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In-Cell Enzymology to Probe His-Heme Ligation in Heme Oxygenase Catalysis^{#,†}

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[†]Atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank (<http://www.rcsb.org/pdb>) under accession codes 4WD4 and 5BQT.

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¹The abbreviations used are: HO, heme oxygenase; BV, biliverdin IX α ; IFP, infrared fluorescent protein; SynHO1, cyanobacterial heme oxygenase-1 from *Synechocystis* sp. 6803; HuHO1, human heme oxygenase-1; AtHO1, heme oxygenase-1 from *Arabidopsis thaliana*; ALA, 5-aminolevulinic acid

ABSTRACT

Heme oxygenase (HO) is a ubiquitous enzyme with key roles in inflammation, cell signaling, heme disposal, and iron acquisition. HO catalyzes the oxidative conversion of heme to biliverdin (BV) using a conserved histidine to coordinate the iron atom of bound heme. This His-heme interaction has been regarded as essential for enzyme activity, since His-to-Ala mutants fail to convert heme to biliverdin *in vitro*. We probed a panel of proximal His mutants of cyanobacterial, human, and plant HO enzymes using a live-cell activity assay based on heterologous co-expression in *E. coli* of each HO mutant and a fluorescent biliverdin biosensor. In contrast to *in vitro* studies with purified proteins, we observed that multiple HO mutants retained significant activity within the intracellular environment of bacteria. X-ray crystallographic structures of human HO1 H25R with bound heme and additional functional studies suggest that HO mutant activity inside these cells does not involve heme ligation by a proximal amino acid. Our study reveals unexpected plasticity in the active site binding interactions with heme that can enable HO activity within cells, suggests important contributions by the surrounding active site environment to HO catalysis, and can guide efforts to understand the evolution and divergence of HO function.

In vitro enzymology with purified proteins is the workhorse of biochemistry and conforms with Arthur Kornberg's 'commandment' to "not waste clean thinking on dirty enzymes".¹ Nevertheless, enzymes function natively within the complex milieu of living cells, and it remains an exciting yet formidable challenge to test whether the functional properties of enzymes deduced *in vitro* with purified proteins are modified by interactions inside living cells.²

Heme oxygenase is a conserved and ubiquitous enzyme that catalyzes the oxygen-dependent cleavage of heme to produce the linear tetrapyrrole biliverdin with concomitant release of iron and CO. HO is expressed by diverse organisms to serve a variety of central biological roles, including iron scavenging, heme disposal, phytochrome chromophore production, cell signaling, oxidative stress relief, and the inflammatory response.³⁻⁵ The vast majority of HO enzymes adopt a unique and highly conserved α -helical fold and utilize cytochrome P450 reductase or reduced ferredoxin as the electron donor to catalyze cleavage of heme at the α -meso position to produce biliverdin IX α (Fig. 1A & 1B).⁶

Although a variety of protein side-chains (e.g. Cys, His, Tyr, Lys, and Met) are utilized within the binding pockets of distinct classes of heme-binding proteins to coordinate the iron atom of heme,⁷ all known heme oxygenases, including the non-canonical heme-degrading enzymes from *S. aureus* (IsdG/I)⁸ and *M. tuberculosis* (MhuD),⁹ use a strictly conserved His residue as the proximal heme ligand.^{6, 10} Coordination of iron by the imidazole side-chain of histidine has been regarded as an essential interaction for HO catalysis, based on the observation that His-to-Ala mutants fail to convert heme to biliverdin *in vitro* despite still binding heme¹¹⁻¹⁴ and, in the case of HmuO from *C. diphtheriae*, catalyzing formation of the verdoheme intermediate.¹⁵ HO activity in Ala mutants is restored, however, upon addition of imidazole,

which can partition into the active site cavity created by the His-to-Ala mutation and functionally recapitulate the His-heme interaction of the WT enzyme.^{13, 15, 16} Furthermore, Cys and Tyr mutants of the proximal heme ligand have also been reported to lack HO activity *in vitro*,¹⁴ despite the ability of these residues to serve as heme ligands in other types of hemoproteins.⁷ These observations have suggested that the His-heme interaction is unique in its ability to support HO activity and is thus required for the conversion of heme to biliverdin within the HO active site.

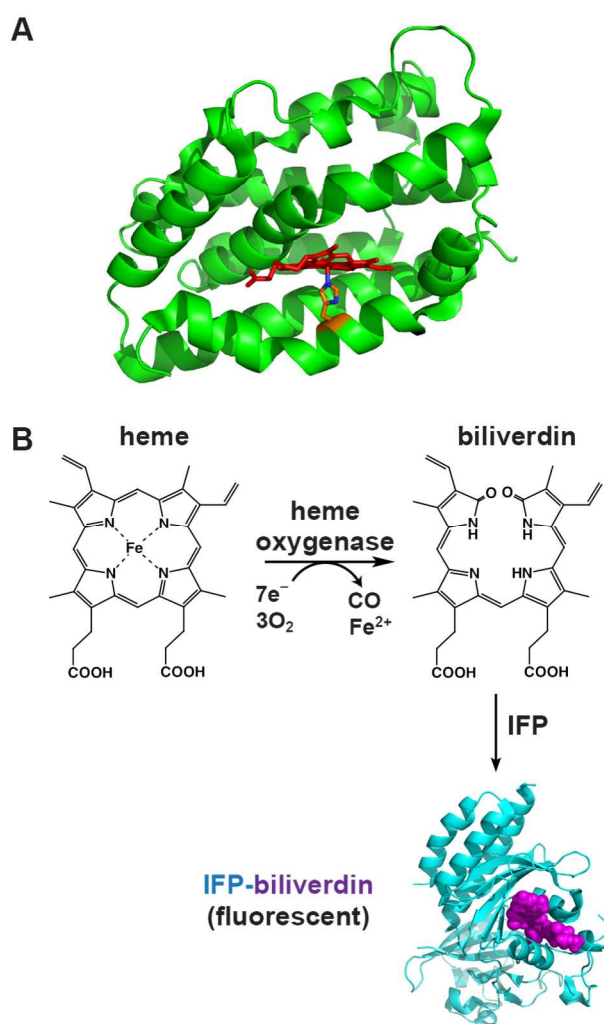


Figure 1. Heme oxygenase structure and reaction. (A) Structure of human HO1 (PDB 1N45) highlighting the α -helical protein fold (green), bound heme (red), and proximal His (orange). (B) HO activity converts heme to biliverdin (BV), which can then be covalently bound by infrared fluorescent protein (IFP, cyan) to generate the fluorescent IFP-BV (bound BV in violet). The structure of IFP-BV

was modeled using the BV-bound X-ray structure of the *D. radiodurans* chromophore-binding domain (PDB 3S7O), from which IFP is derived.

Herein we use an in-cell bacterial model system to probe the functional dependence of diverse HO enzymes on the proximal His-heme interaction and test whether residues other than His at the proximal heme ligand position can support HO catalysis under live-cell conditions. Our results indicate that several His mutants of diverse HO enzymes retain significant activity within live bacteria, and X-ray crystallographic structures of ligand-bound human HO1 H25R suggest that such activity does not involve stable ligation of heme by a proximal amino acid. Our findings reveal unexpected plasticity in the proximal interactions with bound heme that can enable HO activity in live cells and suggest important contributions by the surrounding active site environment to HO catalysis.

MATERIALS AND METHODS

Materials. Biliverdin IX α was purchased from Frontier Scientific Inc. (Logan, UT). DMSO, reduced NADPH, spinach ferredoxin, spinach ferredoxin NADP⁺ reductase, heme (hemin chloride), sodium ascorbate, and bovine liver catalase were purchased from Sigma (St. Louis, MO). IPTG was purchased from Gold Biotechnology Inc. (St. Louis, MO).

Cloning and Site-directed Mutagenesis. Cloning of SynHO1 (*Synechocystis* sp. 6803, NCBI P72849), HuHO1 (NCBI NP_002124.1) residues 1-265, AtHO1 (NCBI AEC07872.1) residues 55-282, and IFP into pET21d (C-terminal hexa-His tag), pET22b (C-terminal hexa-His tag), or pET28a (N- or C-terminal hexa-His tag) vectors was described in a previous publication.¹⁷ The indicated mutations were introduced into the HO proteins using QuikChange II site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). The gene for *E. coli* catalase (*katE*, NCBI

NC_000913.3) was cloned by PCR from Top10 bacteria, and the PCR product was ligated into the NdeI/KpnI sites of a modified pBAD vector (chloramphenicol)¹⁸ in frame with an N-terminal hexa-His tag. Genes for *E. coli* flavodoxin (Genbank M59426) and flavodoxin NADP⁺ reductase (Uniprot 28861) were cloned by PCR from Top10 bacteria, and the PCR products were ligated into the NcoI/XhoI sites of pET21d or pET28a, respectively, in frame with a C-terminal hexa-His tag. The gene for rat outer membrane cytochrome b5 (NCBI NP_085075.1) residues 13-103¹⁹ was ordered as a gBlock[®] from IDT (www.idtdna.com) and sub-cloned by PCR into the NcoI/XhoI sites of pET28a in frame with a C-terminal hexa-His tag. The H39V mutation was introduced by PCR. Sequences of all clones and mutants were confirmed by Sanger sequencing of mini-prep DNA on Applied Biosciences 3130 and 3730 DNA Sequencers at the Protein and Nucleic Acid Chemistry Laboratory at the Washington University School of Medicine.

