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Heme Oxygenase Reveals Its Strategy for Catalyzing Three Successive Oxygenation Reactions

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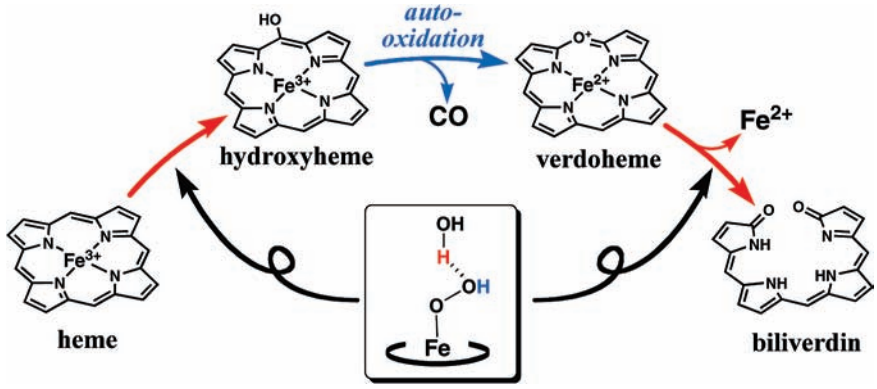

Heme oxygenase (HO) is an enzyme that catalyzes the regiospecific conversion of heme to biliverdin IXα, CO, and free iron. In mammals, HO has a variety of physiological functions, including heme catabolism, iron homeostasis, antioxidant defense, cellular signaling, and O_2 sensing. The enzyme is also found in plants (producing light-harvesting pigments) and in some pathogenic bacteria, where it acquires iron from the host heme.

The HO-catalyzed heme conversion proceeds through three successive oxygenations, a process that has attracted considerable attention because of its reaction mechanism and physiological importance. The HO reaction is unique in that all three O_2 activations are affected by the substrate itself. The first step is the regiospecific self-hydroxylation of the porphyrin α-*meso* carbon atom. The resulting α-*meso*-hydroxyheme reacts in the second step with another O_2 to yield verdoheme and CO. The third O_2 activation, by verdoheme, cleaves its porphyrin macrocycle to release biliverdin and free ferrous iron. In this Account, we provide an overview of our current understanding of the structural and biochemical properties of the complex self-oxidation reactions in HO catalysis.

The first *meso*-hydroxylation is of particular interest because of its distinct contrast with O_2 activation by cytochrome P450. Although most heme enzymes oxidize exogenous substrates by high-valent oxo intermediates, HO was proposed to utilize the Fe−OOH intermediate for the self-hydroxylation. We have succeeded in preparing and characterizing the Fe−OOH species of HO at low temperature, and an analysis of its reaction, together with mutational and crystallographic studies, reveals that protonation of Fe−OOH by a distal water molecule is critical in promoting the unique self-hydroxylation. The second oxygenation is a rapid, spontaneous auto-oxidation of the reactive α-*meso*-hydroxyheme; its mechanism remains elusive, but the HO enzyme has been shown not to play a critical role in it. Until recently, the means of the third O_2 activation had remained unclear as well, but we have recently untangled its mechanistic outline. Reaction analysis of the verdoheme−HO complex strongly suggests the Fe−OOH species as a key intermediate of the ring-opening reaction. This mechanism is very similar to that of the first *meso*-hydroxylation, including the critical roles of the distal water molecule.

A comprehensive study of the three oxygenations of HO highlights the rational design of the enzyme architecture and its catalytic mechanism. Elucidation of the last oxygenation step has enabled a kinetic analysis of the rate-determining step, making it possible to discuss the HO reaction mechanism in relation to its physiological functions.

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Introduction

The biological degradation of heme (iron-protoporphyrin IX) is initiated by a group of enzymes termed heme oxygenases (HOs) that catalyze the regiospecific conversion of heme to biliverdin IXα, CO, and free iron (Figure 1).[1−3] The HO enzyme was first identified in mammals as two isoforms, an inducible HO-1 and a constitutive HO-2.[4] In mammals, electrons required for catalytic turnover are provided by NADPH-cytochrome P450 reductase (CPR),[5] and biliverdin is rapidly reduced by biliverdin reductase to bilirubin. In addition to the HO's well-established capacity in heme catabolism, new results have accumulated on HO's new roles in iron homeostasis, antioxidant defense, cellular signaling, and O_2 sensing.[2,6−9] Higher plants, algae, and cyanobacteria utilize α-biliverdin derived from HO reactions for the synthesis of light-harvesting pigments.[10,11] In some pathogenic bacteria, HO degrades host heme so as to acquire iron essential for their survival and proliferation.[12−14] Physiological electron donors for the bacterial HOs are yet to be identified, while NADPH-ferredoxin reductase and ferredoxin are reported to support some plant HO reactions.[10]

Despite varied physiological functions, the heme degradation by the HO enzymes proceeds through the same mechanism, three successive steps of O_2 activation involving uptake of a total of seven electrons (Figure 1). HO catalysis is unique in that all the O_2 activations are performed by the substrate itself as evidenced by the absence of any other cofactor in HO.[2,3] The first step in HO catalysis is the regiospecific self-hydroxylation of the porphyrin α-*meso* carbon atom. The resulting α-*meso*-hydroxyheme reacts in the second step with another O_2 to yield verdoheme and CO. The third O_2 activation by verdoheme cleaves the porphyrin macrocycle to release biliverdin and free ferrous iron. The first *meso*-hydroxylation has been extensively studied, and the reaction

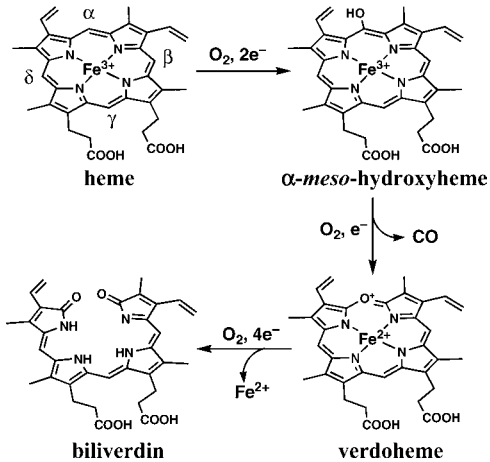

FIGURE 1. Heme degradation sequence catalyzed by HO.

