

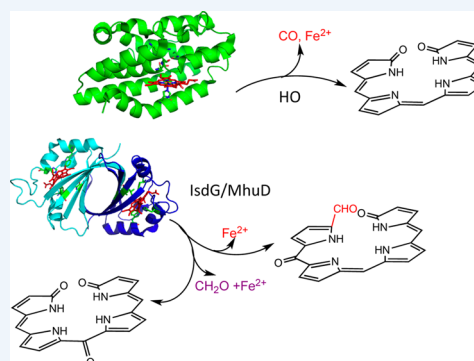
Heme Utilization by Pathogenic Bacteria: Not All Pathways Lead to Biliverdin

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CONSPECTUS: The eukaryotic heme oxygenases (HOs) (E.C. 1.14.99.3) convert heme to biliverdin, iron, and carbon monoxide (CO) in three successive oxygenation steps. Pathogenic bacteria require iron for survival and infection. Extracellular heme uptake from the host plays a critical role in iron acquisition and virulence. In the past decade, several HOs required for the release of iron from extracellular heme have been identified in pathogenic bacteria, including *Corynebacterium diphtheriae*, *Neisseriae meningitides*, and *Pseudomonas aeruginosa*. The bacterial enzymes were shown to be structurally and mechanistically similar to those of the canonical eukaryotic HO enzymes. However, the recent discovery of the structurally and mechanistically distinct noncanonical heme oxygenases of *Staphylococcus aureus* and *Mycobacterium tuberculosis* has expanded the reaction manifold of heme degradation. The distinct ferredoxin-like structural fold and extreme heme ruffling are proposed to give rise to the alternate heme degradation products in the *S. aureus* and *M. tuberculosis* enzymes. In addition, several “heme-degrading factors” with no structural homology to either class of HOs have recently been reported. The identification of these “heme-degrading proteins” has largely been determined on the basis of in vitro heme degradation assays. Many of these proteins were reported to produce biliverdin, although no extensive characterization of the products was performed. Prior to the characterization of the canonical HO enzymes, the nonenzymatic degradation of heme and heme proteins in the presence of a reductant such as ascorbate or hydrazine, a reaction termed “coupled oxidation”, served as a model for biological heme degradation. However, it was recognized that there were important mechanistic differences between the so-called coupled oxidation of heme proteins and enzymatic heme oxygenation. In the coupled oxidation reaction, the final product, verdoheme, can readily be converted to biliverdin under hydrolytic conditions. The differences between heme oxygenation by the canonical and noncanonical HOs and coupled oxidation will be discussed in the context of the stabilization of the reactive $\text{Fe}^{\text{III}}\text{--OOH}$ intermediate and regioselective heme hydroxylation. Thus, in the determination of heme oxygenase activity in vitro, it is important to ensure that the reaction proceeds through successive oxygenation steps. We further suggest that when bacterial heme degradation is being characterized, a systems biology approach combining genetics, mechanistic enzymology, and metabolite profiling should be undertaken.



■ INTRODUCTION

Heme oxidation plays a central role in iron reutilization and cell signaling in mammals, cyanobacteria, and plants.^{1–3} Bacterial pathogens utilize extracellular heme as an iron source via heme-degrading enzymes.^{4–6} The ability to utilize heme as an iron source is essential for virulence and pathogenesis. Similar to their eukaryotic counterparts, heme oxygenases (HOs) from pathogens such as *Corynebacterium diphtheriae*,⁷ *Neisseriae meningitides*,⁸ and *Pseudomonas aeruginosa*⁹ catalyze the conversion of heme to biliverdin with the release of iron and CO (Figure 1A). Distinct from the canonical HOs are the noncanonical IsdG/I (iron surface determinant) proteins of *Staphylococcus aureus* and *Bacillus anthracis* and MhuD (*Mycobacterium tuberculosis* heme utilization) of *Mycobacterium tuberculosis*.^{5,10,11} The *S. aureus* IsdG reaction converts heme to a novel chromophore termed staphylobilin, where the β - or δ -meso-carbon is released as formaldehyde (Figure 1C).^{12,13} In contrast, the structurally related MhuD cleaves heme to a

product called mycobilin that retains the meso-carbon as an aldehyde (Figure 1B).¹⁴

It is important to consider the biological significance of the HOs in the context of pathogenesis. The products of the eukaryotic canonical HOs have been reported to have important antioxidant and antiproliferative/anti-inflammatory properties.^{15–18} Similarly, CO produced as a byproduct of heme utilization may provide an advantage by aiding in the suppression of inflammation. Cystic fibrosis patients with chronic *P. aeruginosa* infections exhale higher levels of CO.¹⁹ The absence of CO production in *M. tuberculosis* and *S. aureus* suggests that infection does not favor CO production. In *M. tuberculosis*, CO can activate the dormancy regulon through the heme-dependent two-component sensor kinases DosS and DosT.²⁰ Thus, avoiding activation of the dormancy genes in an

