1 with C-terminal truncations established that these yeast heme oxygenases, like their mammalian analogues, possess C-terminal lipophilic peptides that anchor them to the membrane (Supporting Information, Figure S1). Truncation of the membrane binding domain can circumvent the difficulties associated with purification of proteins from microsomal membranes (*25*). Ultimately, these modifications should make crystallographic analyses of these proteins feasible.

In this study, we have demonstrated that the products of the *Hmx1* and *CaHmx1* genes cleave heme to biliverdin. This reaction requires dioxygen and a reducing partner to transfer electrons. The expression of human and bacterial heme oxygenases in *E. coli* turned the culture medium green due

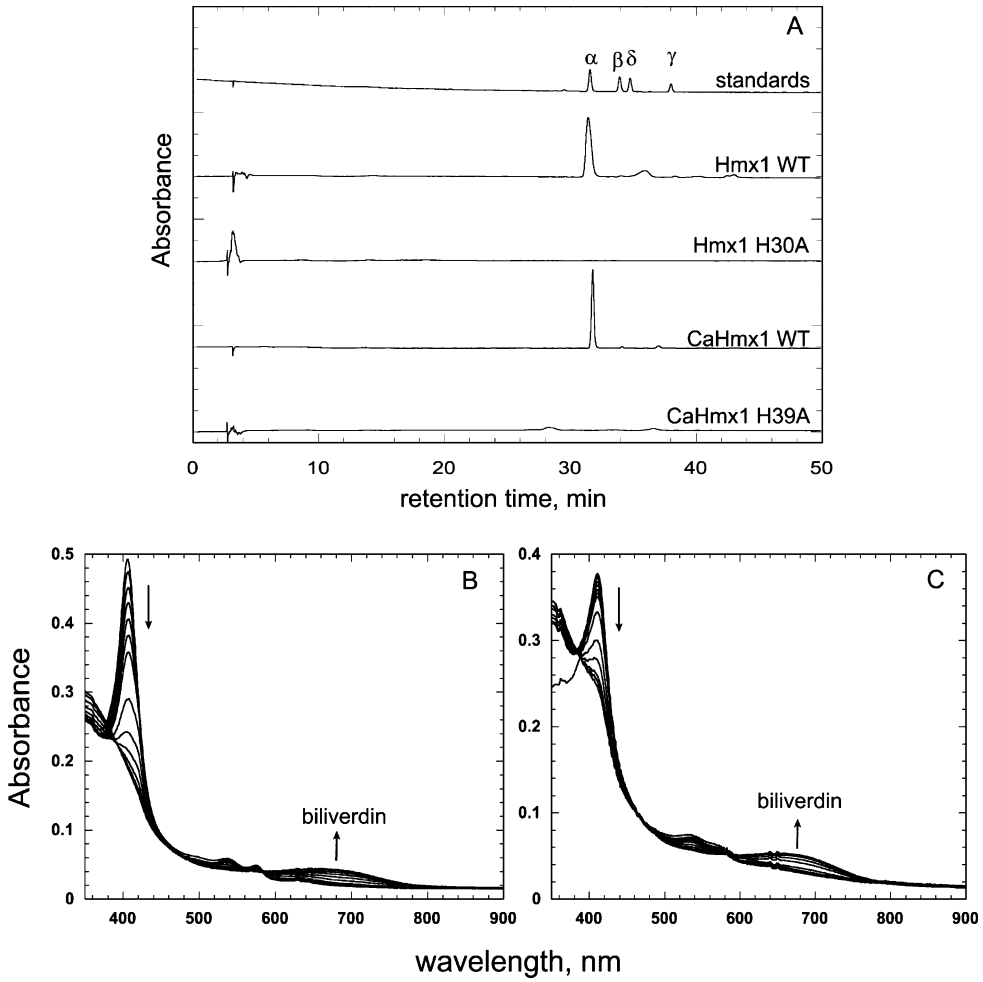

FIGURE 7: Biliverdin formation by yeast heme oxygenases in the presence of ascorbate. (A) HPLC analysis of the biliverdin dimethyl esters isolated from the reactions supported by sodium ascorbate (50 mM). The retention times are for biliverdin IXα (30.1 min), biliverdin IXβ (32.6 min), biliverdin IXδ (33.5 min), and biliverdin IXγ (37.1 min), respectively. The conversions of heme−Hmx1 (B) and heme−CaHmx1 (C) to biliverdin in the presence of ascorbate are also shown.

to the accumulation of biliverdin (*10*, *25*, *28*). Presumably, heme oxidation occurs during the expression in *E. coli* as flavodoxin and flavodoxin reductase support catalytic turnover of the mammalian and bacterial heme oxygenases (*45*). However, the lack of accumulated green chromophore (biliverdin) in cultures expressing the fungal heme oxygenases suggests that Hmx1 and CaHmx1 do not readily accept electrons from *E. coli* flavodoxin and flavodoxin reductase. Nevertheless, several reduction systems, including mammalian and yeast CPR and bacterial flavoprotein/ferredoxin reductase pairs, can support biliverdin production in purified Hmx1 and CaHmx1, albeit at lower rates than those obtainable with ascorbate. These results suggest that the yeast heme oxygenases may have different reduction partners for heme oxygenase activity.

Our RR analysis of the heme−Hmx1 complex shows that, in the oxidized protein, the heme iron is coordinated to a proximal histidine and an aqua ligand with a pK_a around 8. Iron(III)−aqua stretching frequencies are not observable in the RR of hemoproteins, but the iron(III)−hydroxo stretching frequency suggests a comparable distal environment in Hmx1 and other heme oxygenases. In the reduced protein, the aqua ligand is destabilized and the ferrous heme presents an open coordination site trans from a neutral proximal histidine identified by a ν(Fe-His) at 218 cm^{-1}. The similarity in ν(Fe-His) in yeast, mammalian, and bacterial heme oxygenases suggests a common structure of the proximal pocket across species in this family of enzymes.