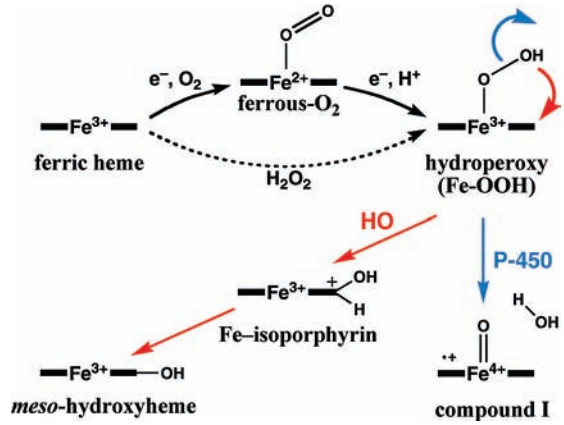

FIGURE 2. Comparison of oxygen activation by HO and cytochrome P450.

mechanism is well understood.[15,16] However, the second and third O_2 activation mechanisms have remained unclear. Recently, we have succeeded in untangling the mechanistic outline and kinetic properties of the third step.[17,18] This Account provides an overview of our current understanding on the structural and biochemical properties of HO catalysis. Inspection of each step highlights rational design of the relationship between enzyme architecture and HO catalysis. Elucidation of the last oxygenation step also allows us to discuss the HO reaction mechanism in relation with its physiological functions.

The First *meso*-Hydroxylation of Heme

The HO catalytic sequence is initiated by regiospecific hydroxylation of the porphyrin α-*meso* carbon atom using one molecule of O_2 and two electrons (Figure 1). Among the three distinct oxygenation steps, the first *meso*-hydroxylation is of particular interest due to its sharp contrast to O_2 activation by cytochrome P450. In both HO and P450, an initial step for the O_2 activation is reduction of the ferric heme iron to the ferrous state, followed by rapid O_2 binding (Figure 2). The subsequent one-electron reduction and protonation of the ferrous-O_2 heme give a ferric hydroperoxy species (Fe−OOH). In P450 as well as peroxidase and catalase, the terminal oxygen of Fe−OOH is thought to be liberated as water to give an oxo ferryl porphyrin π-cation radical or its equivalent, compound I (O═Fe(IV)(Por+·)), as an active species. The Fe−OOH species in HO has been proposed as the hydroxylating reagent of the heme *meso*-carbon. HO compound I artificially generated by *m*-chloroperbenzoic acid never hydroxylates itself but decays to the ferric state through an oxo ferryl porphyrin, compound II.[19] As a surrogate of O_2 and two electrons, H_2O_2 efficiently supports α-*meso*-hydroxyheme formation (Figure 2), whereas most alkyl and acyl hydroperoxides afford the

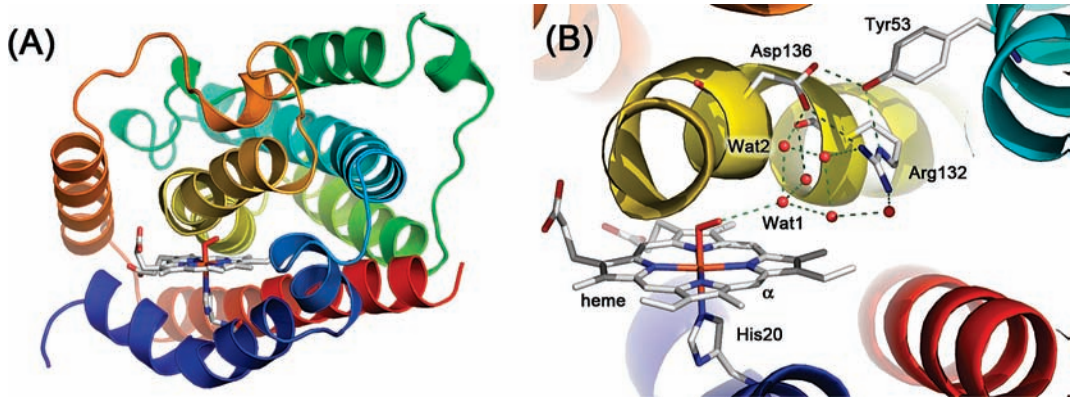

FIGURE 3. Crystal structure of the ferrous-O_2 heme−HmuO complex: (A) overall structure and (B) heme environment (PDB code 1V8X).[26] Red balls represent oxygen atoms of water molecules.

inactive ferryl hemes. A small peroxide, ethyl hydroperoxide, is reported to produce α-*meso*-ethoxyheme in a relatively lower yield, suggesting an intramolecular ethoxy transfer by an Fe−OOEt intermediate.[20,21] The Fe−OOH species of HO has been successfully observed at cryogenic temperatures, and its conversion to α-*meso*-hydroxyheme without detectable intermediates is indicative of self-hydroxylation by Fe−OOH.[22] The iron−isoporphyrin product releases the *meso*-proton immediately to generate *meso*-hydroxyheme (Figure 2).[23]

Crystal structures of heme−HO complexes, especially a ferrous-O_2 complex of HmuO, HO from *Corynebacterium diphtheriae* (Figure 3), reveal salient structural aspects that govern the regiospecific *meso*-hydroxylation.[24−26] The heme group in HO is tightly sandwiched between two helices termed the "proximal" and "distal" helices (Figure 3A, blue and yellow, respectively). The proximal helix contains an axial His ligand, which is neutral and thus does not enhance cleavage of the O−O bond leading to compound I formation unlike the more anionic His ligand in peroxidases.[27] The distal helix of HO is kinked above the heme plane around two conserved Gly residues and is in close contact with the heme group to sterically restrict access to all the *meso* positions except for the α-*meso*-carbon. As a result, the steric constraints imposed by the distal helix direct the terminal oxygen atom of the ferrous-O_2 complex toward the heme α-*meso*-carbon.