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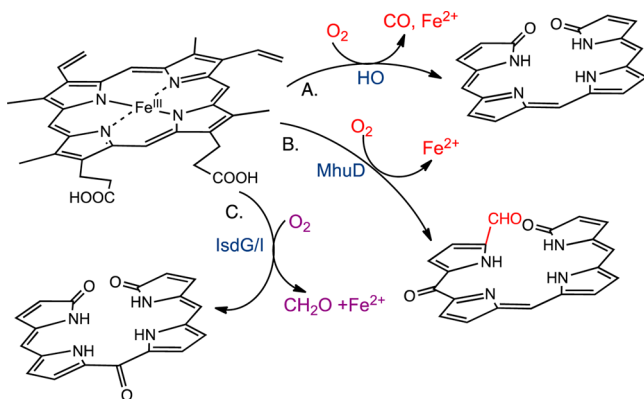


Figure 1. Reaction products of the (A) canonical and (B and C) noncanonical heme oxygenases. The pyrrole substituents have been omitted for simplicity.

immune-competent state is important for survival of the organism. In contrast, CO-releasing molecules have been shown to produce bactericidal effects in *S. aureus*.^{21,22} Induction of colonic HO-1 or exposure to CO in animal models of colitis increases the activity of macrophages and reduces intracellular invasion and bacterial load.^{23,24} Therefore, in gastrointestinal pathogens that cause acute invasive disease, heme utilization may involve a mechanism similar to that of *S. aureus*. Furthermore, mycobilin, staphylobilin, and/or formaldehyde may have as yet unidentified biological functions in the host–pathogen interaction.

In addition to the previously described enzymes, several heme-binding proteins identified in pathogenic bacteria have been reported to be HOs on the basis of *in vitro* assays. However, it is not clear whether heme degradation is due to HO activity or the nonenzymatic “coupled oxidation” of heme described almost a century ago.^{25–27} In the current Account, we discuss the differences between coupled oxidation and heme oxygenation in the context of generating, stabilizing, and directing the $\text{Fe}^{\text{III}}\text{–OOH}$ intermediate toward regioselective heme hydroxylation. In addition, the unique mechanism by which the noncanonical HOs control the intrinsic reactivity of *meso*-hydroxyheme by suppressing CO production and promoting further oxidation of heme is also addressed.

■ COUPLED OXIDATION: A MODEL FOR BIOLOGICAL HEME BREAKDOWN

It was shown some 90 years ago that in the presence of O_2 a mixture of heme in pyridine/water could be degraded by hydrazine or ascorbate.^{26,27} The product “green hemin” was

formed simultaneously with the release of CO. In such studies it was clear that the product of the reaction was not biliverdin but rather verdohemin, which upon addition of KOH and HCl hydrolyzes to biliverdin. Interestingly, the chemically coupled oxidation of hemin in aqueous pyridine gave an equimolar mixture of all four verdohemin isomers, whereas oxidation of hemoglobin and myoglobin yielded primarily biliverdin IXα.^{28,29}

The first step in the reaction was presumed to be heme hydroxylation at the α -*meso* position to form the α -oxy derivative (Figure 2).³⁰ Early studies confirmed that $\text{Fe}^{\text{II}}\text{–oxymesoporphyrin}$ could be converted stepwise to $\text{Fe}^{\text{III}}\text{–mesobiliverdin}$ by two molecules of O_2 .³¹ Chemically synthesized *meso*-hydroxyheme was similarly converted to biliverdin.³² It was initially thought the reaction of verdoheme to biliverdin proceeded through hydrolysis with insertion of an oxygen atom from water,³³ a mechanism at odds with $^{18}\text{O}_2/^{16}\text{O}_2$ -labeling studies identifying the terminal lactam oxygens as being derived from separate O_2 molecules.^{34–36} However, a series of detailed mechanistic studies determined that verdoheme could be oxidatively cleaved to biliverdin (Figure 2).^{37,38}

Analysis of the coupled oxidation of cytochrome *b*₅ variants revealed critical differences between enzymatic and non-enzymatic heme degradation.³⁹ Replacement of the coordinating residue His-63 or His-39 with Val resulted in different end products. The H63V variant was arrested at verdoheme, whereas the final product of the H39V-catalyzed reaction was biliverdin.³⁹ The difference in end products was explained in terms of the relative stability of the $\text{Fe}^{\text{II}}\text{–O}_2$ complexes. Coupled oxidation of the H63V variant could be inhibited by the addition of the H_2O_2 -scavenging enzyme catalase.⁴⁰ This is distinct from the reaction of H_2O_2 with the $\text{Fe}^{\text{III}}\text{–heme:HO-1}$ complex, which generates a coordinated $\text{Fe}^{\text{III}}\text{–OOH}$ intermediate that then reacts to form verdoheme.⁴¹ In contrast, H39V proceeds to $\text{Fe}^{\text{III}}\text{–biliverdin}$ in the presence of catalase, suggesting that the reaction proceeds through a coordinated $\text{Fe}^{\text{II}}\text{–O}_2$ complex. Therefore, although coupled oxidation and heme oxygenation lead to *meso* hydroxylation, they do so by distinct mechanisms. The fact that heme proteins can undergo nonenzymatic conversion of heme to verdoheme with the release of CO indicates that this step in heme degradation is relatively facile. Therefore, detection of CO as defining enzymatic HO activity should be met with caution.⁴²

■ WHAT DEFINES A HEME OXYGENASE?