Protein Expression and Purification. Bacterial transformation, protein expression, and protein purification were performed as previously described.¹⁷ SynHO1, IFP, HuHO1, AtHO1, and rat cyt b5 were expressed in BL21/DE3 *E. coli* bacteria by transformation with mini-prep plasmid DNA and overnight growth and selection at 37° C in 10-ml LB cultures supplemented with 50 µg/ml carbenicillin (pET21d and pET22b) or 30 µg/ml kanamycin (pET28a). For co-expression of IFP and SynHO1, HuHO1, AtHO1, or rat cyt b5, bacteria were transformed with plasmid DNA encoding each desired protein but with orthogonal resistance cassettes (e.g., SynHO1/pET21d plus IFP/pET28a) and selected with both antibiotics. Overnight cultures were diluted 1:100 into 5 ml LB supplemented with appropriate antibiotics and grown at 37° C to an A₆₀₀ ~0.5. Protein expression was induced by addition of 1 mM IPTG (and 1 mM 5-aminolevulinic acid for the IFP only experiment in Fig. 2), and bacteria were grown at 37° C and

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250 rpm for 1-5 hours prior to harvest. Bacteria analyzed in Fig. 2A & 2B were harvested after 5 hours, whereas the bacteria analyzed in Fig. 2C were harvested at the stated time-points of 0-5 hours. Bacteria were harvested by centrifugation and resuspended in 1 ml 50 mM Tris•HCl (pH 8), 100 mM NaCl. For the western blot analyses, samples contained 2 µl of the 1 ml resuspended bacteria in Tris•HCl (Fig. 2A), 10 µl of the 5 ml LB growth medium (Fig. 2C), or 10 µl of the 100 µl reaction mix (Fig. 6C),

For co-expression of katE, IFP, and SynHO1 (WT and His17 mutants), Top10 bacteria were transformed with mini-prep plasmid DNA of the pBAD (chloramphenicol) vector encoding katE with N-terminal hexa-His tag (described above) and a dual-expression pBAD vector (ampicillin) that encoded both IFP (with C-terminal hexa-His tag) and untagged SynHO1 (WT or mutants), each with its own ribosome binding site. Bacteria were selected overnight with both chloramphenicol and ampicillin, diluted 1:100 into fresh LB media supplemented with antibiotics, and grown at 37° C for ~3 hours to an OD₆₀₀ ~0.4. Protein expression was induced by addition of 0.04% arabinose, and bacteria were grown a further 5 hours at 37° C and then harvested. In all cases, protein expression was confirmed by Coomassie-stained SDS-PAGE and/or α-polyhistidine Western blot.

To test whether *in vivo* HO activity by SynHO1 mutants depended on the presence of a hexa-His tag on either SynHO1 or IFP, we modified the IFP/SynHO1 dual-expression pBAD vector described above to remove the hexa-His tag from the IFP cassette and to encode either WT or H17R SynHO1. Top10 bacteria were transformed with this plasmid, and protein expression was induced with 0.04% arabinose. Lysates from bacteria expressing IFP and either WT or H17R SynHO1 gave the expected fluorescence peaks indicative of IFP-BV formation in

both cases, indicating that *in vivo* HO activity by SynHO1 H17R did not depend on the presence of a hexa-His tag.

Heme-bound complexes of the purified SynHO1 WT and mutants were prepared by addition of concentrated hemin chloride (alkaline aqueous solution) to the clarified bacterial lysates, centrifugation (30 min. x 20,000 g) and syringe filtration (0.45 μ M), and passage over a HisTrap HP metal chelation column. Purification was then continued as previously described,¹⁷ using nickel-chelation and size-exclusion chromatography.

Western blots were performed with a rabbit anti-polyhistidine antibody conjugated to an IR800 fluorescent dye (Rockland Inc.) used at 1:10,000 dilution. Blots were imaged on an Odyssey Imaging System (LI-COR Biosciences) using an intensity setting of 5 and quantified using Image Studio Lite (LI-COR Biosciences).

Microscopy. Images of live *E. coli* bacteria were acquired on an Axio Imager.M1 epifluorescence microscope (Carl Zeiss Microimaging, Inc.) equipped with a Hamamatsu ORCA-ER digital CCD camera and running Axiovision 4.8 software. Fluorescence images were acquired with the Zeiss filter set 50 (excitation 625-655 nm, emission 665-715 nm) using identical acquisition times and processed with identical brightness and contrast settings.

UV-Vis Absorbance and Fluorescence Spectroscopy. Fluorescence and absorbance spectra were acquired as previously described.¹⁷ For analysis of bacterial lysates, bacteria were harvested 5 hours post-induction, resuspended as describe above, lysed by sonication, and then centrifuged 10 min. at 16,000 g to clarify. Fluorescence excitation (emission 740 nm) and emission (excitation 630 nm) spectra were acquired on a Cary Eclipse fluorescence spectrometer (Varian

Inc.) using 10-nm slit widths and a PMT detector voltage setting of 800. Spectra in Fig. 2B, Fig. 3, and Fig. 5 were normalized to the maximum intensity observed for WT, which was set to a value of 1. Fluorescence emission spectra were obtained for intact bacteria in Fig. 2C by diluting 30 μ l of the LB growth culture at each time point into 30 μ l PBS and acquiring an emission scan (excitation 630 nm) using the settings above.

UV-Vis absorbance spectra were acquired on a DU 640 spectrophotometer (Beckman Coulter) using 1-cm path length quartz cuvettes and 100 μ l samples. Absorbance spectra of clarified bacterial lysates in Fig. 3A were background corrected by subtracting the absorbance spectrum of an equivalent empty vector culture. Fluorescence scans of resuspended bacterial pellets in 1.7-ml eppendorf tubes were acquired on the 700-nm channel of an Odyssey Imaging System (LI-COR Biosciences) using an intensity setting of 1 and a focal offset of 3 mm.

In vitro HO Activity Assays. Assays were performed as previously described¹⁷. For reactions using ascorbate, 100- μ l samples contained 20 μ M reconstituted WT or H17R SynHO1-heme complex, 10 mM ascorbate, 1 μ M bovine catalase, 100 μ M NADPH, 50 mM Tris•HCl (pH 8), and 100 mM NaCl. For reactions using spinach ferredoxin (Fd) and ferredoxin NADP⁺ reductase (FNR) or *E. coli* flavodoxin (Fld) and flavodoxin NADP⁺ reductase (FNR), 100- μ l samples contained 20 μ M reconstituted WT or H17R SynHO1-heme complex, 20 μ M spinach Fd or *E. coli* Fld, 0.025 U/ml spinach FNR or 40 μ M *E. coli* FNR, 1 μ M bovine catalase, 100 μ M NADPH, 50 mM Tris•HCl (pH 8), and 100 mM NaCl. Reactions were initiated by adding the ascorbate, ferredoxin, or flavodoxin to sample mixtures containing the other components. The complete reaction mix was then transferred to a cuvette, and spectra were acquired at the indicated time points. For the experiments in Fig. 6C, samples also included 20 μ M apo IFP, and

fluorescence emission spectra were acquired at the indicated time points using the instrument settings given above and plotted using the raw fluorescence intensities. *In vitro* activity assays were repeated multiple (≥ 2) times using independently purified protein samples. Similar results were observed in all cases, and representative spectra have been included herein.

HPLC Analysis of Purified SynHO1 Variants. SynHO1 WT or His17 mutants were expressed in *E. coli* (without IFP), isolated using affinity and size-exclusion chromatography as described above, and concentrated to 500 μl using Amicon spin concentrators (Millipore) with a 10-kDa molecular mass cutoff. Protein concentrations were determined by absorbance at 280 nm using a molar extinction coefficient of $21,890 \text{ M}^{-1} \text{ cm}^{-1}$. Protein purity was confirmed by the appearance of a single band at the expected molecular weight using Coomassie-stained SDS-PAGE, and protein masses were confirmed by LC-MS as described below. 20 μl of the final concentrated solution for each SynHO1 WT or His17 mutant protein isolated from bacteria was analyzed by C_{18} reverse-phase HPLC, using a previously described HPLC system, column, and solvent system.¹⁷ Briefly, the 20- μl sample was injected onto the column and eluted using a constant flow rate of 0.3 ml/min., with absorbance monitored at 400 nm. The resulting chromatogram for analysis of each variant shown in Fig. 4 was normalized to the maximum absorbance for each variant.