The unusually acute Fe−O−O angle in oxy-HO (∼110°), first deduced from the resonance Raman analysis, allows van der Waals contact of the terminal oxygen with the α-*meso*-carbon.[26,28] The terminal oxygen atom also interacts with a water molecule (Wat1 in Figure 3B), which is part of a distal hydrogen bond network including a water cluster and a catalytically critical Asp residue (Asp136 in HmuO, Figure 3B). The distal Asp, which is conserved in most mammalian, plant, and bacterial HOs, interacts with the heme ligand through intervening water molecules (Wat1 and Wat2).[24,25,29] Distal Asp in mammalian HO-1 is indispensable for the *meso*-hydroxylation, suggesting direct involvement of the carboxylate moiety in the oxygen activation.[30,31] Some bacterial HOs, however, contain no carboxylate residue in their distal pocket.[32,33] Furthermore, we have found that distal Asp of HmuO can be replaced by Asn without significant loss of the enzyme activity.[34] Our comprehensive study on HmuO concludes that the distal Asp is not involved in the oxygen activation but stabilizes the water network to place catalytically critical Wat1 at a position suitable for efficient Fe−OOH activation.[34] The different effect of the Asp substitutions in HO-1 and HmuO could be due to the varied stability of their hydrogen bond network.[35] Contrary to our conclusion, a crystal structure of a distal Asp mutant of human HO-1 (D140A) showed only a limited perturbation on the distal pocket water network that retains Wat1 at the original position.[36] This structure has led Poulos, Ortiz de Montellano, and their co-workers to propose that distal Asp works only as a hydrogen bond *acceptor* to prevent protonation of Fe−OOH by Wat1, thus stabilizing the Fe−OOH intermediate.[36] However, our direct investigation on the active species affirms the Fe−OOH activation by the proton *donation* in HO-1, as discussed below.[37]

The fleeting Fe(III)−OOH species of HO-1 has been captured by a cryo-reduction method for detailed electron paramagnetic resonance (EPR) and electron−nuclear double resonance (ENDOR) characterization.[22] At liquid nitrogen or helium temperatures, the ferrous-O_2 heme of HO-1 is reduced by irradiation of γ- or β-ray to afford the Fe−OOH species.[22,38,39] The exchangeable terminal proton of Fe−OOH is observed by ENDOR.[22] A nonprotonated peroxo form ($Fe-OO^-$) is not observed for wild type HO-1 even near 4 K where no heavy atom movement is expected to take place.[40] This is in contrast to significant suppression of the peroxo-protonation in P450 below 55 K.[41] The efficient proton donor

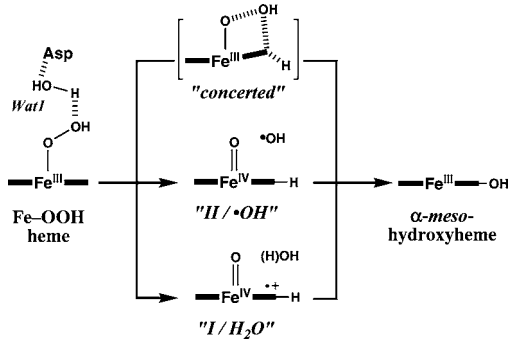

FIGURE 4. Three pathways proposed for the heme *meso*-hydroxylation by HO: (top) a concerted self-hydroxylation, (middle) a recombination of compound II with hydroxyl radical generated transiently, and (bottom) a water addition to compound I.

in HO is the Wat1 molecule which interacts with the heme-bound oxygen in the ferrous-O_2 heme (Figure 3B). The Fe−OO^- species in HO-1 becomes observable by the Asp140 to Ala replacement, consistent with displacement and/or fluctuation of the Wat1 molecule.[37]

ENDOR spectroscopy detects another exchangeable proton upon annealing of wild type Fe−OOH to 200 K.[37] The *protonated* Fe−OOH above 200 K directly converts to Fe(III)−hydroxyheme. The second proton is missing in D140A HO-1, where the distal water network is substantially destabilized. The donor of the second proton is, therefore, again most likely to be Wat1 (Figure 4). The *nonprotonated* Fe−OOH in the Asp mutant does not form the hydroxyheme product, indicating a critical functionality of the second proton. The Fe−OOH conversion in wild type HO-1 exhibits a significant solvent isotope effect as well as a secondary isotope effect upon deuteration of the *meso*-protons.[42] These observations suggest a concerted *meso*-hydroxylation process: proton transfer to Fe−OOH through Wat1 occurring in synchrony with bond formation between the terminal oxygen and the α-*meso* carbon to form the tetrahedral isoporphyrin intermediate (Figures 2 and 4, top). The two protons required for formation and activation of Fe−OOH may be transferred from bulk water to the active site through the distal hydrogen bond network, which is extended to the molecular surface of the enzyme.[24,29]

Quantum chemical calculations, however, estimate a very large activation energy for the highly strained transition state of the concerted hydroxylation mechanism.[43,44] Instead, the calculations favor stepwise mechanisms including an initial O−O bond cleavage followed by rebinding of a liberated OH group to the α-*meso*-carbon (Figure 4, middle and bottom). The O−O bond may be cleaved heterolytically to produce compound I and an OH anion.[44] HO compound I cannot perform the self-hydroxylation as described above, ruling out this

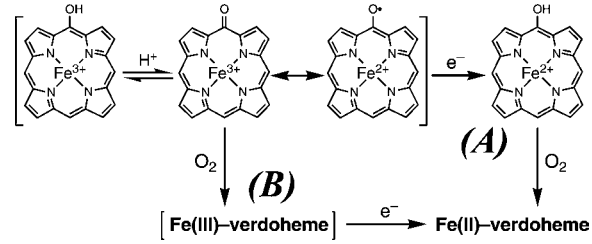

FIGURE 5. Possible resonance structures and reaction pathways of *meso*-hydroxyheme.

compound I pathway (Figure 4, bottom).[19] Alternatively, the O−O bond homolysis can produce compound II and hydroxyl radical (·OH). Compound II alone is inactive for the self-hydroxylation,[20] but the reactivity of the hydroxyl radical transiently generated above the *meso*-carbon is yet to be experimentally evaluated. A recent hybrid quantum mechanical/molecular mechanical study suggests that the addition of the hydroxyl radical takes place with very small activation energy, thereby making the reaction a nonsynchronous but *effectively concerted* pathway.[45] In this scheme, the distal water cluster holds the highly reactive radical in an orientation for the rapid and exclusive attack on the α-*meso*-carbon.[45]

The Second Auto-Oxidation of *meso*-Hydroxyheme

In the second step of the HO catalysis, Fe(III)−α-*meso*-hydroxyheme is converted into Fe(II)−verdoheme and CO by consuming one molecule of O_2 and one electron (Figure 1). This step is distinct in several aspects from the other two HO reaction steps. First, only this second step is *not* inhibited by CO ligation to the iron and it *cannot* be supported by H_2O_2 as a surrogate of O_2 and reducing equivalent(s).[46,47] Second, all the α-, β-, γ-, and δ-isomers of *meso*-hydroxyheme are converted to corresponding isomers of verdoheme.[48] In contrast, the first heme hydroxylation is α-selective, and only the α-verdoheme is converted to biliverdin by HO.[48] Third, the HO enzyme does *not* play a critical role only in this second O_2 activation; that is, even in the absence of HO, hydroxyheme immediately reacts with O_2 to afford verdoheme.[49] These observations describe this second step as a spontaneous auto-oxidation of the reactive α-*meso*-hydroxyheme.