Several structurally unrelated bacterial heme-binding proteins distinct from the canonical and noncanonical HOs have been

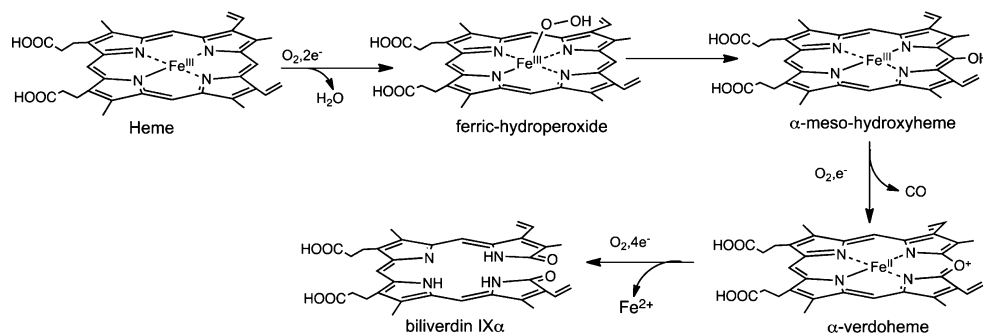


Figure 2. Reaction intermediates in the canonical HO-catalyzed degradation of heme to biliverdin.

reported to have HO activity.^{5,10,42–45} However, this characterization was largely based on a heme toxicity phenotype that could be “complemented” with a gene encoding a canonical HO. The cytoplasmic heme-binding protein ChuS of *Escherichia coli* O157:H7^{42,46} belongs to a structurally related family that includes *Yersinia enterocolitica* HemS and *P. aeruginosa* PhuS.^{46,47} The HO activity of ChuS was based on the detection of CO in the presence of ascorbate. Although the authors ruled out degradation by H₂O₂ by including catalase in the reaction, they did not characterize the products of the reaction. Given the fact that *P. aeruginosa* encodes a previously characterized iron-regulated canonical HemO, the Wilks group revisited the role of the cytoplasmic heme-binding protein PhuS. Bacterial genetics and [¹³C]heme isotopic labeling studies confirmed that heme degradation is driven by the catalytic action of HemO, with PhuS acting as a regulator of extracellular heme flux to HemO.^{48,49} A recent report suggests that in addition to trafficking heme, PhuS is a heme-degrading enzyme that converts heme to verdoheme.⁵⁰ The authors further suggested that verdoheme is trafficked to HemO for conversion to biliverdin. However, it should be noted that these studies were performed in vitro and that the stoichiometry of the reaction was not reported. Furthermore, in the Δ hemO strain, PhuS does not compensate for the loss of HemO by converting heme to verdoheme.⁴⁹

Similarly, several gastroenteric pathogens, including *Helicobacter pylori*, *Vibrio cholera*, *Campylobacter jejuni*, and *Plesiomonas shigelloides*, have been described as encoding HOs.^{43,51,52} In *P. shigelloides* this was based on an inability to efficiently utilize heme in the Δ hugZ (*hutZ* in *V. cholera* and *chuZ* in *C. jejuni*) strain. Furthermore, while complementation of *P. shigelloides* hugZ with a canonical HO from *Synechocystis* PCC6803 alleviated heme toxicity, the HugZ protein showed no detectable HO activity.⁵¹ Therefore, it is extremely important when assigning HO function that a systematic approach combining genetics, metabolite analysis, and enzymology is undertaken.

■ STRUCTURAL DIVERSITY AND THE WIDENING PARADIGM OF HEME OXYGENATION

The canonical HO enzymes from bacteria to mammals have a similar overall α -helical structural fold (Figure 3A).^{53–55} Heme is held between the proximal and distal helices and anchored in the pocket through interactions of the propionates with surface-exposed Arg and Lys residues. Interestingly, HemO from *P. aeruginosa* gives rise to a regioselectivity altered from that of all other HOs as a consequence of the in-plane rotation of the heme within the binding site.⁵³ In addition to the conserved proximal His ligand, all canonical HOs retain an ordered hydrogen-bonding network required for proton delivery and activation of the coordinated Fe^{II}–O₂ (Figure 3B).

