Heme Oxygenase Mechanism: Evidence for an Electrophilic, Ferric Peroxide Species

PAUL R. ORTIZ DE MONTELLANO†

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446

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The action of heme oxygenase is graphically (but perhaps unsuspectingly) familiar to anyone who has observed the gradual discoloration of a bruise from "black and blue" to green and then yellow. The initial dark color is due to heme from the hemoglobin released into the damaged tissue by ruptured blood vessels. Oxidation of the heme to biliverdin by heme oxygenase provides the green tint, and subsequent reduction of biliverdin to bilirubin the yellow color (Figure 1). Heme and biliverdin are highly lipophilic and relatively difficult to eliminate, but bilirubin is readily excreted after conjugation with glucuronic acid.

Heme oxygenase is highly unusual in that it uses heme as both its substrate and prosthetic group. It is also mechanistically distinct from the other classes of hemoproteins, including the cytochromes P450, peroxidases, catalases, nitric oxide synthases, prostaglandin synthases, thromboxane synthases, and prostacyclin synthases. Nevertheless, the reactions catalyzed by heme oxygenase are part of the same heme-dependent reaction manifold that underlies the catalytic action of all hemoproteins, and elucidation of its mechanism can be expected to shed considerable light on the function of all hemoproteins.

Heme Oxygenase

Heme oxygenase is a membrane-bound enzyme that catalyzes the oxidation of heme to biliverdin, CO, and free iron in a reaction that requires O_2, NADPH, and cytochrome P450 reductase.[1−3] The enzyme regiospecifically oxidizes heme at the α-*meso* position (Figure 2).[4,5] The existence of two heme oxygenase isoforms, denoted HO-1 and HO-2, is now well-established,[6−8] and a third form has recently been reported.[9] The molecular masses of human HO-1 and HO-2 are 33 and 36 kDa, and comparison of their protein sequences shows that the two human isoforms are 42% identical.[10] Regions of much higher sequence conservation are found, however, notably in the regions that correspond to HO-1 residues 11−40 and 126−149 (Figure 3).[10,11] In both proteins, the fifth ligand to the heme is a histidine and the distal ligand is a water molecule.[12−14] Recent heme oxygenase studies have been greatly facilitated by expression of active, soluble forms of the protein lacking the membrane binding domain.[15−17]

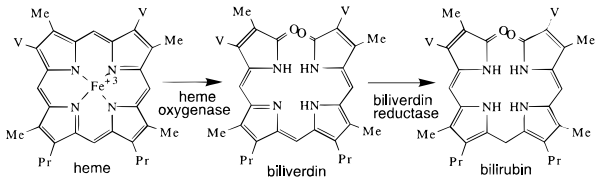

FIGURE 1. Metabolism of heme via biliverdin to bilirubin. The substituent abbreviations are as follows: V, −CH=CH$_2$; Pr, −CH$_2$-CH$_2$CO$_2$H.

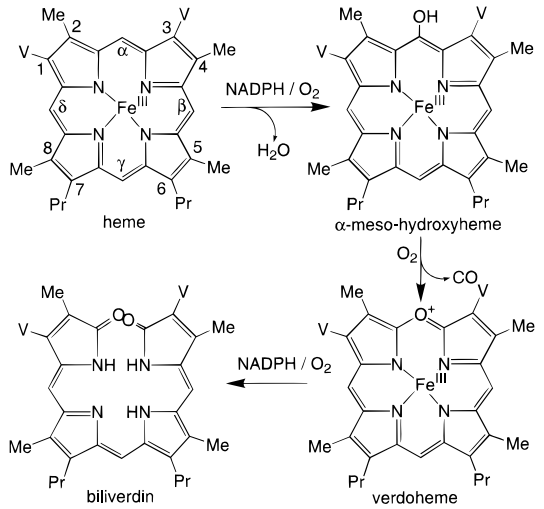

FIGURE 2. Steps in the oxidation of heme to biliverdin catalyzed by heme oxygenase. The substituted carbons and *meso* positions of the porphyrin are labeled.