The Fe(III)− and Fe(II)−hydroxyheme−HO-1 complexes are stable under anaerobic conditions. A resonance Raman study on the Fe(III) complex indicates deprotonation of the α-*meso* hydroxy group to have an oxophlorin-like ring structure rather than a porphyrin-type macrocycle (Figure 5).[46,50] An EPR spectroscopic study on the Fe(III) complex leads to the detection of a minor free radical as well as a major Fe(III) high spin species.[46,47] The CO addition suppresses the iron high spin

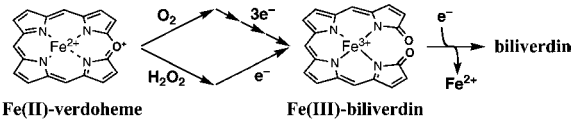

FIGURE 6. Dual pathway of verdoheme ring-opening supported by O_2 and H_2O_2.

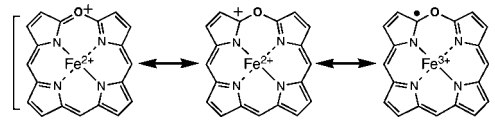

FIGURE 7. Possible resonance structures of ferrous-verdoheme.

EPR signal with concomitant enhancement in the radical signal, suggesting a resonance of Fe(III)-oxophlorin and Fe(II)-oxy radical porphyrin structure (Figure 5). Upon reduction of the iron, the resonance Raman spectral pattern of hydroxyheme becomes similar to that of the protoheme complexes.[46,50] A remarkable D_2O effect of the porphyrin vibration modes reveals protonation of the *meso*-hydroxy group in the Fe(II) complex. Thus, α-*meso*-hydroxyheme undergoes a redox-linked conversion between a keto and an enol form (Figure 5).

The Fe(III)−hydroxyheme−HO complex can afford Fe(II)−verdoheme through two possible pathways (Figure 5). In pathway A, Fe(III)−hydroxyheme is reduced to the Fe(II) state, which then reacts with O_2 to afford Fe(II)−verdoheme. In the alternative pathway B, Fe(III)−hydroxyheme reacts with O_2, prior to reduction of the iron, to give a metastable intermediate, which is normally considered as Fe(III)−verdoheme but is also suggested as a ring-oxidized form of *meso*-hydroxyheme.[47,51] Regardless of the nature of the intermediate, one-electron reduction of the air-oxidized intermediate produces Fe(II)−verdoheme (Figure 5). Under physiologically relevant conditions, pathway B seems dominant because Fe(III)−hydroxyheme reacts with O_2 much faster than its reduction to the ferrous state.[46,52]

The hydroxyheme conversion to verdoheme is a multistep reaction that includes O_2 binding, O−O bond cleavage, CO liberation, and reorganization of the macrocycle. O_2 binding is postulated to take place at the α-pyrrole carbon of Fe(III)−hydroxyheme on the basis of no inhibition occurs upon CO ligation.[3,46] Nevertheless, no intermediate has been observed in the O_2 reaction of the Fe(III) complex, and thus, its mechanism remains unknown. The Fe(II) complex reacts with O_2 to accumulate an intermediate species having an unusual absorption band at 815 nm,[53] characterization of which might clarify the second step mechanism.

The Third Redox-Dependent Ring-Opening of Verdoheme

At the third stage of heme degradation, Fe(II)−verdoheme is converted to biliverdin and the free ferrous ion with consumption of one O_2 molecule and four reducing equivalents (Figure 1). The verdoheme macrocycle is initially cleaved to afford an Fe(III)−biliverdin complex (Figure 6), and subsequent iron reduction liberates the ferrous ion. Biliverdin release from HO is the rate limiting step in HO catalysis but is dramatically accelerated by biliverdin reductase.[54] Under physiological conditions where biliverdin release is not rate limiting, the ring-opening of Fe(II)−verdoheme is considered to be the rate-determining step in the HO catalysis.[54] The third oxygenation step mechanism has just began to be understood. Verdoheme can be nonenzymatically converted to biliverdin by hydrolysis or by redox reactions using O_2 or H_2O_2.[55−57] The hydrolytic pathway proceeds through nucleophilic attack of the hydroxide ion at the positively charged α-pyrrole carbon of the verdoheme ring.[58,59] Subsequent deprotonation of the hydroxide adduct produces the iron−biliverdin complex. The redox-dependent pathway is relatively complex, and little is known about its mechanism. Recently, in O_2-dependent ring-opening, a reaction intermediate has been characterized as an oxidized form of the Fe−biliverdin complex.[60] Nevertheless, even initial binding sites of O_2 and H_2O_2 are yet to be identified.

In spite of this chemical versatility, the HO enzyme was originally reported to exclusively utilize O_2 for biliverdin formation. The enzyme is known to incorporate an oxygen atom of O_2 rather than of water into biliverdin,[61] and the verdoheme−HO complex was reported not to react with H_2O_2.[3,20] While the latter observation is true for *Fe(III)*−verdoheme, we have demonstrated that the *Fe(II)*−verdoheme−HO-1 complex readily reacts with H_2O_2 to afford biliverdin under reducing conditions (Figure 6).[17]

The dual pathway degradation of verdoheme (Figure 6) is initiated by binding of either O_2 or H_2O_2 on Fe(II)−verdoheme to allow direct observation of ring-opening intermediates.[17] Although these intermediates are yet to be identified, their reduction produces biliverdin through Fe(III)−biliverdin (Figure 6). O_2- and H_2O_2-binding may occur either on the verdoheme iron or at the α-pyrrole carbon due to possible resonance of Fe(II)−verdoheme involving redox state change of iron (Figure 7). The peroxide reaction should be less complicated than the oxygen reaction because it requires only one electron reduction to yield Fe(III)−biliverdin instead of multiple reduction steps (Figure 6). In the peroxide reaction, the H_2O_2 binding would produce the Fe−OOH or ring−OOH verdoheme complexes (Figure 8). These species may be depro-