In contrast, IsdG/I and MhuD have an overall fold distinct from that of the classical HOs in which the two monomers adopt ferredoxin-like α/β -sandwich folds that come together to form a β -barrel at the homodimer interface (Figure 4A).^{11,56} Each monomer binds heme in a hydrophobic cleft on either side of the β -barrel, with the coordinating His residue provided by the surrounding helix/loops. In IsdI, heme is ligated through His-76 and the propionates are anchored through interactions with Arg-21 and Arg-25 (Figure 4B). The ordered hydrogen-bonding network required for activity in the canonical HO enzymes is absent, with Asn-6 being the only residue capable of hydrogen bonding to the ligand. However, the resting-state

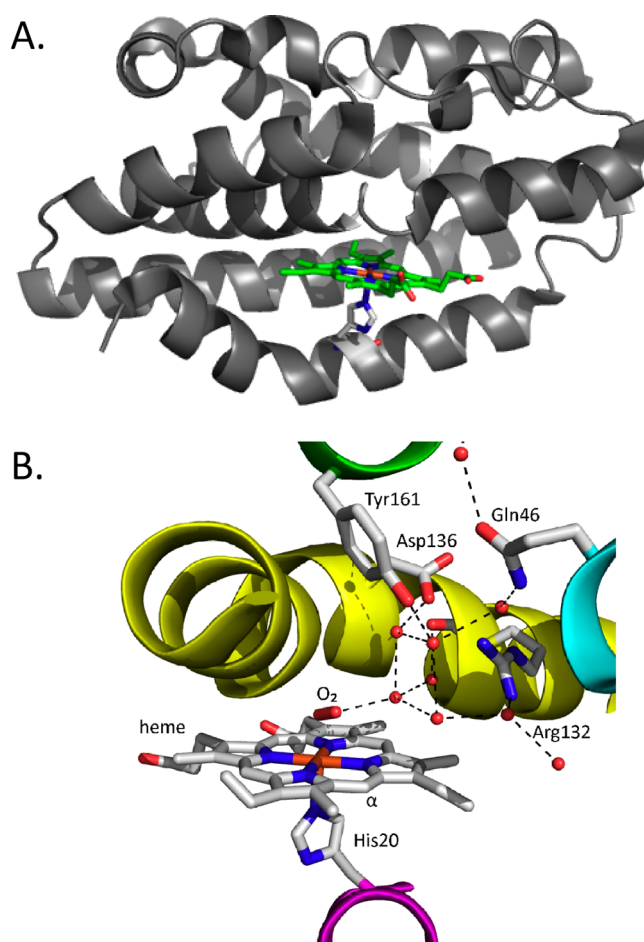


Figure 3. Structure of the bacterial canonical HOs. (A) Overall structural fold of the δ/β -regioselective *P. aeruginosa* HemO [Protein Data Bank (PDB) code 1SK7]. (B) Active site structure with the *C. diphtheriae* Fe^{II}–O₂ heme–HmuO complex (PDB code 1V8X), where the oxygen atoms of the structural waters are represented as red balls and the bound O₂ is shown in stick format.

heme in IsdI undergoes significant distortion from planarity through steric interactions of the heme β -, γ -, and δ -meso-carbon atoms with Trp-66, Phe-22, and Val-79, respectively (Figure 4B).⁵⁶ Furthermore, mutation of Trp-66 to Tyr leads to less heme ruffling and reduced enzyme activity.⁵⁷ Therefore, in contrast to the canonical HOs, where the interactions of the coordinated Fe^{III}–OOH with the hydrogen-bonding network modulate the heme reactivity, in the noncanonical IsdG-like proteins this is achieved through sterically induced heme ruffling.

■ OXYGEN ACTIVATION AND HEME HYDROXYLATION: THE INITIAL STEP IN HEME DEGRADATION

The initial step in the canonical HOs is the reduction of the Fe^{III}–heme:HO complex to the Fe^{II}–O₂ complex.^{58–60} Although the absorption spectrum of the HO-1 Fe^{II}–O₂ complex is similar to that of oxymyoglobin,⁶¹ the Raman spectrum shows a unique oxygen-isotope shift suggesting that the bound O₂ is highly bent.⁶² The structure of the bacterial Fe^{II}–O₂ heme–HmuO complex confirmed that the 110° Fe–O–O bond angle is due to interaction of the O₂ ligand with the distal helix (Figure 3B).⁶³ Reduction of the Fe^{II}–O₂ complex to the activated Fe^{III}–OOH complex leads to the formation of α -