Mass Spectrometry. Mass spectrometry analysis of purified WT and H17 mutant (K, R, M and A) SynHO1 proteins was performed at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (<http://mass-spec.stanford.edu>). Samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) on an Agilent 1100 binary

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pump HPLC system coupled to a Thermo-Fisher LTQ XL ion trap mass spectrometer. The LC column was a 30 x 2.1 mm Zorbax SB-C₈, with initial conditions of 98% A buffer (0.1% formic acid in water)/ 2% B buffer (0.1% formic acid in acetonitrile) ramped to 95% B buffer in 20 min. at a flow rate of 0.3 ml/min. Samples were analyzed by ESI in the positive ion mode with a mass range of 400-2000 m/z. The injection volume was 20 µl for all samples. The Biomass Deconvolution algorithm was used to determine the intact molecular weight of each protein. All proteins had deconvoluted masses within 1-4 Da of the expected molecular weight for the specified WT or mutant protein minus the start Met residue, consistent with N-terminal processing of these proteins in *E. coli* by methionine aminopeptidase.²⁰

Mass spectrometry analysis of affinity-isolated SynHO1 was performed at the Fingerprints Proteomics Facility, College of Life Sciences, University of Dundee, Dundee, United Kingdom. Ni-CAMTM HC Resin (Sigma-Aldrich, St. Louis, MO) was used to isolate WT SynHO1 bearing a C-terminal hexahistidine tag and expressed from BL21/DE3 *E. coli*. Resin was incubated with clarified bacterial lysates, washed five times with potassium phosphate buffer (pH 7.5, 0.5M NaCl, 10 mM imidazole), and eluted with identical buffer containing 0.5 M imidazole. The eluted protein was separated by 10% SDS-PAGE. A gel slice spanning the entire lane was excised, trypsinized, and analyzed by tandem mass spectrometry on an LTQ Orbitrap and the Mascot search engine to identify *E. coli* proteins.

X-ray Crystallography: Protein expression and purification of HuHO1 H25R. Guided by prior structural studies of HuHO1,^{21, 22} the truncated HuHO1 H25R gene encoding residues 1-233 was cloned into a pET28a vector in frame with the N-terminal hexa-His tag and thrombin cleavage site. This plasmid was transformed into BL21 DE3 *E. coli* cells, and bacteria were selected

overnight in kanamycin. Cells were grown to $OD_{600} = 0.5$, and expression was induced with 1 mM IPTG for 18 hours at 30 °C. Cells were lysed by sonication, and the clarified supernatant was subsequently loaded onto a His-Trap HP metal chelating column (GE Healthcare). Protein was eluted with a linear gradient of imidazole between 0 and 500 mM. Relevant fractions were pooled and then dialyzed against 25 mM HEPES, 150 mM NaCl pH 7.2 and simultaneously treated overnight with thrombin to cleave off the hexa-His tag. The protein was passed again over a His-Trap column to recover the untagged protein, although this time only the flow-through was recovered. Protein was passed through a PD-10 desalting column (Sephadex G-25) to exchange the buffer to 10 mM KH_2PO_4 (pH 7.4). The protein was then applied to a Hi-Trap Q XL anion-exchange column (GE Healthcare) and eluted with a gradient of 0-250 mM KCl to remove bound biliverdin from the protein. The heme-HO1 and biliverdin-HO1 complexes were prepared by mixing the respective ligand with purified protein at a molar ratio of 2:1 (ligand:protein). Excess ligand was removed by passage through a PD-10 column in 10 mM KH_2PO_4 (pH 7.4) buffer. Homogeneous samples for crystallization trials were obtained after a chromatographic step in a Superdex-75 size-exclusion column.

Protein Crystallization. Suitable crystals of H25R HuHO1 in complex with heme or biliverdin were obtained by hanging drop vapor diffusion. Crystals of HuHO1 in complex with biliverdin were obtained in the reported crystallization solution of HuHO1 in complex with heme²³ by mixing 5 μ l of protein solution (9 mg/ml) with 5 μ l of a solution composed of 100 mM HEPES (pH 7.5), 2.08 M ammonium sulfate, and 0.9% 1,6-hexanediol at 20 °C. In contrast, we failed to obtain single crystals of H25R in complex with heme under these conditions. We therefore explored modified conditions by using commercial screens containing additives (Hampton) in an

ORYX8 instrument (Douglas Instruments, UK). Single crystals of HuHO1 (13 mg ml⁻¹) in complex with heme were obtained by mixing 0.45 µl of protein solution with 0.45 µl of a solution composed of 100 mM HEPES (pH 7.5), 2.08 M ammonium sulfate, and 0.9% 1,6-hexanediol, and with 0.10 µl of a detergent solution of 1.4 mM Big-CHAP at 20 °C. Suitable crystals grew to full size within a few weeks at 20 °C, after which they were transferred to mother liquor supplemented with 20% glycerol and subsequently frozen and stored in a vessel containing liquid N₂.

Diffraction Data Collection and Structure Refinement. Diffraction data from single crystals of HuHO1 complexes were collected at beamline BL5A of the Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K). Diffraction images recorded by the CCD detector were processed with the program MOSFLM and merged and scaled with the program SCALA or AIMLESS of the CCP4 suite.²⁴ The structures were determined by molecular replacement using the coordinates of wild-type HuHO1 in complex with heme (PDB code, 1N45) with the program PHASER.²⁵ The models were refined with the programs REFMAC5²⁶ and manually built with COOT.²⁷ Validation was carried out with PROCHECK.²⁸ Data collection and structure refinement statistics are given in Table S1. Structure factors and model coordinates of ligand-bound HuHO1 H25R were deposited with the RCSB Protein Data Bank under accession codes 5BTQ (biliverdin) and 4WD4 (heme).

RESULTS

In-cell enzymology of HO catalysis by proximal His ligand mutants. Nearly all studies of HO activity have been performed *in vitro* using purified enzymes, reconstituted HO-heme

complexes, and cytochrome P450 reductase, ferredoxin, or ascorbate as the electron donor. Indeed, *in vitro* studies have enabled sophisticated detailed investigations of HO structure and reaction mechanism. We sought to test and extend the conclusions from prior *in vitro* studies regarding the dependence of HO activity on the proximal His-heme interaction, using a live-cell model system that involves heterologous co-expression in *E. coli* of an HO enzyme together with a fluorescent biliverdin biosensor.

E. coli strains B and K-12 do not express an HO and thus do not degrade endogenous heme to biliverdin.²⁹ However, HO enzymes from many diverse organisms are enzymatically active when heterologously expressed in *E. coli*,^{17, 30-35} providing a model system for HO activity in living cells. The endogenous bacterial electron donor that supports heterologous HO activity within *E. coli* remains unknown but is thought to be flavodoxin.³⁶ We previously showed that HO co-expression in *E. coli* with infrared fluorescent protein (IFP),³⁷ an engineered version of the chromophore-binding domain of the *D. radiodurans* phytochrome that specifically binds biliverdin IX α ,^{38, 39} provides a sensitive fluorescent reporter of HO activity by cyanobacterial (*Synechocystis* sp. 6803, SynHO1), human (HuHO1), and plant (*A. thaliana*, AtHO1) HO1 enzymes.¹⁷ In this live-cell system (Fig. 1B), recombinant HO and IFP expression are induced in *E. coli*, and HO activity is monitored by testing the intact live bacteria or clarified bacterial lysates (typically 3-5 hours post-induction) for the unique fluorescence excitation (686 nm) and emission (708 nm) peaks characteristic of mature biliverdin-bound IFP. Consistent with the absence of HO activity in *E. coli*, expression of IFP alone gave no detectable fluorescence peaks for IFP-BV, including growth conditions in which endogenous heme biosynthesis was stimulated with exogenous 5-aminolevulinic acid (Fig. 2A & 2B and Fig. S1).

We first tested a broad panel of His17 mutants of SynHO1, since this residue was previously identified as the proximal ligand for bound heme.^{40, 41} In contrast to prior *in vitro* studies of diverse HO enzymes that failed to detect conversion of heme to BV by proximal His mutants, we observed that bacteria expressing IFP and six different SynHO1 His17 mutants (K, R, M, C, Q, and A) gave the expected fluorescence indicative of BV-bound IFP (Fig. 2 and Fig. S1). These results suggested that the proximal His-heme interaction, despite conservation in all known heme-degrading enzymes, might not be strictly required for HO activity within live cells. Nevertheless, three mutants (Y, E, and F) showed little or no evidence of BV formation, suggesting that bulky or negatively charged side-chains at this position may inhibit HO activity (see below).