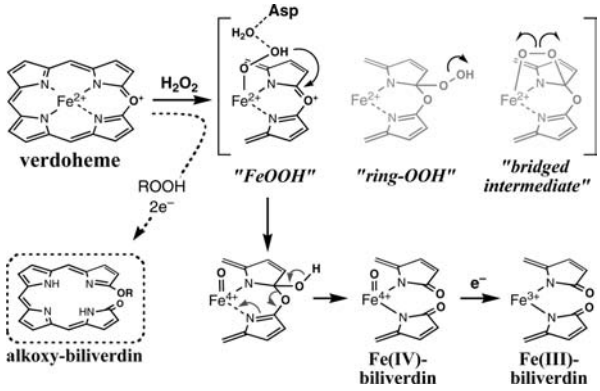

FIGURE 8. Proposed mechanism for the ring-opening of verdoheme.

tonated to form a bridged intermediate. All the three possible intermediates could generate biliverdin upon O−O bond cleavage.

The ring-opening of verdoheme also is supported by small alkyl hydroperoxides such as CH_3OOH to yield the corresponding alkoxy-biliverdin (Figure 8).[18] Alkoxy incorporation is only possible through the Fe−OOR intermediate whose terminal alkoxy group is transferred into the α-pyrrole carbon. Therefore, the corresponding OH transfer of Fe−OOH is likely in the H_2O_2 pathway (Figure 8). This Fe−OOH mechanism is very similar to that of the HO first step, *meso*-hydroxylation of heme (Figure 4). As found for the *meso*-hydroxylation, the distal Asp is critical in the third oxygenation supported by H_2O_2 and also by O_2.[17] The effects of the distal Asp substitutions are essentially the same for the O_2 and H_2O_2 pathways. On the basis of these observations, a water-mediated activation of Fe−OOH has been proposed both in the O_2- and H_2O_2-dependent ring-opening of verdoheme (Figures 4 and 8).

Contrary to our mechanistic proposal, a reported crystal structure of the Fe(II)−verdoheme−human HO-1 complex shows no water in the distal heme pocket.[62] The verdoheme iron in the crystal is five-coordinate without an exogenous ligand; however, spectroscopic studies unambiguously indicate that the Fe(II)−verdoheme iron is six-coordinate low-spin, possibly with a water (hydroxide) ligand.[50,63] In order to reassess the verdoheme structure, we have recently crystallized a verdoheme−HmuO complex.[64] In our structure refined to 2.0 Å resolution, spherical electron density corresponding to an aqua-ligand and water molecules are clearly observed at the verdoheme distal side, consistent with our proposed water-assisted oxygen activation for the verdoheme opening.[64]

TABLE 1. Kinetic Parameters for the O_2 and H_2O_2 Reactions of HO

	O_2	H_2O_2	ref
heme	6.9×10^6	1.3×10^3	66 and 67
hydroxyheme	3.9×10^5	—[b]	53
	—[a]		46
verdoheme	2.5×10^2	1.3×10^4	17

[a] Too fast to measure. [b] Not reactive.

Implications of Redox-Dependent Pathway of the Third Oxygenation Step

Our mechanistic studies can answer a fundamental question of the HO catalysis: why does nature favor cleavage of the verdoheme macrocycle through energy and resource (NADPH) consuming redox pathways (Figure 6) rather than a spontaneous hydrolysis pathway? Our results indicate mechanistic similarities between the redox-dependent verdoheme reaction with the first HO step, *meso*-hydroxylation of heme (Figures 4 and 8).[17,18] Both the first and third oxygenations are facilitated by the common oxygenase-type structure of HO including the distal Asp and the water cluster (Figure 4). If HO hydrolyzed the verdoheme ring, the enzyme active site would have to involve a hydrolase-like structure, elevating basicity of a water molecule, in addition to the oxygenase-type structure. Such a molecular design appears to be practically impossible so that the similarity of the first and third steps is important to catalyze the multiple reactions by the single HO enzyme. It should be noted that oxygen activation at the HO second step does not require the help from the enzyme.[49]

The redox pathway is also advantageous over the verdoheme hydrolysis for the physiological functions of HO, O_2 sensing and cytoprotection against oxidative stress. The third verdoheme degradation is considered to be the major rate-determining step of the HO catalysis,[54] and its redox pathway involves the anomalously slow reaction with O_2 (Figure 6, Table 1). Due to the rate-limiting O_2 reaction, the HO catalytic activity responds to changes in O_2 concentration so as to function as a physiological O_2 sensor.[9,17] The slow O_2 reaction can be circumvented through the relatively faster H_2O_2 reaction (Figure 6, Table 1).[17] Elevation of the H_2O_2 level which occurs under severe oxidative stress would produce more biliverdin and bilirubin, both of which are potent antioxidants, to augment the cytoprotective feature of the HO reaction.[7,17,65] Although H_2O_2 is also reactive with the ferric *heme*−HO complex (Figure 2, Table 1), its primary target should be the verdoheme complex. The ferric heme complex is reduced to the ferrous state much faster than its reaction with H_2O_2.[54]

Concluding Remarks

The structure and mechanism of HO have been studied for more than three decades. At a relatively earlier stage of HO research,

an Fe−OOH species was proposed as an active intermediate in the first heme *meso*-hydroxylation.[20] Recent enormous progress has allowed for the direct characterization of the FeOOH species to explore the unique O_2 activation by HO in detail. Further research on the first step is expected to establish a mechanism in harmony both with experimental and theoretical studies. While the mechanism of the second oxygenation remains elusive, we have succeeded in elucidating, at long last, the outline of the third oxygenation mechanism. Further studies should include characterization of the reaction intermediates observed (Figure 6) and determination of the O_2 binding site of Fe(II)−verdoheme. Analysis of reactions of the verdoheme intermediate, especially with endogenous substances, is also of physiological interest. As suggested for H_2O_2, the major rate-limiting step may be perturbed by small molecules that modulate the activity and function of HO. A comprehensive analysis of verdoheme reactivity may be helpful in understanding actions and responses of HO *in vivo* under a variety of cellular conditions.