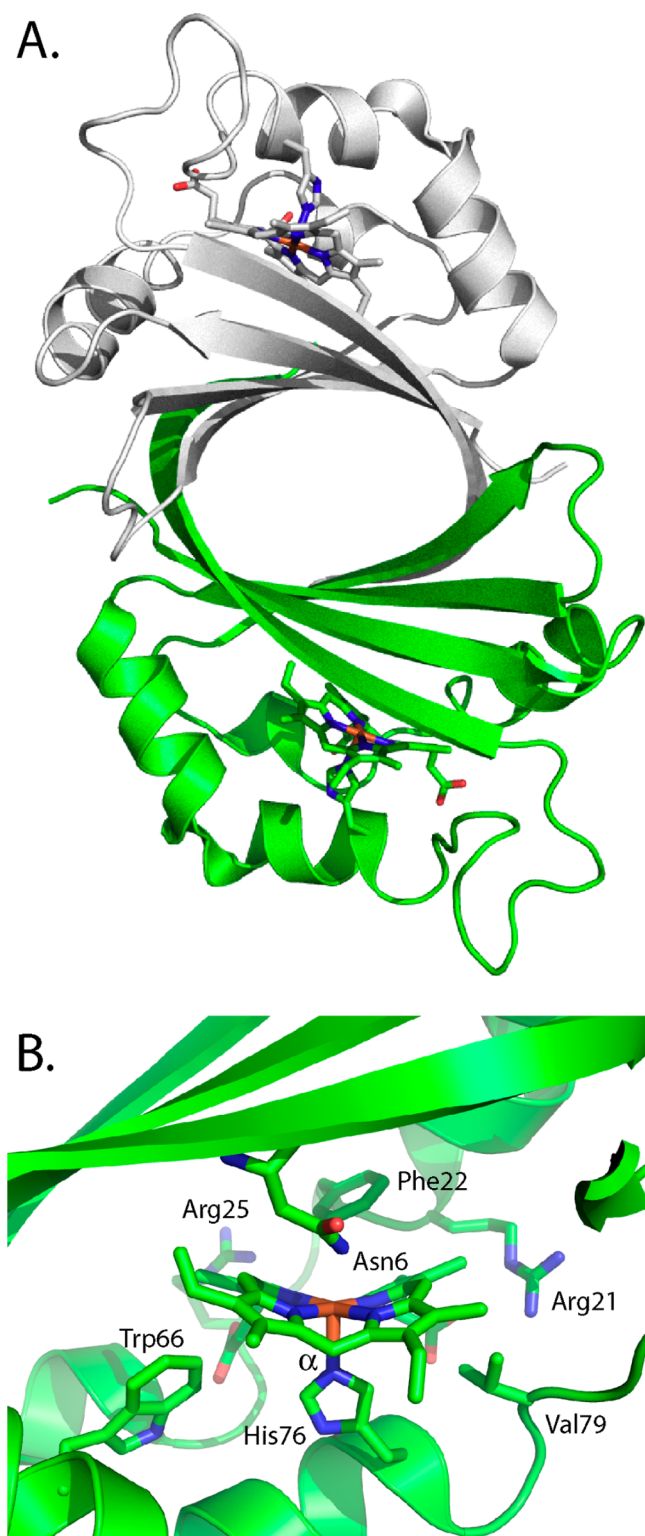


Figure 4. Structure of the IsdI of *S. aureus*. (A) Overall structural fold of the heme-bound IsdI homodimer. (B) Active site of heme-IsdI. The α -meso-carbon is labeled. PDB code 3LGM.

meso-hydroxyheme,⁶⁴ which in the presence of oxygen is rapidly converted to ferric verdoheme.⁴¹

In contrast, the noncanonical enzymes degrade heme to bilin products distinct from those for the canonical enzymes in which either the meso-carbon is retained as an aldehyde (as in MhuD) or released as formaldehyde (as in IsdG/I).^{13,14} It is

proposed that the conserved structural fold and resulting heme distortion account for the alternate reaction products.^{13,14}

■ THE YIN AND YANG: STERIC VERSUS ELECTRONIC CONTRIBUTIONS TO HEME HYDROXYLATION

Regioselective hydroxylation of heme in the canonical HOs results from a combination of steric constraints placed on the activated oxygen species and electronic perturbation of the heme. On the basis of the fact that H_2O_2 can substitute for O_2 and reducing equivalents, the meso hydroxylation of heme was proposed to proceed by either (a) nucleophilic addition of the terminal oxygen of the ferric peroxo ($\text{Fe}^{\text{III}}\text{-OO}^-$) species to the α -meso-carbon or (b) electrophilic addition of the terminal oxygen of the protonated ($\text{Fe}^{\text{III}}\text{-OOH}$) species.⁴¹ However, ethyl hydroperoxide yielded α -meso-ethoxyheme, ruling out nucleophilic addition.⁶⁵ Cryogenic electron-nuclear double resonance (ENDOR) and electron paramagnetic resonance (EPR) studies confirmed $\text{Fe}^{\text{III}}\text{-OOH}$ to be the activated intermediate following one-electron reduction of the $\text{Fe}^{\text{II}}\text{-O}_2$ complex. Temperature annealing experiments further identified $\text{Fe}^{\text{III}}\text{-OOH}$ as the immediate precursor of α -meso-hydroxyheme (Figure 2).⁶⁴ The activation of $\text{Fe}^{\text{II}}\text{-O}_2$ to $\text{Fe}^{\text{III}}\text{-OOH}$ is a common step in heme-containing monooxygenases and HOs.⁶⁶ However, the reactions diverge significantly, as in contrast to the self-hydroxylation by heme oxygenases, the P450-type monooxygenases proceed to Compound I, a ferryl ($\text{Fe}^{\text{V}}\text{=O}$) porphyrin π -radical cation.