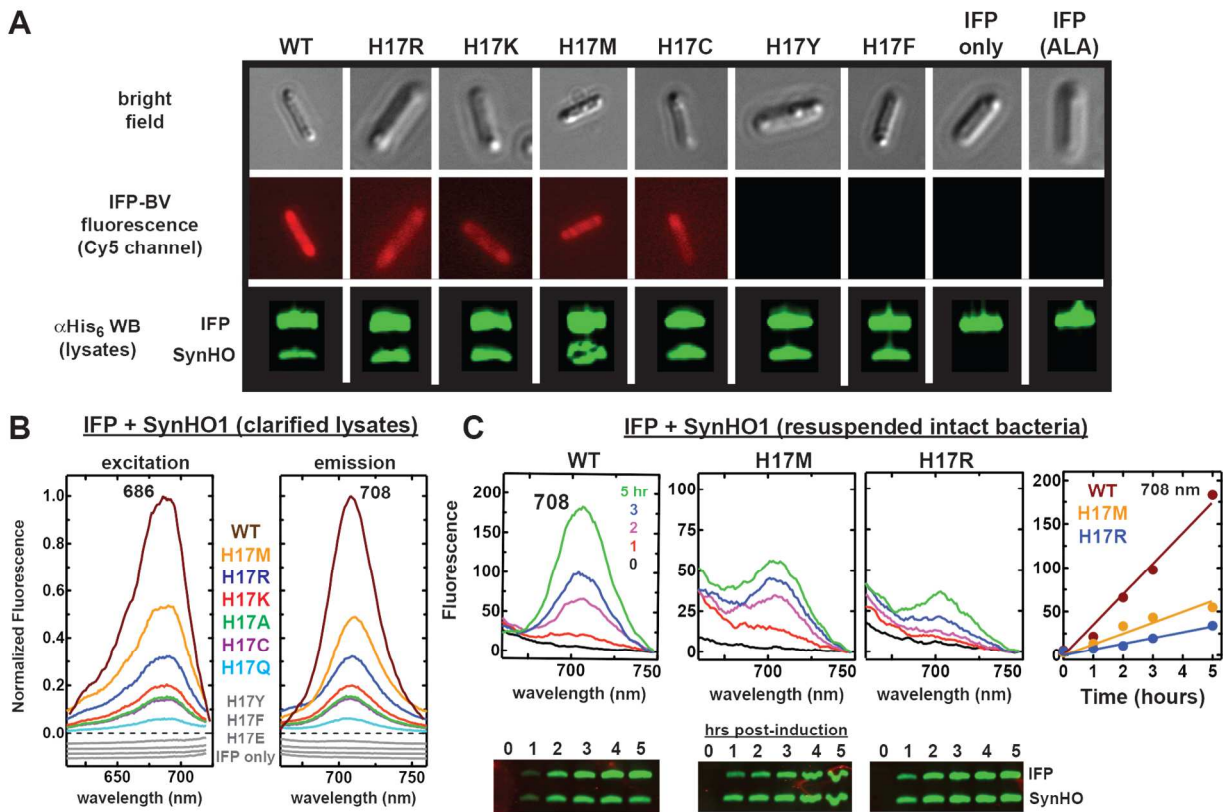


Figure 2. *In vivo* tests of catalytic activity by SynHO1 variants. (A) Bright field and fluorescence images (Zeiss filter set 50: excitation 640/30 and emission 690/50 nm) of live *E. coli* bacteria (5 hours post-induction) expressing IFP alone ($\pm$ 1 mM 5-aminolevulinic acid, ALA) or in combination with the indicated SynHO1 variant. Fluorescence images used identical acquisition times, brightness, and contrast

settings. Below each fluorescence image is an anti-polyhistidine western blot probing expression of the His-tagged IFP and SynHO1 variant in samples harvested 5 hours post-induction. (B) Fluorescence excitation and emission spectra of clarified bacterial lysates expressing IFP alone or co-expressed with the indicated SynHO1 variant. Spectra for each variant (harvested 5 hours post-induction) were normalized to the maximum intensity for the WT enzyme. Spectra in gray were offset from each other to avoid overlap. (C) Fluorescence emission spectra of resuspended intact bacteria harvested at the indicated time points post-induction. Spectra are plotted with the raw fluorescence intensities. Below each spectrum is an anti-polyhistidine western blot probing expression of the His-tagged IFP and SynHO1 variant in samples harvested at the indicated time points post-induction. The right-most panel is a plot of the fluorescence intensity at 708 nm as a function of time for each variant, fit with a linear function (slopes given in text).

We observed a hierarchy of fluorescence intensities across the variants, despite similar protein expression levels, with the largest IFP-BV signal observed for the WT enzyme in all cases (Fig. 2 & Fig. S2). A time-dependent analysis of BV production in live bacteria confirmed that the activity (k_{obs}) of the WT enzyme (35 fluorescence units/hr) is at least 3 to 5-fold greater than the most active H17M (12 units/hr) and H17R mutants (7 units/hr) (Fig. 2C). We emphasize, however, that this comparison provides only a crude estimate of the relative in-cell activities of the WT and mutant enzymes. Indeed, the specific rate-limiting step or steps that underpin the observed rate of increase in IFP-BV fluorescence within bacteria remain obscure and could include rate-limiting contributions that are independent of turnover by the WT or mutant HO enzymes, such as heme biosynthesis or IFP expression. We note, however, that absorbance spectra of clarified lysates from bacteria expressing WT or H17R SynHO1 without IFP (Fig. 3A) displayed relative biliverdin absorbance intensities (H17R/WT absorbance ratio at 390 nm = 0.5) that qualitatively matched the relative IFP-BV fluorescence intensities in Fig. 2B (H17R/WT fluorescence ratio at 708 nm = 0.3). We also observed that IFP quantitatively forms IFP-BV within the dead time (5-10 s) required to mix apo IFP with either free or HO-bound BV and acquire fluorescence spectra. These observations suggest that expression of IFP and formation of IFP-BV do not limit the detection of BV production by WT or mutant HO enzymes in bacteria. Overall, we conclude that the WT His-heme interaction maximizes HO activity but

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that several H17 mutants retain significant (but lower) in-cell activity, in contrast to prior *in vitro* studies of proximal His mutants that reported no activity. Additional activity tests for SynHO1 proximal His mutants confirmed the absence of detectable activity *in vitro* (see below).

To test the generality of our findings with SynHO1, we next tested whether proximal His mutants of HuHO1^{21, 32} and AtHO1⁴² were active when expressed within *E. coli*. Co-expression of IFP with HuHO1 His25 mutants (R, K, M, and C) but not AtHO1 His86 mutants (R, M, and K) resulted in fluorescence peaks indicative of BV-bound IFP (Fig. 3B & 3C). The HuHO1 results extend our observations with SynHO1 that some HO mutants can retain activity within live cells in the absence of a proximal His-heme interaction. The AtHO1 data, however, indicate that this functional plasticity within *E. coli* bacteria is not a general property of all HO enzymes and that proximal His mutations can differentially affect the activity of discrete HO proteins, possible due to differing interactions with redox partners (see additional discussion below).

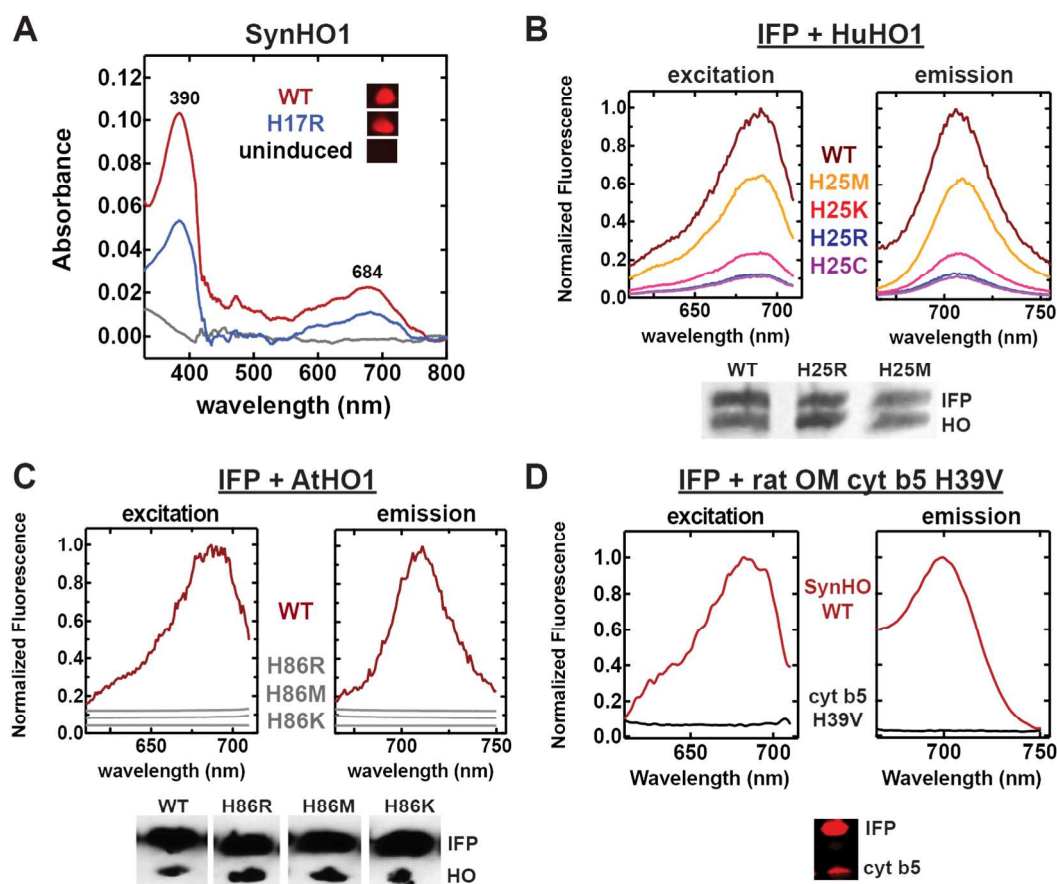


Figure 3. Testing in-cell HO activity by HO1 and rat outer membrane cytochrome b5 variants. (A) UV-Vis absorbance spectra of clarified lysates from uninduced bacteria or bacteria expressing SynHO1 WT or H17R) 4 hours after IPTG induction. (B & C) Fluorescence excitation and emission spectra of clarified bacterial lysates expressing IFP alone or co-expressed with the indicated HuHO1 (B) or AtHO1 (C) variant. Spectra for each variant (harvested and lysed 5 hours post-induction) were normalized to the maximum intensity for the WT enzyme. Spectra in gray were offset from each other to avoid overlap. (D) Fluorescence excitation and emission spectra of clarified bacterial lysates co-expressing IFP and either SynHO1 WT or rat OM cyt b5 H39V. Spectra for both proteins (5 hours post-induction) were normalized to the maximum intensity for SynHO1. Also shown for each species is an anti-polyhistidine western blot probing expression of the His-tagged proteins in samples harvested at the indicated time points.