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BIOGRAPHICAL INFORMATION

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Masao Ikeda-Saito received his B.E. (1973), M.E. (1975), and Ph.D. (1978) degrees in Biophysical Engineering from Osaka University. In 1975, he joined the laboratory of Dr. T. Yonetani in the Biochemistry and Biophysics Department at the University of Pennsylvania. He became a faculty member of the same department in 1981. In 1989, he moved to Case Western Reserve University School of Medicine where he became a Professor of Physiology and Biophysics in 1996 prior to his appointment as a professor at Tohoku University in 1998. He was a recipient of the Japanese Chemical Society Award for Creative Work (2007).

FOOTNOTES

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REFERENCES

1 Tenhunen, R.; Marver, H. S.; Schmid, R. Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* **1969**, *244*, 6388–6394.

2 Yoshida, T.; Kikuchi, G. Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme oxygenase system. *J. Biol. Chem.* **1978**, *253*, 4230–4236.

3 Ortiz de Montellano, P. R. Heme oxygenase mechanism - Evidence for an electrophilic, ferric peroxide species. *Acc. Chem. Res.* **1998**, *31*, 543–549.

4 Maines, M. D.; Trakshel, G. M.; Kutty, R. K. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J. Biol. Chem.* **1986**, *261*, 411–419.

5 Schacter, B. A.; Nelson, E. B.; Marver, H. S.; Masters, B. S. S. Immunochemical evidence for an association of heme oxygenase with the microsomal electron transport system. *J. Biol. Chem.* **1972**, *247*, 3601–3607.

6 Maines, M. D. The heme oxygenase system: A regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 517–554.

7 Poss, K. D.; Tonegawa, S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10925–10930.

8 Suematsu, M.; Ishimura, Y. The heme oxygenase-carbon monoxide system: A regulator of hepatobiliary function. *Hepatology* **2000**, *31*, 3–6.

9 Williams, S. E.; Wootton, P.; Mason, H. S.; Bould, J.; Iles, D. E.; Riccardi, D.; Peers, C.; Kemp, P. J. Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* **2004**, *306*, 2093–2097.

10 Cornejo, J.; Willows, R. D.; Beale, S. I. Phytobilin biosynthesis: Cloning and expression of a gene encoding soluble ferredoxin-dependent heme oxygenase from Synechocystis sp. PCC 6803. *Plant J.* **1998**, *15*, 99–107.

11 Davis, S. J.; Kurepa, J.; Vierstra, R. D. Arabidopsis thaliana HY1 locus, required for phytochrome-chromophore biosynthesis, encodes a protein related to heme oxygenases. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6541–6546.

12 Schmitt, M. P. Utilization of host iron sources by *Corynebacterium diphtheriae*: Identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J. Bacteriol.* **1997**, *179*, 838–845.

13 Wandersman, C.; Stojiljkovic, I. Bacterial heme sources: The role of heme, hemoprotein receptors and hemophores. *Curr. Opin. Microbiol.* **2000**, *3*, 215–220.

14 Genco, C. A.; Dixon, D. W. Emerging strategies in microbial haem capture. *Mol. Microbiol.* **2001**, *39*, 1–11.

15 Colas, C.; Ortiz de Montellano, P. R. Autocatalytic radical reactions in physiological prosthetic heme modification. *Chem. Rev.* **2003**, *103*, 2305–2332.

16 Unno, M.; Matsui, T.; Ikeda-Saito, M. Structure and catalytic mechanism of heme oxygenase. *Nat. Prod. Rep.* **2007**, *24*, 553–570.

17 Matsui, T.; Nakajima, A.; Fujii, H.; Mansfield Matera, K.; Migita, C. T.; Yoshida, T.; Ikeda-Saito, M. O_2- and H_2O_2-dependent verdoheme degradation by heme oxygenase: Reaction mechanisms and potential physiological roles of the dual pathway degradation. *J. Biol. Chem.* **2005**, *280*, 36833–36840.

18 Matsui, T.; Omori, K.; Jin, H.; Ikeda-Saito, M. Alkyl peroxides reveal the ring opening mechanism of verdoheme catalyzed by heme oxygenase. *J. Am. Chem. Soc.* **2008**, *130*, 4220–4221.

19 Matsui, T.; Kim, S. H.; Jin, H.; Hoffman, B. M.; Ikeda-Saito, M. Compound I of heme oxygenase cannot hydroxylate its heme *meso*-carbon. *J. Am. Chem. Soc.* **2006**, *128*, 1090–1091.

20 Wilks, A.; Ortiz de Montellano, P. R. Rat liver heme oxygenase. High level expression of a truncated soluble form and nature of the *meso*-hydroxylating species. *J. Biol. Chem.* **1993**, *268*, 22357–22362.

21 Wilks, A.; Torpey, J.; Ortiz de Montellano, P. R. Heme oxygenase (HO-1). Evidence for electrophilic oxygen addition to the porphyrin ring in the formation of α-meso-hydroxyheme. *J. Biol. Chem.* **1994**, *269*, 29553–29556.

22 Davydov, R. M.; Yoshida, T.; Ikeda-Saito, M.; Hoffman, B. M. Hydroperoxy-heme oxygenase generated by cryoreduction catalyzes the formation of α-*meso*-hydroxyheme as detected by EPR and ENDOR. *J. Am. Chem. Soc.* **1999**, *121*, 10656–10657.

23 Evans, J. P.; Niemevz, F.; Buldain, G.; Ortiz de Montellano, P. R. Isoporphyrin intermediate in heme oxygenase catalysis: Oxidation of α-meso-phenylheme. *J. Biol. Chem.* **2008**, *283*, 19530–19539.

24 Schuller, D. J.; Wilks, A.; Ortiz de Montellano, P. R.; Poulos, T. L. Crystal structure of human heme oxygenase-I. *Nat. Struct. Biol.* **1999**, *6*, 860–867.

25 Sugishima, M.; Omata, Y.; Kakuta, Y.; Sakamoto, H.; Noguchi, M.; Fukuyama, K. Crystal structure of rat heme oxygenase-1 in complex with heme. *FEBS Lett.* **2000**, *471*, 61–66.