The stabilization of the O_2 ligand in the canonical HOs is a combination of the conserved distal Gly-Gly motif and the hydrogen-bonding network provided through bridging H_2O molecules (Figure 3B).^{53–55,63,67} NMR studies confirmed that the ordered water molecules are required for the donation of a proton to the coordinated $\text{Fe}^{\text{II}}\text{-O}_2$.^{68,69} Disruption of the hydrogen-bonding network upon mutation of Asp-140 in HO-1 resulted in destabilization of the $\text{Fe}^{\text{III}}\text{-OOH}$ intermediate and accelerated decay to the $\text{Fe}^{\text{V}}\text{=O}$ species.^{70,71} This was supported by EPR and ENDOR experiments establishing that Asp-140 forms a hydrogen bond with the ordered water molecule that not only stabilizes the $\text{Fe}^{\text{II}}\text{-O-O}$ species but also functions as an efficient conduit to transfer protons required for the formation and activation of $\text{Fe}^{\text{III}}\text{-OOH}$.⁷² The positioning of the nearby water was proposed to promote nucleophilic attack by acting as a hydrogen-bond acceptor and/or to control the steric interactions with the α -meso-carbon.⁷³ Interestingly, mutation of the equivalent Asp-136 in HmuO did not lead to complete loss of the water network or activity, highlighting the critical nature of the hydrogen bond to the distal oxygen.⁷⁴ Collectively, the data support a theory wherein the water promotes nucleophilic attack by acting as a hydrogen-bond acceptor⁷³ or alternatively constrains the bent end-on geometry of $\text{Fe}^{\text{III}}\text{-OOH}$ for interaction with the α -meso-carbon.^{63,74} Furthermore, while the conserved Asp is absent in the *N. meningitidis* and *P. aeruginosa* HemOs, the hydrogen-bonding network is conserved.^{53,55}

In contrast to the water-driven oxygen activation of the $\text{Fe}^{\text{III}}\text{-OOH}$ intermediate, Rivera and co-workers proposed that the initial hydroxylation was facilitated by the heme.^{75,76} Previous studies of model porphyrins have shown a correlation between the sum of the squares of the g values ($\sum g^2$) and the electronic structure of the heme in which a $\sum g^2$ value of ~ 14 is indicative of the $(d_{xz}d_{yz})^4(d_{xy})^1$ state with the unpaired electron residing in the d_{xy} orbital.⁷⁶ The unique EPR fingerprint of low-spin models of the $\text{Fe}^{\text{III}}\text{-OOH}$ HO intermediate and ^{13}C NMR

spectrum of the $\text{Fe}^{\text{III}}\text{--OH}$ HO complex led the authors to propose such an electronic structure for the $\text{Fe}^{\text{III}}\text{--OOH}$ HO intermediate. However, Mössbauer analysis indicated that the $\text{Fe}^{\text{III}}\text{--OOH}$ complex assumed a $(d_{xy})^2(d_{xz},d_{yz})^3$ electronic state common to most low-spin ferric species.⁶⁶ Interestingly, the $(d_{xy})^1$ electron configuration, which places a large amount of unpaired electron density on the *meso*-carbons,⁷⁷ does provide a rationale for heme degradation by the noncanonical enzymes.

In earlier mechanistic studies of the canonical HOs, O–O bond homolysis followed by addition of hydroxyl radical ($\cdot\text{OH}$) to the *meso*-carbon was ruled out on the basis of the indiscriminate nature of the hydroxyl radical.⁴¹ However, recent quantum mechanics/molecular mechanics (QM/MM) studies have suggested that the most favorable pathway is a nonsynchronous concerted mechanism wherein O–O bond homolysis guided by the distal-pocket water cluster leads to attack of $\cdot\text{OH}$ at the *meso*-carbon.⁷⁸

In contrast, the ruffled heme in the noncanonical heme–IsdG/I has been proposed to promote hydroxylation through modulation of the heme electronic structure.^{56,57} An ^1H NMR study of the $\text{Fe}^{\text{III}}\text{--OH}$ and $\text{Fe}^{\text{III}}\text{--CN}^-$ complexes of IsdI revealed large upfield shifts for the *meso*-H and smaller downfield shifts for the methyl groups, suggesting that the heme exhibits predominantly the $(d_{xz},d_{yz})^4(d_{xy})^1$ electron configuration with the electron density residing on the *meso*-carbons.⁷⁹ This is consistent with the inactive IsdI W66Y variant, which shows reduced $(d_{xz},d_{yz})^4(d_{xy})^1$ character and less heme ruffling.⁵⁷ The authors concluded that the heme ruffling in the Isd-like proteins promotes oxidation without the assistance of the hydrogen-bonding network required in the canonical HOs.