To calibrate the level of in-cell HO activity by proximal His mutants of SynHO1 and HuHO1 relative to other heme-binding proteins previously reported to produce BV from heme in the presence of reducing agents, we co-expressed IFP in bacteria with the H39V mutant of rat outer membrane cytochrome b5. This axial His mutant of rat cyt b5 was previously shown to convert heme to biliverdin *in vitro* in a catalase-insensitive manner, indicating low-level HO activity.⁴³ In contrast to the significant in-cell BV production observed by IFP-BV fluorescence

(Fig. 2 and Fig. 3B) or BV absorbance (Fig. 3A) for SynHO1 or HuHO1 mutants, we were unable to detect BV production in bacteria co-expressing IFP and rat cyt b5 H39V (Fig. 3D). This observation supports our conclusion that several (but not all) proximal His mutants of SynHO1 and HuHO1 retain significant HO activity much greater than that expected for low-level or non-HO specific background activity.

Proximal His mutants of SynHO1 contain bound biliverdin when purified from E. coli. To further test our conclusion that several SynHO1 proximal His mutants remain active in *E. coli* and to confirm production of the expected biliverdin IX α , we expressed WT and each of four His17 mutants (R, M, K, and F) of SynHO1 in *E. coli* (without IFP) and purified each protein using affinity and size-exclusion chromatography. Prior work has suggested that product release from HO enzymes is slow in the absence of HO-interacting proteins that bind biliverdin (e.g., biliverdin reductase).⁴⁴ We therefore reasoned that expression of each active HO mutant in *E. coli* without IFP would lead to a stable HO-BV complex that could be chromatographically isolated and subsequently analyzed for bound product.

We first acquired UV-Vis absorbance spectra of each HO protein purified from bacteria to assess the presence of bound biliverdin based on its characteristic absorbance spectrum. Spectra of WT and three active His17 mutants (R, M, and K) displayed absorbance maxima at 390 and 685 nm (Fig. 4A), as expected for the presence of bound biliverdin and as previously reported for WT HO from diverse organisms.^{15, 35, 44, 45} The spectrum of the H17F mutant showed no evidence of bound BV, consistent with the IFP fluorescence data in Fig. 2 that suggested the absence of HO activity.

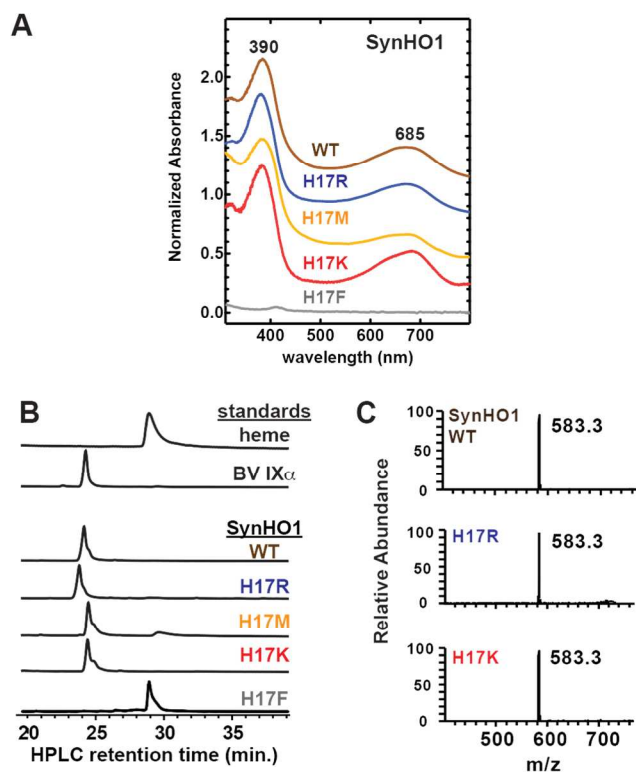


Figure 4. Spectroscopic and chromatographic analysis of SynHO1 variants isolated from bacteria. (A) UV-Vis absorbance spectra of purified proteins. (B) Reverse-phase C₁₈ HPLC chromatograms (absorbance at 400 nm) of SynHO1 variants isolated from bacteria and tetrapyrrole standards. The y-axis in each chromatogram was normalized to the maximum absorbance for each variant or standard (C) Mass spectra of SynHO1 variants isolated from bacteria, corresponding to an LC column retention time of 24-25 min. Each spectrum was normalized to the 583.3 m/z peak for each variant.

We next analyzed the isolated proteins by reverse-phase C₁₈ HPLC and confirmed that biliverdin IX α co-purified with the WT and the R, M, and K mutants, based on observation of a 400 nm-absorbing peak in the chromatogram of each injected protein sample that migrated with a retention time identical to a commercial biliverdin IX α standard (Fig. 4B). Purified SynHO1 WT, H17R, and H17K were further analyzed by liquid chromatography-mass spectrometry, which identified ion peaks in each spectrum with identical chromatographic retention times and m/z 583.3, as expected for biliverdin IX α (Fig. 4B & 4C). HPLC analysis of the H17F mutant revealed the presence of bound heme but not biliverdin, supporting the conclusion that this mutant is inactive in bacteria but suggesting that it retains the ability to bind heme (Fig. 4B). We

note that the experiments in Fig. 4 were carried out in order to confirm product identity only and were not intended to assess relative amounts of BV production.

Over-expression of E. coli catalase does not ablate in-cell activity of SynHO1 mutants. Prior work has indicated that certain heme-bound proteins can display HO-like activity called coupled oxidation that differs from canonical HO catalysis in that it depends on unbound hydrogen peroxide and can be inhibited by catalase.^{43, 46} *E. coli* produces endogenous hydrogen peroxide as a by-product of aerobic metabolism and growth.⁴⁷ Although bacteria produce anti-oxidant enzymes such as catalase (katG and katE) and alkyl hydroperoxide reductase (ahpCF) that scavenge hydrogen peroxide and thus protect *E. coli* during aerobic growth,⁴⁸ we considered whether in-cell production of biliverdin by proximal His mutants of SynHO1 and HuHO1 might reflect coupled oxidation from endogenous hydrogen peroxide rather than canonical HO activity.

Catalase is often included with *in vitro* HO activity assays to distinguish true HO activity (catalase insensitive) from coupled oxidation (catalase sensitive).^{46, 49} To perform an analogous test of the in-cell activity of proximal His mutants of SynHO1, we cloned the *E. coli* catalase gene *katE* and over-expressed *katE* in bacteria along with IFP and either WT, H17K, or H17R SynHO1. If BV production by SynHO1 proximal His mutants were due to coupled oxidation, then we expected *katE* over-expression to substantially reduce or ablate activity by the mutants relative to WT activity. We observed robust *katE* expression similar to IFP and SynHO1 (Fig. 5A). Nevertheless, the SynHO1 proximal His mutants remained active (Fig. 5B), with IFP-BV fluorescence intensities relative to WT that were indistinguishable from those in the absence of *katE* (Fig. 2B). This result suggests that catalysis by WT HO and proximal His mutants in

bacteria proceeds by similar reaction mechanisms that do not involve coupled oxidation by hydrogen peroxide.

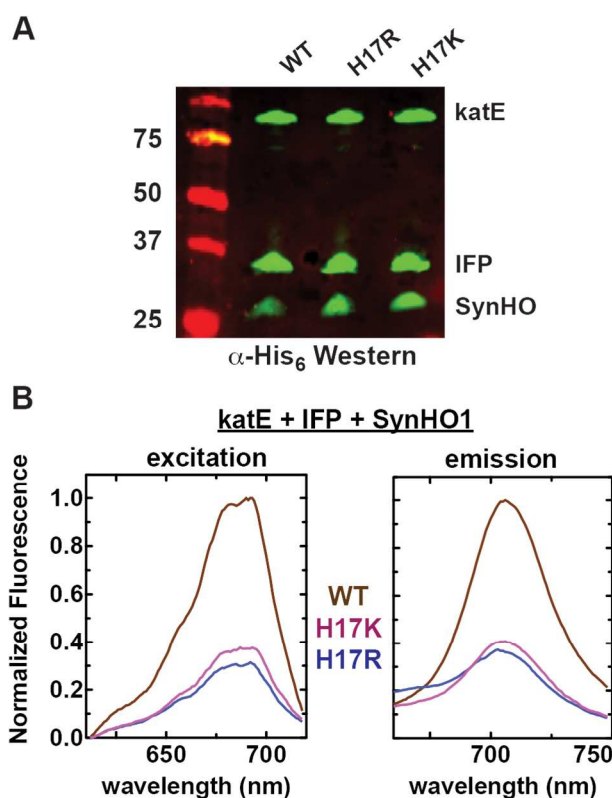


Figure 5. Catalase over-expression does not ablate *in vivo* activity of SynHO1 proximal His mutants. (A) Anti-polyhistidine Western blot of soluble bacterial lysates showing expression of *E. coli* katE, IFP, and SynHO1. All proteins contained an N-terminal (katE, 85 kDa) or C-terminal (IFP, 36 kDa; SynHO1, 28 kDa) hexa-His tag. (B) Fluorescence excitation and emission spectra of soluble lysates from *E. coli* bacteria expressing katE, IFP, and SynHO1. Spectra for each variant (harvested 5 hours post-induction) were normalized to the maximum intensity for the WT enzyme.