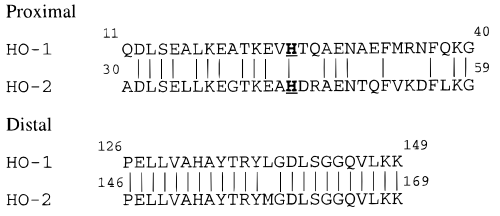

FIGURE 3. Two regions of human HO-1 and HO-2 with highest sequence identity. The proximal iron ligand is underlined.

First Phase: Heme α-*meso*-Hydroxylation

Formation of the Activated Oxygen Species. The first two steps in catalytic turnover of the heme−heme oxygenase complex are reduction of the heme to the ferrous state by NADPH−cytochrome P450 reductase and binding of oxygen to give the $Fe^{II}-O_2$ complex with an absorption maximum at 410 nm.[3] In the presence of CO, the Fe^{II}−CO complex (λ_{max} = 418 nm) is formed.[18,19] The spectroscopic resemblance of heme oxygenase to myoglobin suggests that the ferrous dioxygen complex may resemble that in oxymyoglobin, but its properties have not been

Paul R. Ortiz de Montellano received his education at MIT and Harvard and is currently a Professor of Chemistry, Pharmaceutical Chemistry, and Pharmacology at the University of California in San Francisco. His core research interests focus on the structure, mechanism, inhibition, and function of hemoproteins. His principal collaborators on Saturday afternoons continue to be Boito, Gounod, Puccini, and Verdi.

† Fax: (415) 502-4728. E-mail: ortiz@cgl.ucsf.edu.

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extensively studied. However, an abnormal oxygen isotope shift in the resonance Raman spectrum of the HO-1 ferrous−O_2 complex has led to the proposal that the iron−oxygen−oxygen bond is bent, bringing the terminal oxygen of the complex closer to the heme periphery.[20] The authors propose that tilting of the oxygen ligand is due to steric interactions with active site residues. Independent support for an interaction of distal ligands with the protein is provided by ^{1}H NMR studies which suggest that the cyano ligand is tilted away from a perpendicular to the heme plane by interactions with the protein.[21]

Substitution of H_2O_2 for O_2 and Reducing Equivalents. The Fe^{II}−O_2 complex must be reduced further for heme oxidation to occur.[3] Since two-electron reduction of O_2 produces a species equivalent in oxidation state to H_2O_2, we examined the possibility that H_2O_2 might substitute in heme oxygenase catalysis for O_2 and two reducing equivalents. Indeed, incubation of the HO-1−heme complex with 1 equiv of H_2O_2 results in rapid formation of the HO-1−verdoheme complex.[15] Addition of cytochrome P450 reductase and NADPH to this verdoheme complex produces the expected biliverdin IXα.[15] Later studies confirmed that H_2O_2 similarly supports the HO-2-catalyzed conversion of heme to verdoheme.[22] H_2O_2 thus supports the oxidation of the heme to verdoheme but not the conversion of verdoheme to biliverdin.

Reaction of the HO-1−heme complex with 1 equiv of H_2O_2 under anaerobic conditions produces a distinct intermediate that is stable in the absence of oxygen.[23] This intermediate is identical by absorption and resonance Raman spectroscopy to the complex formed anaerobically between synthetic α-*meso*-hydroxyheme and HO-1.[24] The competence of α-*meso*-hydroxyheme as a precursor of verdoheme definitively identifies it as a key intermediate in heme oxygenase-mediated heme catabolism.[23−26] The conversion of α-*meso*-hydroxyheme to biliverdin in model systems supports this conclusion.[27−30]

Reaction with a Peracid. The nature of the species that hydroxylates the heme is further defined by studies with *m*-chloroperbenzoic acid.[15] Reaction with this peracid produces an intermediate with the spectroscopic properties of a ferryl (Fe^{IV}=O) species.[31] The ferryl intermediate accounts for only one of the two oxidizing equivalents of H_2O_2. The second oxidizing equivalent is used to oxidize the protein because a transient EPR detectable radical is simultaneously formed. The ferryl species reverts to the ferric state when it is reduced with ascorbic acid or phenol.[15] If guaiacol (*o*-methoxyphenol) is used, the telltale color change due to peroxidation of this substrate is observed. The key finding, however, is that the ferryl intermediate is not converted to verdoheme. Furthermore, addition of 1 equiv of H_2O_2 to the enzyme after reaction with 1 equiv of *m*-chloroperbenzoic acid also does not produce verdoheme, which indicates that the ferryl intermediate protects the heme from the reaction with H_2O_2 that produces verdoheme. Clearly, the ferryl species is not an intermediate in the normal heme oxygenase reaction!