■ OXIDATION OF MESO-HYDROXYHEME: A NEW PARADIGM

Beyond the initial hydroxylation step, the mechanism of the noncanonical HOs diverges from that of the canonical HOs. In mechanistic studies of the canonical HOs, the $\text{Fe}^{\text{III}}\text{--meso-hydroxyheme}$ intermediate was shown to decay to $\text{Fe}^{\text{III}}\text{--verdoheme}$ in the presence of oxygen.^{60,65,80,81} EPR and Raman studies showed that $\text{Fe}^{\text{III}}\text{--meso-hydroxyheme}$ exists as a resonance hybrid of keto (oxophlorin), phenolate, and ferrous neutral radical structures (Figure 5).^{80,82} Under aerobic conditions, the neutral radical reacts with oxygen to give a ferrous peroxy radical intermediate that interacts with the ferrous iron or via internal electron transfer to give the $\text{Fe}^{\text{III}}\text{--OOH}$ (Figure 5). The $\text{Fe}^{\text{III}}\text{--OOH}$ species decays to an unstable ferryl alkoxy radical that extrudes the *meso*-carbon as CO. The resulting radical is oxidized to the $\text{Fe}^{\text{IV}}\text{=O}$ intermediate, and the carbocation is trapped as verdoheme.

Skaar and co-workers invoked a similar mechanism for IsdG in which the conversion of heme to staphylobilin proceeds through either $\text{Fe}^{\text{III}}\text{--dihydroxyheme}$ (Figure 6A) or $\text{Fe}^{\text{III}}\text{--meso-hydroxyverdoheme}$ (Figure 6B).¹² The common intermediate in both pathways $\text{Fe}^{\text{III}}\text{--hydroxyverdoheme}$ is converted to 5-oxo-bilirubin (ring cleavage at the β -*meso*-carbon) or 15-oxo-bilirubin (ring cleavage at the δ -*meso*-carbon) via a similar mechanism as the canonical HOs. However, neither the stoichiometry of the reaction or verification of the $\text{Fe}^{\text{III}}\text{--dihydroxyverdoheme}$ intermediate has been shown.

In contrast, Ikeda-Saito and co-workers have shown that heme oxidation by MhuD does not give rise to CO, precluding verdoheme as an intermediate.¹⁴ The MhuD-catalyzed reaction cleaves the porphyrin ring with retention of the α -*meso*-carbon

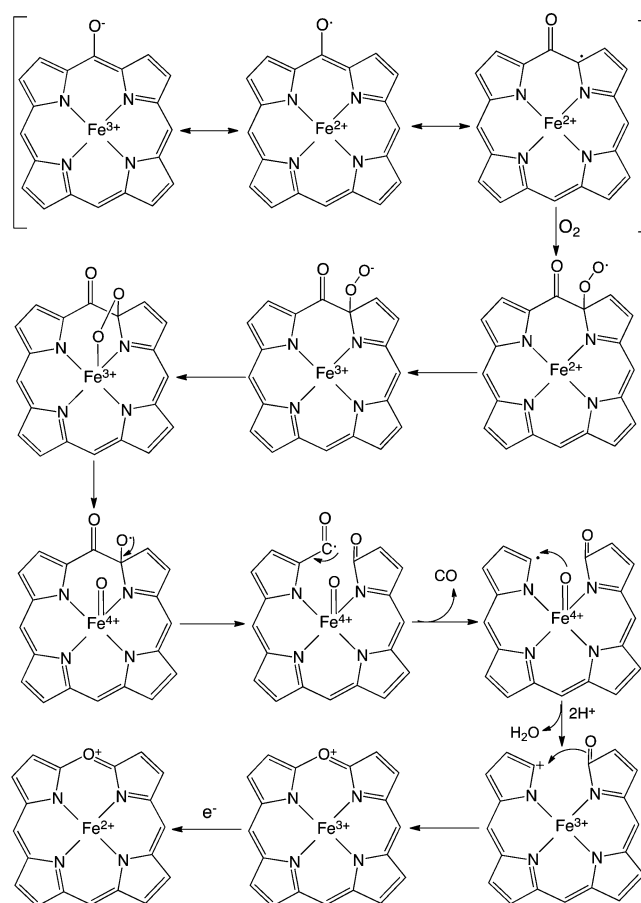


Figure 5. Proposed mechanism for the conversion of *meso*-hydroxyheme to verdoheme in the canonical HOs.