Proximal His mutants of SynHO1 are inactive in vitro. Prior *in vitro* studies of proximal His-to-Ala HO mutants from multiple organisms,^{12, 16, 50} and His-to-Cys or Tyr mutants in HuHO1,¹⁴ have reported little or no conversion of heme to biliverdin by the purified proteins. Because our in-cell results contrast with these prior *in vitro* studies and because *in vitro* activity measurements with several of mutants herein (e.g., K, R, and M) have not been reported previously, we reconstituted heme-bound complexes with several of our purified SynHO1 mutants and assessed their activity *in vitro*.

We first acquired UV-Vis absorbance spectra of the ferric heme complexes of WT and H17 mutants of SynHO1. A prominent Soret peak centered near 404 nm was observed in the spectrum of WT and each of the four purified mutants of SynHO1 (Fig. 6A), and both the WT and H17R complexes had absorption bands at 500 and 630 nm (Fig. 6A, inset), consistent with a high-spin complex in all cases.⁴⁰ The Soret peaks of the mutants had broader line widths than WT, which may suggest a mixture of coordination states.¹⁴

We first tested the HO activity of the WT and mutant enzymes using 10 mM ascorbate as the reducing agent, and we included 1 μ M bovine catalase to prevent heme degradation via coupled oxidation. Addition of ascorbate to the WT SynHO1-heme complex resulted in a time-dependent decrease in Soret peak intensity and concomitant rise in UV-Vis absorbance at 686 nm (Fig. 6B), as expected for HO-catalyzed conversion of heme to BV and as previously reported for WT SynHO1.⁴⁰ In contrast, ascorbate addition to the SynHO1 H17R-heme complex resulted in no significant decrease in Soret peak intensity and no detectable increase in 686-nm absorbance over a similar 90-min. timescale (Fig. 6B), providing no evidence for conversion of heme to BV (see below). Very similar results were observed for the H17K and H17M mutants with ascorbate (Fig. S3A), and exogenous imidazole did not stimulate activity by H17R (Fig. S3B). Since SynHO1 is thought to use ferredoxin as its cognate redox partner *in vivo*,³⁰ we also tested the ability of spinach ferredoxin and ferredoxin NADP⁺ reductase to support activity of the H17R mutant *in vitro*. As with ascorbate, we observed no significant changes in the Soret peak intensity with no evidence for biliverdin production (Fig. S3C).

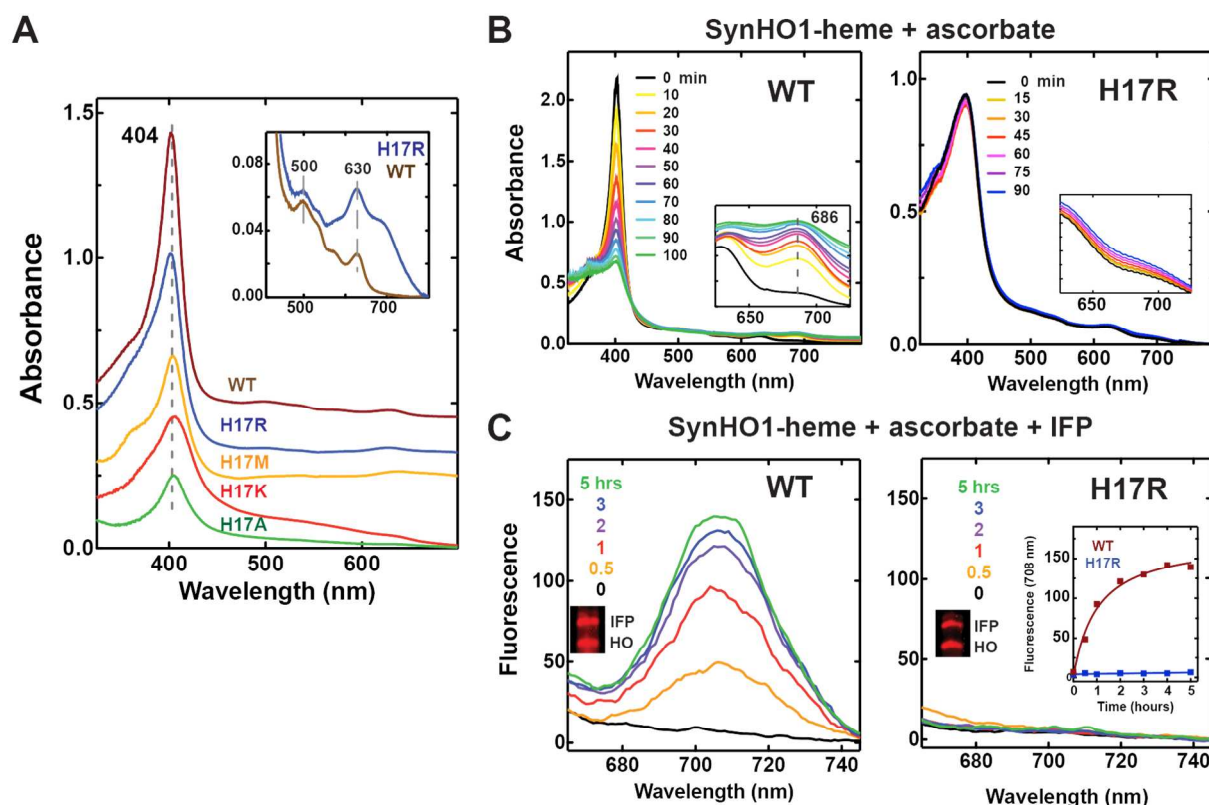


Figure 6. UV-Vis absorbance spectra and HO activity assays with purified SynHO1-heme complexes. (A) UV-Vis absorbance spectra of 20 μ M purified SynHO1-heme complexes. Inset shows a y-axis expansion for the WT and H17R spectra. (B) Time-dependent (min.) UV-Vis absorbance changes for 20 μ M WT and H17R SynHO1-heme complexes after addition of 10 mM ascorbate and 1 μ M catalase. (C) Time-dependent (min.) fluorescence emission spectra for 20 μ M WT and H17R SynHO1-heme complexes after addition of 10 mM ascorbate, 1 μ M catalase, and 20 μ M apo IFP. The inset is a plot of the fluorescence intensity at 708 nm as a function of time for WT and H17R.

We extended the *in vitro* tests of WT and H17R catalysis with ascorbate by adding purified apo IFP and measuring the time-dependent rise in IFP-BV fluorescence. WT SynHO1 showed a steady increase in IFP-BV fluorescence (Fig. 6C) over a similar time-scale as the loss of Soret peak intensity observed by UV-Vis absorbance in Fig. 6B. However, no IFP-BV fluorescence was observed *in vitro* for the H17R mutant over 5 hours (Fig. 6C), in contrast to the steady increase in IFP-BV fluorescence observed for this mutant in live bacteria over a similar time-scale and at similar protein levels (Fig. 2C).

Prior study of proximal His mutants of HuHO1 suggested that these proteins have more negative redox potentials compared to WT HuHO1, resulting in a diminished driving force for reduction of the mutants compared to WT by a common redox partner.¹⁴ We therefore considered whether the more negative redox potential of *E. coli* flavodoxin (-455 mV)⁵¹ compared to commonly used *in vitro* electron donors such as spinach ferredoxin (-420 mV)⁵², cytochrome P450 reductase (-270 mV)¹⁴, or ascorbate (-282 mV)⁵³ might account for our observation of enhanced HO activity by proximal His mutants within live *E. coli* compared to *in vitro* conditions. As noted above, the endogenous *E. coli* flavodoxin has been proposed as the *in vivo* redox partner for heterologous HO activity.³⁶

We cloned the *E. coli* genes for flavodoxin and flavodoxin NADP⁺ reductase (FNR), expressed these proteins in *E. coli*, purified them to homogeneity using affinity and size-exclusion chromatography, and then tested their ability to support HO activity *in vitro*. The purified proteins had the same SDS-PAGE migrations and UV-Vis absorbance spectra as previously reported.³⁶ We observed a time-dependent decrease in the Soret peak for the heme complex of WT SynHO1 in the presence of *E. coli* flavodoxin, FNR, NADPH, and catalase, as expected for HO-catalyzed heme degradation (Fig. S3D). The heme complex of SynHO1 H17R, however, showed no evidence of heme degradation (Fig. S3D), suggesting that the more negative *E. coli* flavodoxin redox potential alone does not account for the enhanced in-cell mutant activity.

X-ray crystallographic structures of HuHO1 H25R with bound heme or biliverdin. To directly assess the consequences of mutating the proximal His on HO active site architecture and interactions with bound ligands, we determined X-ray crystallographic structures of HuHO1

H25R bound to either heme or BV. Data collection and refinement statistics are given in Table S1. Although crystallographic studies have been described for the HuHO1 H25A mutant,⁵⁴ the previously reported structure did not contain bound ligand, and coordinates and structure factors were not deposited with the RCSB Protein Data Bank (PDB). Our work thus presents the first comprehensive structural study of an HO proximal His mutant with bound heme substrate and biliverdin product.