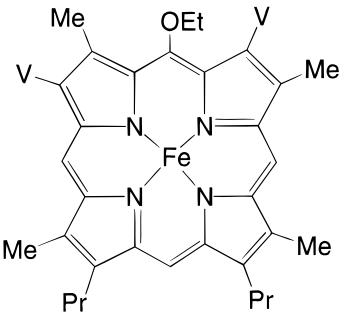

FIGURE 4. Product formed in the HO-1-catalyzed oxidation of heme supported by ethyl hydroperoxide.

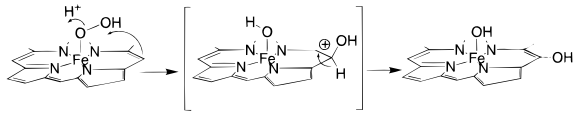

FIGURE 5. Electrophilic oxidation of the porphyrin by the HO-1 ferric peroxide.

Reaction with Alkyl Hydroperoxides. The reactions of the HO-1−heme complex with *tert*-butyl hydroperoxide, cumene hydroperoxide, and ethyl hydroperoxide have also been examined.[15,32] Reaction with the first two peroxides follows a course similar to the reaction with *m*-chloroperbenzoic acid in that a ferryl species is formed, although the reaction is accompanied by some degradation of the heme to non-biliverdin products. The reaction with ethyl hydroperoxide also gives rise to a ferryl species, but reduction of this intermediate with ascorbic acid to prevent oxidative degradation of the prosthetic group, followed by isolation, HPLC purification, and complete spectroscopic characterization, shows that the heme is partially converted to iron α-*meso*-ethoxyheme (Figure 4).[32]

The formation of α-*meso*-ethoxyheme mimics the formation of α-*meso*-hydroxyheme, the first step of the normal reaction. The α-*meso*-ethoxy product is unsuitable for the downstream reactions of the catalytic process because, unlike α-*meso*-hydroxyheme, it cannot be deprotonated (see below). The ethyl hydroperoxide reaction specifically rules out a mechanism in which the terminal oxygen of a ferric peroxo anion (Fe^{III}−OO^-) adds as a nucleophile to the porphyrin because the terminal oxygen in the ethyl hydroperoxide complex is blocked. This conclusion, in conjunction with the finding that the ferryl species is also not involved, narrows the mechanism to electrophilic addition of the ferric peroxide (Fe^{III}−OOH) terminal oxygen to the porphyrin (Figure 5).

Oxidation of α-*meso*-Methyl Hemes. Given that α-*meso*-hydroxylation is the first committed step in the conversion of heme to biliverdin, it should be possible to block, or divert, the reaction by placing substituents at the α-*meso* position. For these studies we first used α-*meso*-methylmesoheme and a closely related symmetric analogue (Figure 6). Surprisingly, the α-*meso*-methyl derivatives were oxidized without the formation of CO to the same biliverdin products as those obtained from the *meso*-unsubstituted hemes![33,34] The failure to form CO rules out oxidative removal of the methyl group followed

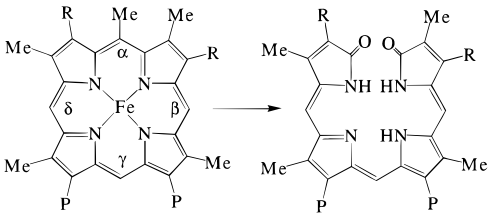

FIGURE 6. Oxidation of α-*meso*-methylhemes to biliverdins: R is a methyl or an ethyl.

by normal α-*meso*-hydroxylation. Thus, the mechanism responsible for α-*meso*-hydroxylation and CO formation can be subverted by α-*meso*-substitution to one which still produces the biliverdin but not CO. Extensive efforts to isolate and identify the α-*meso*-carbon containing product formed in this reaction, including studies with a ^{13}C-labeled *meso*-methyl group, have excluded acetaldehyde and acetic acid but have not successfully identified the fragment.[35] It is possible that the fragment eliminated in the oxidation of α-*meso*-methyl-substituted hemes is a reactive species that binds covalently to the protein.