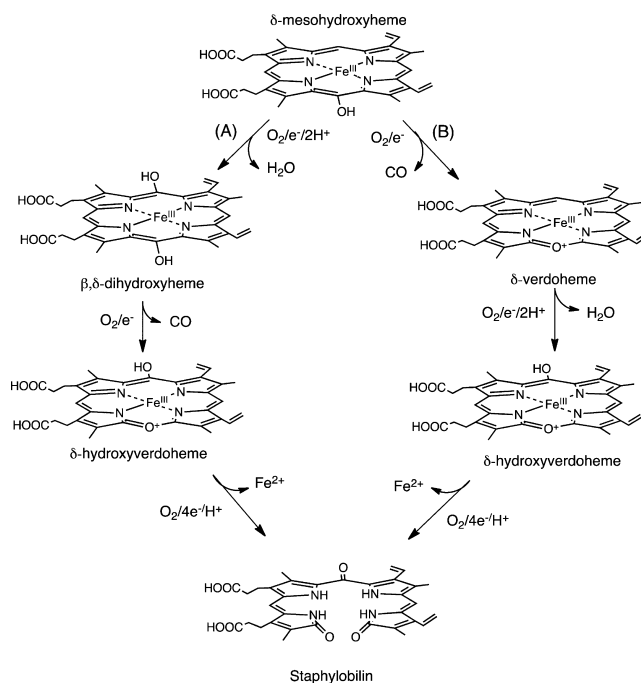


Figure 6. Proposed mechanism for the conversion of heme to staphylobilin via a verdoheme intermediate. Adapted from ref 12.

as an aldehyde and modification of the adjacent *meso*-carbon to a carbonyl at either the β -*meso*-carbon (mycobilin-a) or δ -*meso*-

carbon (mycobilin-b).¹⁴ On the basis of the structural homology between MhuD and IsdG, the authors proposed that the reaction mechanism for IsdG is similar to that for MhuD, and in subsequent studies they have shown that the conversion of heme to staphylobilin proceeds with the release of the *meso*-carbon as formaldehyde.¹³ Furthermore, ¹⁸O₂-labeling studies confirmed that three O atoms from ¹⁸O₂ are incorporated into staphylobilin and mycobilin, in contrast to the two O atoms in biliverdin.

Although the detailed mechanism of the noncanonical HO reaction has yet to be elucidated, the unique protein fold and heme electronic structure must play a significant role. In the canonical HOs, the ferrous neutral radical form of Fe^{III}-*meso*-hydroxyheme reacts directly with O₂ to generate verdoheme with the release of CO (Figure 5). This rapid autoxidation of *meso*-hydroxyheme to verdoheme is suppressed in the IsdG-like proteins. Suppression of the autoxidation step is most likely due to the extreme heme ruffling and altered electronic configuration of the *meso*-hydroxyheme or equivalent intermediate.⁷⁹

Despite the similarity between the MhuD and IsdG/I aldehyde intermediates, the enzymes have distinct regioselectivities. The diagonal modification at the β - and δ -*meso*-carbons in IsdG/I was proposed to occur because the extreme heme ruffling places the terminal oxygen of the putative Fe^{III}-OOH in proximity of both the β - and δ -*meso*-carbons. In contrast, MhuD cleaves the heme at the α -*meso* carbon with carbonyl modification of either the adjacent β - or δ -*meso*-carbon. The regioselectivity is most likely a combination of an in-plane rotation that places the α -*meso*-carbon at the site of activation and the orientational disorder around the α/γ -axis that places either the β - or δ -*meso*-carbon in a position for oxidation to the carbonyl.¹³ This alternate heme orientation is supported in the diheme MhuD structure, where heme I is rotated 90° from that of the heme in IsdI and heme II in MhuD.¹¹

It is unclear at the present time whether the alternate regioselectivity in the MhuD and IsdG proteins plays a role in determining whether the aldehyde is retained or released, respectively. Furthermore, it is not known whether the initial *meso*-carbon hydroxylation leads to ring opening prior to the carbonyl modification or vice versa. Although the mechanistic details of the noncanonical HO-catalyzed reaction have yet to be revealed, this class of heme-degrading enzymes represents a paradigm shift in oxidative heme cleavage.

■ CONCLUDING REMARKS

For decades the biological degradation of heme was thought to be restricted to oxidative cleavage of heme to biliverdin with the release CO. Although monooxygenases and heme oxygenases share an O₂-activation mechanism similar to that of the Fe^{III}-OOH, they diverge through either an oxoferryl species or “self” heme hydroxylation, respectively. In HO, heme hydroxylation is a combination of the decreased tendency to undergo O–O bond cleavage while simultaneously activating the heme. This is achieved by the conformational flexibility of the heme binding site and the extensive hydrogen-bonding network. Where the IsdG-like proteins lie along this activation pathway is of great interest and will provide further insight into the role of structural distortion in heme reactivity.

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Notes

The authors declare no competing financial interest.

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