The overall structure that we obtained of heme-bound HuHO1 H25R at 2.95 Å resolution is nearly identical to that previously determined for the WT HuHO1-heme complex (PDB code 1N45),^{21, 22} with a root mean square deviation (RMSD) between backbone atoms for the two structures of 0.33 Å. The electron density map of the HuHO1 H25R-heme structure shows well-defined density to support the modeled atomic positions for bound heme and active site residues, with no indication of alternative ligand or side-chain conformations (Fig. 7A).

In contrast to heme-bound WT HuHO1 in which His25 is positioned to coordinate the iron atom of heme (Fe-Ne2 distance, 2.0 Å), the Arg25 side-chain in the H25R mutant is oriented away from the parent His25 position such that it does not serve as an axial heme ligand and instead adopts a conformation within hydrogen-bonding distance (3.0-3.4 Å) of the Thr21 side-chain and Lys18 backbone oxygen atoms (Fig. 7B). This change is accompanied by three additional rearrangements within the H25R active site: 1) a 22° tilt in the orientation of bound heme relative to WT, 2) a ~1 Å displacement of the C-terminal half of the proximal backbone helix toward the bound heme, and 3) reorientation of the Glu29 side-chain, which in WT HuHO1 is hydrogen-bonded to the Nδ1 atom of His25, to place a carboxylate oxygen atom within 3.0 Å of the heme iron (Fig. 7B). This 3-Å distance, however, is at least 1 Å longer than expected for coordination of iron by a carboxylate group.⁵⁵ Outside of these changes, the positions of the

remaining residues appear to be conserved between the active sites of H25R and WT HuHO1 with bound heme. Although the WT structure reported a water molecule as a distal axial ligand of heme,²² at the 2.95-Å resolution of our structure we did not observe electron density for a distal water molecule.

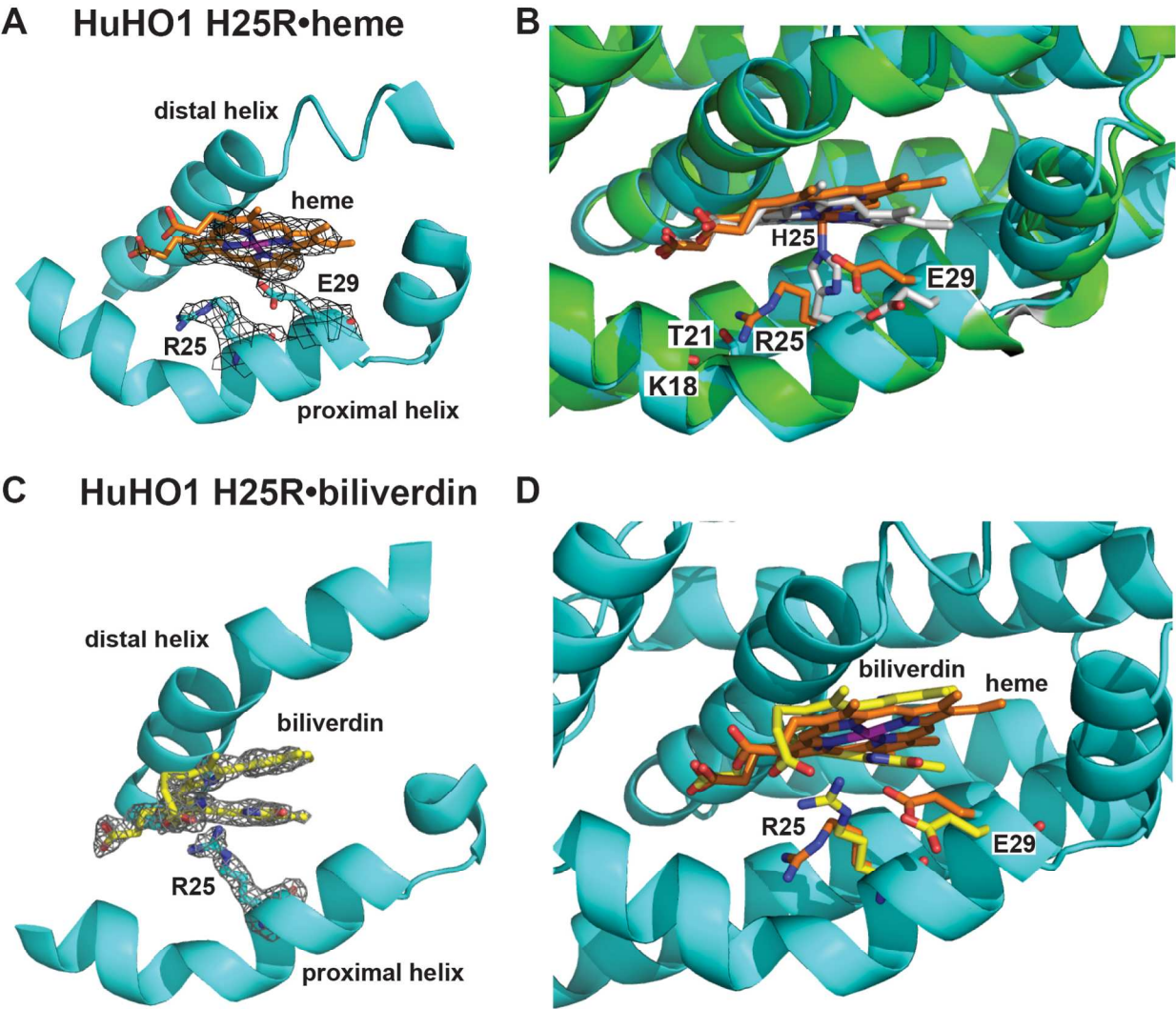


Figure 7. X-ray structural studies of HuHO1 H25R. (A) Sigma-A-weighted $2F_o - F_c$ electron density map (contoured at 1σ) from the 2.95-Å resolution structure of heme (orange) bound to HuHO1 H25R (determined herein, PDB 4WD4). (B) Superposition of the HuHO1 H25R•heme structure (protein in cyan, heme in orange) and the previously published WT HuHO1•heme structure (PDB 1N45, protein backbone in green, side-chains in white, heme in white). (C) Sigma-A-weighted $2F_o - F_c$ electron density map (contoured at 1.2σ) from the 2.08-Å resolution structure of biliverdin IX α (yellow) bound to HuHO1 H25R (determined herein, PDB 5BTQ). (D) Superposition of the heme- and biliverdin-bound structures

of HuHO1 H25R (heme in orange, biliverdin in yellow, residues from heme-bound structure in orange, residues from BV-bound structure in yellow). See Table S1 for data acquisition and refinement statistics

We considered whether the reorientation of Glu29 in the H25R mutant and the possible interactions between this residue and bound heme might be a common feature of HuHO1 His25 mutants and contribute to their in-cell activity (Fig. 3A). We prepared the H25M/E29A and H25R/E29A double mutants and co-expressed them in *E. coli* with IFP. Lysates from these bacteria displayed the expected fluorescence peak for IFP-BV formation (Fig. S4A), indicating that the double mutants retain HO activity and suggesting that in-cell catalysis by His25 mutants does not depend critically on interactions between heme and Glu29.

To assess the effect of proximal His mutation on the orientation of bound product and its interactions with the surrounding active site, we determined a 2.08 Å resolution structure of biliverdin-bound HuHO1 H25R. The overall structure was very similar to heme-bound WT HuHO1 (backbone RMSD of 0.38 Å), with well-defined electron density to support the modeled orientations of active site residues and bound BV and no indication of alternative conformations (Fig. 7C). The orientation of BV bound to H25R is very similar to that of bound heme, although the terminal pyrrole moieties are offset in a helical conformation that avoids steric clashes between the lactam oxygen atoms (Fig. 7D). This configuration is nearly identical to previous structures of BV bound to rat HO1⁵⁶ (Fig. S4B) and HmuO⁵⁷ from *C. diphtheriae*. In addition, one of the propionate groups of BV bound to H25R has adopted an alternate conformation compared to bound heme that places it within hydrogen-bonding distance (3.2 Å) of the side-chain of Arg25, which is repositioned relative to the heme-bound structure (Fig. 7D). Glu29 also adopts an altered position with bound BV that differs from the H25R-heme complex (Fig. 7D) and is closer to that in the WT H25R-heme complex.

We note that the BV position observed in our HuHO1 H25R structure is in striking contrast to the very different position and orientation described previously for BV bound to WT HuHO1⁴⁵, in which BV was suggested to partition into a deeper and more sequestered binding pocket within the HO protein core. The prior study, however, reported limited electron density to support the modeled conformation and suggested that BV might be disordered between multiple conformations. We did not observe electron density for this alternative, deeper orientation of BV. The conformation of BV modeled in our H25R HuHO1 structure is supported by clear electron density and, as noted above, is very similar to the reported positions of BV bound to WT rat HO1 and *C. diphtheriae* HmuO.

DISCUSSION

Role of His-heme coordination and additional active site interactions in HO catalysis.