Regiochemistry of Heme Oxidation. The finding that α-*meso*-methylmesoheme is oxidized to mesobiliverdin IXα led us to examine the oxidation of the other three *meso*-methylmesoheme regioisomers.[34] The required *β*-, *γ*-, and *δ*-*meso*-methylmesohemes were synthesized, and their regiochemistry was assigned by ^1H NMR.[36] Given the high specificity of HO-1 for oxidation of the α-*meso*-carbon, we expected that the enzyme would oxidize the *β*-, *γ*-, and *δ*-*meso*-methylmesohemes at the α-position to give the corresponding methyl-substituted mesobiliverdin IXα isomers. Contrary to expectation, *γ*-*meso*-methylmesoheme, as shown by mass spectrometric analysis of the product, is oxidized exclusively at the *γ*-*meso* position to the unsubstituted mesobiliverdin IX*γ*. The *δ*-*meso*-methyl isomer is oxidized at both the *δ*-*meso* position, yielding mesobiliverdin IX*δ*, and at an unsubstituted (presumably α) position to give a methyl-substituted mesobiliverdin IX. Finally, the *β*-*meso*-methyl derivative is a very poor substrate even though its α-*meso* position is not blocked.

Modeling studies based on the crystal structures of myoglobin and hemoglobin have led to the proposal that the oxidation regiochemistry is determined by steric orientation of the iron-bound dioxygen ligand.[37,38] This steric "steering" determines the extent to which the terminal oxygen is located above each of the *meso* positions. Myoglobin and hemoglobin were used as models for the heme oxygenase reaction because coupled oxidation of their heme groups produces low yields of biliverdin isomers.[5,39] In sperm whale myoglobin, coupled oxidation of the heme occurs exclusively at the α-*meso* position, but in human hemoglobin the oxidation occurs at both the α- and *β*-*meso* positions.[38,40] Free heme in solution is oxidized at all four *meso* positions.[41] However, the finding that the regiochemistry of heme oxidation is drastically altered by *meso*-methyl substitution is incompatible with a dominant role for steric steering in governing the oxidation regiospecificity. A simple steric mechanism cannot explain complete inversion of the oxidation regiochemistry to favor the *γ*- over the α-position upon introduction of a methyl group at the *γ*-position. A steric mechanism is also incompatible with oxidation of the *δ*-*meso*--position when a substituent is placed at that position, although steric effects may contribute to the poor oxidation of the *β*-*meso*-methyl isomer. Thus, steric steering is not viable as a mechanism for controlling the HO-1 heme oxidation regiochemistry (but might still be valid for the coupled oxidation of myoglobin and hemoglobin).

Electronic Effects on the Reaction Regiochemistry. If steric effects do not control the oxidation regiochemistry, the reaction must be controlled by electronic effects. This inference is supported by the changes in heme oxidation regiochemistry due to methyl substitution. If heme oxidation occurs by electrophilic addition to a *meso* carbon, the reaction should be facilitated by electron donating substituents at that carbon. Thus, as observed, a methyl group on the α-*meso* carbon reinforces oxidation of that position. A methyl at the *γ*-*meso* carbon also increases its reactivity toward an electrophilic oxygen and, if this preference overrides the mechanism that normally channels the reaction to the α-position, could result in exclusive *γ*-*meso* carbon oxidation. Methyl substitution at the *δ*-*meso* position would similarly increase its reactivity toward oxidation, as observed, but the fact that oxidation occurs at both the *δ*- and α-positions indicates that the *δ*-*meso* methyl is not as effective as the *γ*-*meso* methyl in overriding the factors that normally favor α-*meso* oxidation.

Although the above picture is internally consistent, the possibility exists that a *meso* substituent perturbs the physical structure of the heme group, such as by causing a puckering or ruffling of the porphyrin,[42,43] and thus introduces a bias for oxidation of the substituted site. Furthermore, the argument for electronic control of the regiochemistry would be considerably strengthened if substitution of a *meso* electron withdrawing rather than donating group were to alter the oxidation regiochemistry in a different and consistent manner. The four *meso*-formyl mesoheme regioisomers were therefore examined as HO-1 substrates.[44] The key finding of these studies is that the formyl-substituted carbon is never the site of the oxidation reaction. Thus, each of the four *meso*-formyl-mesoheme isomers is oxidized with essentially quantitative formation of CO to a biliverdin that retains the *meso*-formyl group. The difference between the site of oxidation in the methyl- versus formyl-substituted series of hemes provides persuasive evidence that electronic effects control the regiochemistry of heme oxidation by HO-1 and confirms that the reaction involves electrophilic addition to the porphyrin ring.