Based on sequence alignments with human HO-1, the proximal histidine ligands, His30 in Hmx1 and His39 in CaHmx1, are conserved (Figure 1). Studies of biliverdin production by the corresponding site-directed mutants strongly support the inference that these histidine residues are the proximal ligands for the heme (Figure 7A). Reconstitution of the His to Ala mutants with hemin only gave rise to very weak Soret absorptions and suggested that the anchoring of heme within the substrate pocket is significantly dependent on the proximal histidine. Attempts at characterizing these heme−protein complexes using RR spectroscopy were hampered by substantial fluorescence background. Moreover, the observation of poorly resolved porphyrin skeletal modes suggests the presence of nonspecific heme−protein complexes. Circular dichroism spectra, however, indicated that the proteins retained the same fold as the parent enzymes. The sequence alignments also revealed that an alanine is present in Hmx1 and CaHmx1 at the position that corresponds to Gly139 in human HO-1. The peroxidase activity of Hmx1 and CaHmx1, however, is negligible, in sharp contrast to the G139A mutant of human HO-1 (*40*). This suggests that the hydrogen-bonding patterns in the fungal enzyme active sites differ from those in the mammalian protein.

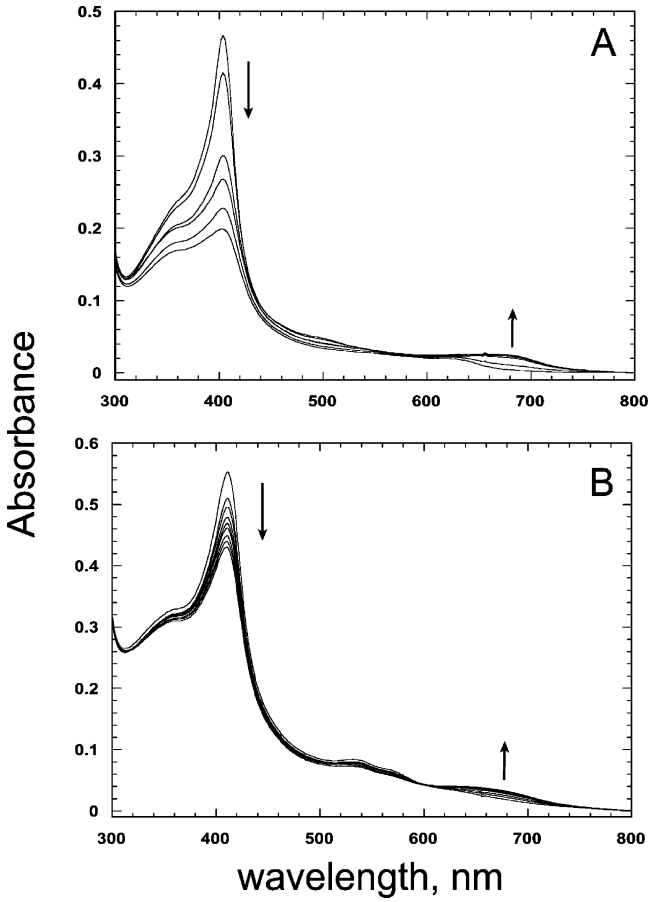

FIGURE 8: UV/visible spectroscopic monitoring for the time course of the reactions of heme−Hmx1 and heme−CaHmx1 with H_2O_2. (A) H_2O_2-dependent conversion of the ferric heme−Hmx1 complex to the verdoheme. (B) Aerobic H_2O_2-dependent conversion of the ferric heme−CaHmx1 complex to the verdoheme.

Several genes that are involved in iron metabolism of *S. cerevisiae* have orthologues in *C. albicans*: *FRE* (ferrireductase-encoding gene), *FTR/FET* (encoding components of the high affinity ferrous uptake system), *SIT/ARN* (siderophore transporters), *CTR* (encoding copper transporters required for reductive uptake of iron), and *Hmx1* (*46*). This indicates that both yeast species have common features in their mechanisms of iron acquisition and metabolism. Although CaHmx1 is the orthologue of Hmx1 in *C. albicans*, the main physiological role of CaHmx1 is almost certainly to mediate the use of extracellular heme as an iron source, whereas Hmx1 may primarily be involved in reutilization of intracellular heme iron (*1*, *6*). The heme uptake system in *C. albicans* mediates specific binding of heme at the cell surface, transport into the cell, and subsequent degradation by CaHmx1 (*6*). The presence of an iron uptake system in *C. albicans* has important implications for pathogenic fungal infection, as CaHmx1 allows *C. albicans* to utilize exogenous heme as an iron source (*6*). The enzymatic mechanism and regulation of CaHmx1 could therefore provide targets for treatment of *Candida* infections.

In the transcriptional response to iron deprivation, *S. cerevisiae* expresses a number of genes involved in the mobilization of intracellular stores of iron including Hmx1 (*1*). In addition to recycling of heme iron, Hmx1 may have an important role in lowering the regulatory pools of heme when cells are starved for iron (*1*). *S. cerevisiae* is a prototypical eukaryotic cell important for elucidating biological pathways controlling iron absorption, distribution, and utilization. The characterization of its heme oxygenase is critical to understanding these pathways as a model for the cellular processing of iron.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE data, UV−visible spectra at different pH values, HPLC of biliverdin products obtained with various reductants, far UV−CD spectra, and pyridine verdohemochrome data. This material is available free of charge via the Internet at http://pubs.acs.org.

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