Understanding the active site features of heme oxygenase that underpin its ability to catalyze conversion of heme to biliverdin remains an on-going challenge. Mutagenesis studies of HO catalysis *in vitro* have supported the view that the proximal His-heme interaction makes an essential contribution to the chemical conversion of heme to biliverdin within the HO active site.^{6, 58} This role has been proposed to reflect the unique electronic effects of the His-heme interaction that tune the redox potential of bound heme and prevent O-O bond cleavage in the ferric heme-hydroperoxy intermediate, as occurs in peroxidases, favoring hydroxylation of the α -meso carbon of the bound heme.⁵⁸⁻⁶⁰ A second model also posits that the His-heme interaction may contribute to positioning the bound heme relative to active site groups on the distal porphyrin face that are critical for catalysis.^{14, 15}

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In contrast to *in vitro* studies, we observed significant HO activity by diverse proximal His mutants of SynHO1 and HuHO1 within live bacteria. Our work provides direct evidence that the His-heme interaction is not strictly required for HO activity by cyanobacterial and mammalian enzymes under intracellular conditions within bacteria and uncovers surprising plasticity in the proximal active site interactions with heme that support HO catalysis in live cells. This conclusion is consistent with prior *in vitro* work that suggested that *D. melanogaster* HO retains the conserved proximal His residue but catalyzes heme degradation in the absence of stable His-heme coordination.⁶¹ A corollary of our findings is that the surrounding active site environment also makes important contributions to HO catalysis, a conclusion supported by extensive *in vitro* work that has revealed critical interactions between heme and active site groups and water molecules on the distal face of heme.^{6, 59, 62, 63} The lack of in-cell activity by a subset of SynHO1 His 17 mutants (Y, E, and F) may reflect unfavorable interactions between these bulky residues and heme and/or propagated effects that disrupt favorable interactions within the remainder of the active site or with redox partners. The inactivity of the H17F mutant, despite still binding heme (Fig. 4B), supports the conclusion that in-cell BV production by R, M, and K mutants reflects specific HO activity within the active sites of these mutants, rather than non-specific heme degradation due to remnant heme association with a protein binding pocket, as has been reported for other heme binding proteins *in vitro*.^{43, 46} If such non-specific activity were the general explanation for remnant in-cell activity by R, M, and K mutants, then we would have most simply expected to have observed in-cell activity for all of the mutants tested herein, which is not what we observed (see summary of models and tests in Supporting Information).

Although our results indicate that proximal His mutants of multiple HO enzymes can remain active within the intracellular environment, the proximal His is nevertheless strictly

conserved as an axial ligand in all known heme-degrading enzymes. Indeed, all proximal His mutants had diminished activity relative to the WT enzyme (Fig. 2 and Fig. 3). This observation suggests that the proximal His-heme interaction optimizes enzyme function and that Nature has consistently selected for this optimal arrangement. As noted above, our results provide only a crude estimate of the in-cell activity of SynHO1 mutants relative to WT. In our view, the most telling observation of our study is that several mutants retain significant activity in live cells (irrespective of whether mutant activity is 25% or 5% of WT), whereas no activity can be detected with purified proteins *in vitro*. This remnant activity by several proximal His mutants is consistent with the general view that enzymatic catalysis arises from many enzyme-substrate interactions within the active site environment, rather than from a single dominant interaction.⁶⁴

⁶⁵ Although the conserved His-heme interaction may optimize positioning and redox potential within the HO active site, our results indicate that HO activity can persist in the absence of His-heme coordination under intracellular conditions within *E. coli*. It remains possible that certain proximal His mutants could also show detectable activity *in vitro* at reaction times substantially longer than those sampled herein (≤ 5 hours, Fig. 6B & 6C). Nevertheless, the central observation remains that certain HO mutants display enhanced in-cell activity relative to the *in vitro* conditions explored herein.

Our failure to detect HO activity by proximal His mutants of AtHO1 provides evidence that HO enzymes can display differing in-cell dependences on the proximal His-heme interaction. This difference between AtHO1 versus SynHO1 and HuHO1, despite conservation of additional active site features such as the distal GG motif,¹⁷ may reflect the structural and functional effects of other active site residues that differ between these enzymes. No crystallographic structures have been reported for AtHO1, and it remains a future challenge to

understand the differences in AtHO1 active site architecture that result in the observed functional differences from SynHO1 and HuHO1.

Recent works have identified HO-like proteins in several organisms, including *A. thaliana* (AtHO2) and *P. falciparum* (PfHO), that show overall sequence homology to known heme oxygenases but lack the conserved His residue and instead feature an Arg (AtHO2) or Lys (PfHO) at the position of the proximal heme ligand.^{42, 66} Both AtHO2 and PfHO bind porphyrins but fail to convert heme to biliverdin *in vitro* or within *E. coli*, and mutation of the proximal Arg (AtHO2) or Lys (PfHO) to His does not confer HO activity in either protein.^{17, 31, 67} These observations suggest that sequence divergence in these proteins beyond loss of the proximal His ligand also contributes to the observed inactivity, underscoring the important roles contributed by additional active site interactions beyond the His-heme coordination to HO catalysis.

Why do proximal His mutants of SynHO1 and HuHO1 show activity in bacterial cells but no activity in vitro? As noted above, neither coupled oxidation nor differences in reduction potentials appear to explain the observed activity differences between these two environments. Based on reports that exogenous imidazole could rescue the activity of His-to-Ala mutants *in vitro*, we also considered whether free histidine or the His side-chain of another protein not present *in vitro* might partition into the mutant HO active site within *E. coli* and enable catalysis, akin to prior reports of imidazole rescue of Src kinase mutants inside cells.^{68, 69} Two observations suggest that this mechanism is not the origin of the observed activity differences: exogenous imidazole does not stimulate activity by SynHO1 H17R *in vitro* (Fig. S3B), and our X-ray structure of HuHO1 H25R suggests insufficient cavity volume beneath the bound heme to accommodate an imidazole group as an axial ligand (Fig. 7A). Finally, to identify protein-protein

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3 interactions that might be critical for HO activity *in vivo*, we affinity-isolated WT SynHO1 from
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5 bacteria and used mass spectrometry to identify more than 200 co-associating proteins (Table
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7 S2). Multiple redox proteins, but not *E. coli* flavodoxin or ferredoxin, were among the identified
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9 candidates, and we have on-going experiments to test the possible role of these proteins in
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11 supporting HO catalysis.
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15 It thus remains a future challenge to elucidate the biochemical mechanism that enhances
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17 HO activity by proximal His mutants within *E. coli* compared to *in vitro* conditions. We
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19 speculate that there may be specific adaptor proteins in bacteria that enhance the interaction and
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21 transfer of electrons between HO and its redox partners. Indeed, recent work has suggested that
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23 HuHO2 and cytochrome P450 reductase (CPR) interact only weakly and transiently *in vitro*,⁷⁰
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25 and scaffolding proteins in other systems have been shown to increase both enzyme specificity
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27 and reaction rate.⁷¹ Our hypothesis is consistent with a prior study of the HuHO1-CPR fusion
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29 protein that reported that covalently tethering CPR and HuHO1, akin to how a scaffolding
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31 protein might facilitate interactions between these proteins via non-covalent binding interactions,
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33 accelerated the rate of biliverdin formation *in vitro* compared to the reaction involving
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35 intermolecular association between the separate HO and CPR proteins.³² Prior work has also
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37 suggested that electron transfer reactions can show heightened sensitivity to the effects of
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39 covalent tethering or physical scaffolds that enhance interactions between the electron donor and
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41 acceptor groups.⁷² Alternatively or in addition, there may be synergistic interactions between
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43 multiple bacterial reductases that cooperatively enhance HO activity compared to solution
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45 conditions *in vitro*. Finally, it remains an important future challenge to test and extend the
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47 conclusions of our study of HO activity in bacteria to other cell types, including the native host
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cells of specific HO enzymes. Future work can also test whether the activity of non-canonical HO enzymes such IsdG/I or MhuD can persist in live cells without His-heme coordination.

Closing perspective. Several recent perspectives have highlighted the importance of developing tools and approaches to test our understanding of enzyme function inside cells and the heterogeneous environment of stable and transient protein-protein interactions within which enzymes operate natively.^{2, 73, 74} Indeed, we note other recent examples of enzymes lacking key residues that appear inactive in solution but show modest activity in the presence of additional intracellular interaction partners or in reconstituted systems *in vitro*.^{75, 76} In our case, use of a fluorescent biliverdin biosensor was critical to detecting the lower activity of HO mutants compared to WT. Our results can guide on-going efforts to fully understand active site contributions to HO catalysis,⁶ to understand the evolution and functional divergence of HO enzymes in biology,^{6, 10, 17, 31, 67} to engineer novel heme proteins with desired functionalities,⁷⁷ and to develop in-cell probes of enzymatic function in other systems.⁷⁸

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION AVAILABLE

Summary of models and tests regarding the origin of in-cell activity, fluorescence images and photos of intact bacteria expressing SynHO1 variants (Fig. S1), quantification of protein expression in Western blots in Fig. 2 (Fig. S2), *in vitro* activity assays of SynHO1 variants with different redox partners (Fig. S3), in-cell studies of HO activity by H17R/E29A and E17M/E29A SynHO1 mutants (Fig. S4A), structural overlay of HuHO1 H25R•BV and rat HO1•BV X-ray structures (Fig. S4B), X-ray crystallographic data collection and refinement statistics (Table S1), and *E. coli* proteins identified by mass spectrometry as associated with WT SynHO1 affinity-purified from bacterial lysates (Table S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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