26 Unno, M.; Matsui, T.; Chu, G. C.; Couture, M.; Yoshida, T.; Rousseau, D. L.; Olson, J. S.; Ikeda-Saito, M. Crystal structure of the dioxygen-bound heme oxygenase from *Corynebacterium diphtheriae*: Implications for heme oxygenase function. *J. Biol. Chem.* **2004**, *279*, 21055–21061.

27 Takahashi, S.; Wang, J.; Rousseau, D. L.; Ishikawa, K.; Yoshida, T.; Host, J. R.; Ikeda-Saito, M. Heme-heme oxygenase complex. Structure of the catalytic site and its implication for oxygen activation. *J. Biol. Chem.* **1994**, *269*, 1010–1014.

28 Takahashi, S.; Ishikawa, K.; Takeuchi, N.; Ikeda-Saito, M.; Yoshida, T.; Rousseau, D. L. Oxygen-bound heme-heme oxygenase complex - Evidence for a highly bent structure of the coordinated oxygen. *J. Am. Chem. Soc.* **1995**, *117*, 6002–6006.

29 Hirotsu, S.; Chu, G. C.; Unno, M.; Lee, D. S.; Yoshida, T.; Park, S. Y.; Shiro, Y.; Ikeda-Saito, M. The crystal structures of the ferric and ferrous forms of the heme complex of HmuO, a heme oxygenase of *Corynebacterium diphtheriae*. *J. Biol. Chem.* **2003**, *279*, 11937–11947.

30 Fujii, H.; Zhang, X.; Tomita, T.; Ikeda-Saito, M.; Yoshida, T. A role for highly conserved carboxylate, aspartate-140, in oxygen activation and heme degradation by heme oxygenase-1. *J. Am. Chem. Soc.* **2001**, *123*, 6475–6484.

31 Lightning, L. K.; Huang, H.; Moenne-Loccoz, P.; Loehr, T. M.; Schuller, D. J.; Poulos, T. L.; Ortiz de Montellano, P. R. Disruption of an active site hydrogen bond converts human heme oxygenase-1 into a peroxidase. *J. Biol. Chem.* **2001**, *276*, 10612–10619.

32 Schuller, D. J.; Zhu, W.; Stojiljkovic, I.; Wilks, A.; Poulos, T. L. Crystal structure of heme oxygenase from the Gram-negative pathogen *Neisseria meningitidis* and a comparison with mammalian heme oxygenase-1. *Biochemistry* **2001**, *40*, 11552–11558.

33 Friedman, J.; Lad, L.; Li, H.; Wilks, A.; Poulos, T. L. Structural basis for novel δ-regioselective heme oxygenation in the opportunistic pathogen *Pseudomonas aeruginosa*. *Biochemistry* **2004**, *43*, 5239–5245.

34 Matsui, T.; Furukawa, M.; Unno, M.; Tomita, T.; Ikeda-Saito, M. Roles of distal Asp in heme oxygenase from *Corynebacterium diphtheriae*, HmuO: A water-driven oxygen activation mechanism. *J. Biol. Chem.* **2005**, *280*, 2981–2989.

35 Li, Y.; Syvitski, R. T.; Chu, G. C.; Ikeda-Saito, M.; La Mar, G. N. Solution ^{1}H NMR investigation of the active site molecular and electronic structures of substrate-bound, cyanide-inhibited HmuO, a bacterial heme oxygenase from *Corynebacterium diphtheriae*. *J. Biol. Chem.* **2003**, *278*, 6651–6663.

36 Lad, L.; Wang, J.; Li, H.; Friedman, J.; Bhaskar, B.; Ortiz de Montellano, P. R.; Poulos, T. L. Crystal structures of the ferric, ferrous, and ferrous-NO forms of the Asp140Ala mutant of human heme oxygenase-1: Catalytic implications. *J. Mol. Biol.* **2003**, *330*, 527–538.

37 Davydov, R.; Kofman, V.; Fujii, H.; Yoshida, T.; Ikeda-Saito, M.; Hoffman, B. M. Catalytic mechanism of heme oxygenase through EPR and ENDOR of cryoreduced oxy-heme oxygenase and its Asp 140 mutants. *J. Am. Chem. Soc.* **2002**, *124*, 1798–1808.

38 Denisov, I. G.; Ikeda-Saito, M.; Yoshida, T.; Sligar, S. G. Cryogenic absorption spectra of hydroperoxo-ferric heme oxygenase, the active intermediate of enzymatic heme oxygenation. *FEBS Lett.* **2002**, *532*, 203–206.

39 Garcia-Serres, R.; Davydov, R. M.; Matsui, T.; Ikeda-Saito, M.; Hoffman, B. M.; Huynh, B. H. Distinct reaction pathways followed upon reduction of oxy-heme oxygenase and oxy-myoglobin as characterized by Mössbauer spectroscopy. *J. Am. Chem. Soc.* **2007**, *129*, 1402–1412.

40 Davydov, R.; Chemerisov, S.; Werst, D. E.; Rajh, T.; Matsui, T.; Ikeda-Saito, M.; Hoffman, B. M. Proton transfer at helium temperatures during dioxygen activation by heme monooxygenases. *J. Am. Chem. Soc.* **2004**, *126*, 15960–15961.

41 Davydov, R.; Makris, T. M.; Kofman, V.; Werst, D. E.; Sligar, S. G.; Hoffman, B. M. Hydroxylation of camphor by reduced oxy-cytochrome P450cam: Mechanistic implications of EPR and ENDOR studies of catalytic intermediates in native and mutant enzymes. *J. Am. Chem. Soc.* **2001**, *123*, 1403–1415.

42 Davydov, R.; Matsui, T.; Fujii, H.; Ikeda-Saito, M.; Hoffman, B. M. Kinetic isotope effects on the rate-limiting step of heme oxygenase catalysis indicate concerted proton transfer/heme hydroxylation. *J. Am. Chem. Soc.* **2003**, *125*, 16208–16209.

43 Kumar, D.; de Visser, S. P.; Shaik, S. Theory favors a stepwise mechanism of porphyrin degradation by a ferric hydroperoxide model of the active species of heme oxygenase. *J. Am. Chem. Soc.* **2005**, *127*, 8204–8213.

44 Kamachi, T.; Yoshizawa, K. Water-assisted oxo mechanism for heme metabolism. *J. Am. Chem. Soc.* **2005**, *127*, 10686–10692.