How is the α-directing electronic effect imposed in normal HO-1 catalysis? If the α-*meso* regioselectivity of hydroxylation is not inherent to the heme group, as indicated by formation of all four isomers by nonenzymatic coupled oxidation,[41] and the active site does not sterically channel the process, how is the normal heme

oxidation regiochemistry controlled? The data on the *meso*-methyl and *meso*-formyl hemes suggest that the reaction is controlled by selective enhancement of the electron density at the α-*meso* position. NMR studies of the rat HO-1−heme complex indicate that there are large differences in electron density at the two β(peripheral)-carbons in a given pyrrole ring rather than the more conventional pattern of similar electron densities at the β-carbons of a single pyrrole ring but large differences between the β-carbons of adjacent pyrrole rings.[21] Thus, the normal pattern is for similar electron densities at positions 1 and 2 of the heme but not at positions 2 and 3 (Figure 2), in contrast to the finding with the heme in HO-1 that positions 1 and 2 differ considerably in electron density.[21] Patterns of electron density similar to that seen with HO-1 have been observed with iron porphyrins bearing electron donating or withdrawing *meso* substituents.[45] The unusual electron distribution in the HO-1-heme complex thus suggests that an anionic group, perhaps a carboxylate anion, located near the α-*meso* position induces an electronic asymmetry in the heme that favors α-*meso* carbon oxidation.

The symmetry of the heme group suggests that the α- and γ-*meso* carbons are related in that elevation of the electron density at one of these carbons results in some elevation in electron density at the other. The electron densities at the β- and δ-*meso* carbons may be similarly related to each other. This relationship between the *meso* carbons on opposite sides of the heme can be used to explain the observation that substitution of a methyl at the γ-*meso* carbon results in exclusive γ-*meso* oxidation, whereas substitution of a methyl at the δ-*meso* carbon results in oxidation at both the δ- and α-*meso* positions. In the case of the γ-*meso* substituent, the methyl reinforces the electron density at the γ-*meso* carbon caused by the factors that normally enhance the electron density at the α-*meso* and, to a lesser extent, γ-*meso* carbon. In the case of the δ-*meso* carbon, the enhancement of the electron density at the δ-*meso* carbon is counterbalanced by the normal enhancement of the electron density at the α- and γ-*meso* positions, resulting in oxidation at multiple sites.[34]

Second Phase: α-*meso*-Hydroxyheme to Verdoheme

The conversion of α-*meso*-hydroxyheme to verdoheme is an oxygen-dependent process because the HO-1−α-*meso*-hydroxyheme complex, whether obtained by reconstitution of the apoenzyme with synthetic α-*meso*-hydroxyheme or by oxidation of the heme complex with H_2O_2, is stable under anaerobic conditions.[23,24] EPR analysis of the anaerobic HO-1−α-*meso*-hydroxyheme complex shows the presence of a rhombic signal at $g = 6.07$ and 5.71 attributable to the ferric iron and a signal at $g = 2.008$ due to an organic radical. Addition of CO to the anaerobic system suppresses the $g = 5-6$ signals and enhances the signal at $g = 2.008$ (Figure 7).[23] A ferric heme species without an organic radical is thus in equilibrium with a

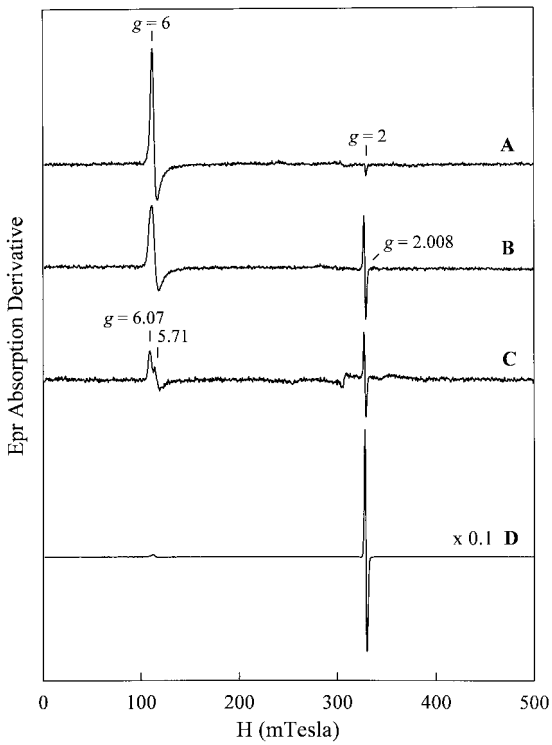

FIGURE 7. EPR of the radical in the α-*meso*-hydroxyheme−HO-1 complex: (A) the ferric heme−HO-1 complex, (B) the α-*meso*-hydroxyheme−HO-1 complex formed anaerobically with 1 equiv of H_2O_2; (C) the α-*meso*-hydroxyheme complex after subtraction of the spectrum of unreacted heme from trace B; and (D) the spectrum in B after the addition of CO to form the Fe^{II}−CO complex. The spectra are from Liu et al.[23]

ferrous heme species with an organic radical (see Figure 8), so that coordination of CO to the ferrous iron shifts the equilibrium toward the radical containing species. The formation of a CO complex is supported by a CO-mediated shift in the Soret maximum of the HO-1−α-*meso*-hydroxyheme complex from 405 to 408 nm. Independent support for this equilibrium is provided by resonance Raman evidence that deprotonation of the ferric α-*meso*-hydroxyheme produces an oxophlorin- rather than porphyrin-like structure.[24] These results argue for the equilibrium indicated in Figure 8. Matera et al. reported a radical signal at $g = 2.004$ when oxygen was added to a solution of the HO-1−α-*meso*-hydroxyheme under an anaerobic atmosphere of nitrogen and CO and attributed it to a ferrous heme with an oxygen bound to the porphyrin radical.[24] These experimental results establish a mechanism in which a deprotonated, free radical form of α-*meso*-hydroxyheme binds molecular oxygen to give a peroxy radical species (Figure 8).

Exposure to oxygen of an anaerobic solution of the HO-1−α-*meso*-hydroxyheme complex produced from the heme complex with 1 equiv of H_2O_2 results in immediate conversion of the substrate to ferric verdoheme.[23] Resonance Raman and absorption spectroscopies indicate that this ferric verdoheme is reduced to ferrous verdoheme by dithionite or NADPH/cytochrome P450 reductase. Thus, the only requirement for the HO-1-catalyzed conversion of ferric α-*meso*-hydroxyheme to ferric verdoheme is one

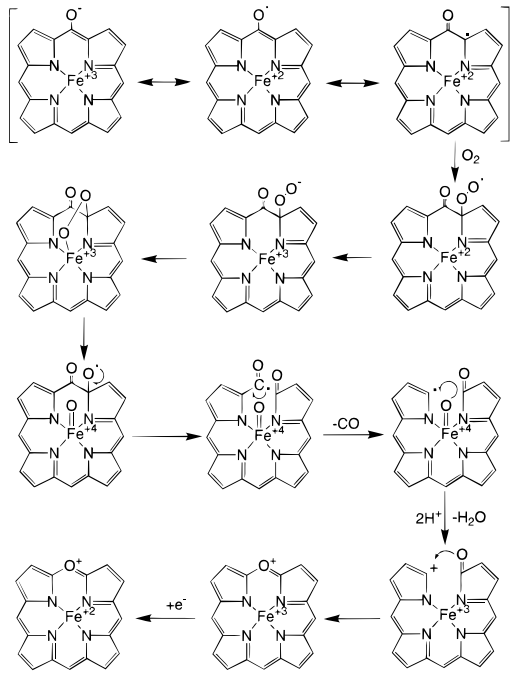

FIGURE 8. Proposed mechanism for the HO-1-catalyzed conversion of ferric α-*meso*-hydroxyheme (shown deprotonated) to ferrous verdoheme.