45 Chen, H.; Moreau, Y.; Derat, E.; Shaik, S. Quantum mechanical/molecular mechanical study of mechanisms of heme degradation by the enzyme heme oxygenase: The strategic function of the water cluster. *J. Am. Chem. Soc.* **2008**, *130*, 1953–1965.

46 Mansfield Matera, K.; Takahashi, S.; Fujii, H.; Zhou, H.; Ishikawa, K.; Yoshimura, T.; Rousseau, D. L.; Yoshida, T.; Ikeda-Saito, M. Oxygen and one reducing equivalent are both required for the conversion of α-hydroxyhemin to verdoheme in heme oxygenase. *J. Biol. Chem.* **1996**, *271*, 6618–6624.

47 Liu, Y.; Moenne-Loccoz, P.; Loehr, T. M.; Ortiz de Montellano, P. R. Heme oxygenase-1, intermediates in verdoheme formation and the requirement for reduction equivalents. *J. Biol. Chem.* **1997**, *272*, 6909–6917.

48 Zhang, X.; Fujii, H.; Mansfield Matera, K.; Migita, C. T.; Sun, D.; Sato, M.; Ikeda-Saito, M.; Yoshida, T. Stereoselectivity of each of the three steps of the heme oxygenase reaction: hemin to *meso*-hydroxyhemin, *meso*-hydroxyhemin to verdoheme, and verdoheme to biliverdin. *Biochemistry* **2003**, *42*, 7418–7426.

49 Morishima, I.; Fujii, H.; Shiro, Y.; Sano, S. Studies on the iron(II) *meso*-oxyporphyrin π-neutral radical as a reaction intermediate in heme catabolism. *Inorg. Chem.* **1995**, *34*, 1528–1535.

50 Takahashi, S.; Mansfield Matera, K.; Fujii, H.; Zhou, H.; Ishikawa, K.; Yoshida, T.; Ikeda-Saito, M.; Rousseau, D. L. Resonance Raman spectroscopic characterization of α-hydroxyheme and verdoheme complexes of heme oxygenase. *Biochemistry* **1997**, *36*, 1402–1410.

51 Migita, C. T.; Fujii, H.; Mansfield Matera, K.; Takahashi, S.; Zhou, H.; Yoshida, T. Molecular oxygen oxidizes the porphyrin ring of the ferric α- hydroxyheme in heme oxygenase in the absence of reducing equivalent. *Biochim. Biophys. Acta* **1999**, *1432*, 203–213.

52 Sakamoto, H.; Omata, Y.; Hayashi, S.; Harada, S.; Palmer, G.; Noguchi, M. The reactivity of α-hydroxyhaem and verdohaem bound to haem oxygenase-1 to dioxygen and sodium dithionite. *Eur. J. Biochem.* **2002**, *269*, 5231–5239.

53 Sakamoto, H.; Takahashi, K.; Higashimoto, Y.; Harada, S.; Palmer, G.; Noguchi, M. A kinetic study of the mechanism of conversion of α-hydroxyheme to verdoheme while bound to heme oxygenase. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 578–583.

54 Liu, Y.; Ortiz de Montellano, P. R. Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1. *J. Biol. Chem.* **2000**, *275*, 5297–5307.

55 Fuhrhop, J. H.; Kruger, P. 1- or 19-Methoxy-, 1- or 19-amino- and 1- or 19-thio-deoxybiliverdins. *Liebigs Ann. Chem.* **1977**, 360–370.

56 Saito, S.; Itano, H. A. Verdohemochrome IXα: Preparation and oxidoreductive cleavage to biliverdin IXα. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1393–1397.

57 Sano, S.; Sano, T.; Morishima, I.; Shiro, Y.; Maeda, Y. On the mechanism of the chemical and enzymic oxygenations of α-oxyprotohemin IX to Fe.biliverdin IXα. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 531–535.

58 Latos-Grazynski, L.; Wojaczynski, J.; Koerner, R.; Johnson, J. J.; Balch, A. L. Verdoheme reactivity. Remarkable paramagnetically shifted ^{1}H NMR spectra of intermediates from the addition of hydroxide or methoxide with Fe(II) and Fe(III) verdohemes. *Inorg. Chem.* **2001**, *40*, 4971–4977.

59 Koerner, R.; Latos-Grazynski, L.; Balch, A. L. Models for verdoheme hydrolysis. Paramagnetic products from the ring opening of verdohemes, 5-oxaporphyrin complexes of iron(II), with methoxide ion. *J. Am. Chem. Soc.* **1998**, *120*, 9246–9255.

60 Nguyen, K. T.; Rath, S. P.; Latos-Grazynski, L.; Olmstead, M. M.; Balch, A. L. Formation of a highly oxidized iron biliverdin complex upon treatment of a five-coordinate verdoheme with dioxygen. *J. Am. Chem. Soc.* **2004**, *126*, 6210–6211.

61 Docherty, J. C.; Schacter, B. A.; Firneisz, G. D.; Brown, S. B. Mechanism of action of heme oxygenase. A study of heme degradation to bile pigment by ^{18}O labeling. *J. Biol. Chem.* **1984**, *259*, 13066–13069.

62 Lad, L.; Ortiz de Montellano, P. R.; Poulos, T. L. Crystal structures of ferrous and ferrous-NO forms of verdoheme in a complex with human heme oxygenase-1: Catalytic implications for heme cleavage. *J. Inorg. Biochem.* **2004**, *98*, 1686–1695.

63 Damaso, C. O.; Bunce, R. A.; Barybin, M. V.; Wilks, A.; Rivera, M. The ferrous verdoheme-heme oxygenase complex is six-coordinate and low-spin. *J. Am. Chem. Soc.* **2005**, *127*, 17582–17583.

64 Matsui, T.; Unno, M.; Ikeda-Saito, M. Unpublished results.

65 Baranano, D. E.; Rao, M.; Ferris, C. D.; Snyder, S. H. Biliverdin reductase: A major physiologic cytoprotectant. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 16093–16098.

66 Migita, C. T.; Mansfield Matera, K.; Ikeda-Saito, M.; Olson, J. S.; Fujii, H.; Yoshimura, T.; Zhou, H.; Yoshida, T. The oxygen and carbon monoxide reactions of heme oxygenase. *J. Biol. Chem.* **1998**, *273*, 945–949.

67 Matsui, T.; Ikeda-Saito, M. Unpublished results.