molecule of O_2. These results are consistent with the report that conversion of myoglobin-bound α-*meso*-hydroxyheme to verdoheme does not require exogenous reducing equivalents,[28] although the myoglobin- and HO-1-catalyzed reactions may differ. Matera et al. have argued that the HO-1-catalyzed conversion of α-*meso*-hydroxyheme to biliverdin requires one electron in addition to one molecule of oxygen.[24] However, they monitored the formation of verdoheme not as the ferric product but as the ferrous−CO complex. There is complete agreement that formation of ferrous verdoheme requires one electron—the question is whether the electron must be introduced prior to the formation of ferric verdoheme. The formation of ferric verdoheme from the α-*meso*-hydroxyheme−HO-1 complex in the complete absence of reducing equivalents clearly establishes that an electron is not required for the reaction. However, in the presence of both cytochrome P450 reductase and NADPH, two possible reaction pathways are possible. Ferric verdoheme may be formed and then reduced to the ferrous state, or an electron may be introduced prior to the extrusion of CO, resulting directly in the formation of ferrous verdoheme. The physiological ratio of these two pathways will depend on the relative rates of the various steps in the two pathways.

The minimal mechanism for the conversion of α-*meso*-hydroxyheme to verdoheme requires deprotonation of α-*meso*-hydroxyheme followed by oxygen binding to give a ferrous peroxy radical (Figure 8). Interaction of the peroxy radical with the ferrous iron, or an analogous interaction after internal transfer of an electron to give the ferric hydroperoxide, produces a ferryl alkoxy radical. The alkoxy radical provides a trigger for elimination of the α-*meso*-carbon as CO. Elimination of the CO generates

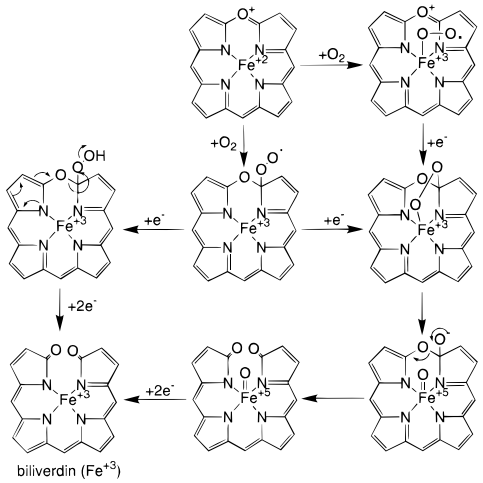

FIGURE 9. Hypothetical mechanism(s) for the conversion of verdoheme to biliverdin. Two alternatives are shown that differ in the sequence in which electrons are introduced and the initial site of oxygen binding.

a carbon radical that can be oxidized internally to the cation by electron transfer to the ferryl species. Intramolecular trapping of the cation then yields ferric verdoheme. No direct evidence exists for the individual steps after binding of oxygen to the porphyrin radical, but the mechanism is unlikely to differ much except perhaps for changes in the order of the steps.

Third Phase: Conversion of Verdoheme to Biliverdin

Little information is available on the conversion of ferrous verdoheme to biliverdin, although the origin of the biliverdin oxygen atoms provides basic information on the reaction mechanism. Under an atmosphere of $^{16}O-^{16}O$ and $^{18}O-^{18}O$, some of the biliverdin produced by HO-1 incorporates one atom each of ^{16}O and ^{18}O.[46] The two oxygen atoms incorporated into biliverdin thus derive from different molecules of oxygen.[47] This rules out a hydrolytic mechanism for the conversion of verdoheme to biliverdin, in accord with the finding that the reaction requires reducing equivalents and O_2.[15,48,49]

A schematic mechanism for this phase of the process includes reduction of verdoheme to the ferrous state, binding of dioxygen, and a second one-electron reduction to give a peroxy intermediate (Figure 9). A hypothetical sequence of steps involving dioxygen bond cleavage to give either an alkoxy anion or a radical, followed by ring cleavage and a two-electron reduction, produces ferric biliverdin. The principal uncertainty in the mechanism is the stage at which the two electrons required to produce biliverdin are introduced. The formation of an $Fe^{V}=O$ species is shown, but if such an intermediate is involved, it is likely to be $Fe^{IV}=O$ in conjunction with a porphyrin radical of some kind. Other variations of this mechanism are possible (Figure 9). In the last step of the reaction, the iron chelated by biliverdin appears to be reduced to the ferrous state. Evidence for this is provided by the finding that coupled oxidation of the heme−heme oxy-

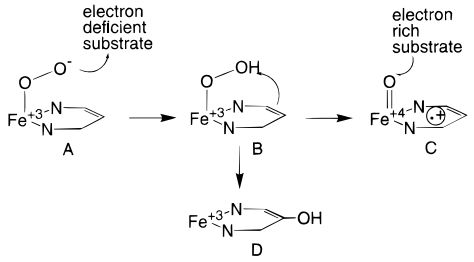

FIGURE 10. Manifold of species produced in the reaction of a heme group with oxygen and two reducing equivalents. The rate of conversion of A to B limits the lifetime (and therefore reactivity) of the ferric peroxo anion. The rate of formation of the ferryl species C via the ferric peroxide B competes with intramolecular hydroxylation to give D. The ferric peroxide B can also be formed directly with H_2O_2.

genase complex produces the ferric biliverdin complex, which is not suitable for reduction by biliverdin reductase, whereas the same reaction supported by NADPH−cytochrome P450 reductase produces biliverdin that is readily accepted as a substrate by biliverdin reductase.[18]

Implications of the Electrophilic Reaction of a Heme Ferric Peroxide Complex

Studies of the catalytic mechanisms of hemoproteins that employ either oxygen or H_2O_2 as a cosubstrate have provided evidence for two types of oxygen reactivity. The most common oxidative intermediate is a ferryl species (Figure 10, structure C) coupled with either a porphyrin radical cation (e.g., horseradish peroxidase) or a protein radical (e.g., cytochrome *c* peroxidase).[50,51] In peroxidases, stepwise electron transfer to this species reduces first the porphyrin or protein radical, and subsequently the ferryl species. A similar but less well-characterized ferryl intermediate is thought to be the primary oxidizing species of cytochrome P450.[52] However, cytochrome P450 enzymes transfer the ferryl oxygen to their substrates rather than simply serving as electron acceptors.

A less common reactive species is the ferric peroxo anion expected from two-electron reduction of O_2 at the iron atom (Figure 10, structure A). Protonation of this intermediate yields a ferric peroxide (Figure 10, structure B), but the peroxo anion can directly react as a nucleophile with highly electrophilic substrates. Thus, addition of the peroxo anion to an aldehyde followed by homolytic scission of the dioxygen bond is currently accepted as the mechanism for the carbon−carbon bond cleavages catalyzed by cytochrome P450 enzymes such as aromatase, lanosterol 14-demethylase, and sterol 17-lyase.[52] A similar nucleophilic addition of the ferric peroxo anion to a carbon−nitrogen double bond has been invoked in the mechanism of the nitric oxide synthases.[53]

The hydroxylation of heme by a ferric peroxide catalyzed by heme oxygenase is the first example of a new hemoprotein reactivity. This reactivity resembles that associated with flavin or pteridine hydroperoxide intermediates, in which the terminal oxygen of what is essentially an alkylhydroperoxide (ROOH) reacts electrophilically with heteroatoms or aromatic rings. As found in heme oxygenase, the ferric peroxide intermediate can similarly react with the porphyrin framework of the heme (Figure 10, structure B). The rapid reaction of the ferric peroxide to give α-*meso*-hydroxyheme highlights a fundamental aspect of hemoprotein mechanisms that involve ferryl species: i.e., the ferric peroxide precursor must decay to the ferryl species fast enough to avoid self-oxidation of the heme group. This inference is strengthened by the fact that hydroxylation of the heme group, as evidenced by biliverdin formation, has been observed under appropriate conditions with a diversity of hemoproteins.[54,55] The hydroxylation reaction thus appears to be inherent to the heme ferric peroxide complex rather than to a specific protein environment. Heme hydroxylation functions as a self-destructive "clock" reaction for heme ferric peroxides against which other catalytic processes must compete. This self-destructive ("apoptotic") process built into the ferric peroxide intermediate suggests that it is unlikely to function effectively as an electrophilic oxidizing agent for any but highly reactive exogenous substrates because they are unlikely to compete effectively with both ferryl formation and intramolecular heme hydroxylation.

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