Autocatalytic Radical Reactions in Physiological Prosthetic Heme Modification

Christophe Colas and Paul R. Ortiz de Montellano*

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

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Christophe Colas received his B.S. in Molecular and Cell Biology in 1990 at the Ecole Normale Supérieure de Lyon, France. The latter included predoctoral stays at the Weizmann Institute of Sciences in Rehovot, Israel, with Prof. R. Arnon and M. Wilcheck, as well as at the CEA of Saclay, France, with Prof. C. Hirth. He earned a position as a high school and college chemistry teacher after succeeding at the Agrégation de Chimie in 1991. Then, he received his Ph.D. in Bioorganic Chemistry in 1998 with Prof. M. Goeldner from the Université Louis Pasteur in Strasbourg, France, where he studied photosensitive suicide inhibitors of butyrylcholinesterase. He is now a postdoctoral fellow with Prof. P. Ortiz de Montellano at the University of California in San Francisco, where his work focuses on investigating the mechanism of covalent attachment of the heme to the apoenzyme in peroxidases.

Paul R. Ortiz de Montellano is a Professor of Chemistry, Pharmaceutical Chemistry, and Pharmacology at the University of California, San Francisco. He is interested in the structure, mechanism, specificity, inhibition, and engineering of hemoproteins and other enzymes that catalyze oxidation–reduction reactions.

I. General Introduction

The complex of iron with a porphyrin, usually protoporphyrin IX, is a highly versatile prosthetic group that has been utilized by nature in the assembly of a large variety of electron storage proteins, ligand transport proteins, enzymes, and receptors. The flexibility provided by the three biologically accessible oxidation states of the iron, the modulation of these states and their chemical properties by the

* Corresponding author. Tel: (415) 476-2903. Fax: (415) 502-4728 or 476-0688. E-mail: ortiz@cgl.ucsf.edu.

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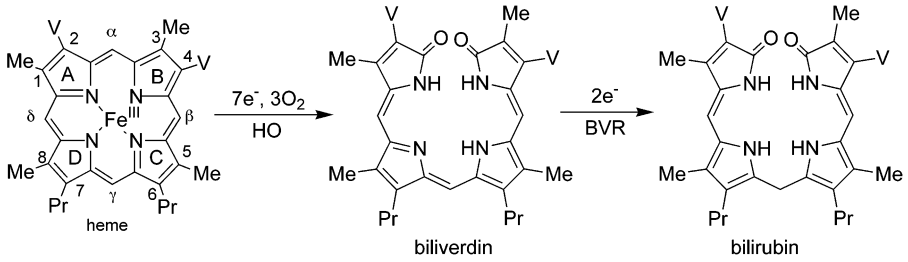
Scheme 1. The Conversion of Heme to Biliverdin and Bilirubin[a]



[a] The substituents are abbreviated as follows throughout this review: V, vinyl; Pr, propionic acid; HO, heme oxygenase; BVR, biliverdin reductase. The ring numbering and labeling nomenclature employed in this review is indicated in the figure.

four nitrogen ligands of the porphyrin plus one or two axial ligands, and the interplay of the π-system of the porphyrin with the iron d-orbitals make possible the rich chemistry associated with the heme group. However, the same factors that contribute to the versatility of the heme group also make it susceptible to autocatalytic modification. Self-catalyzed transformations of the prosthetic heme in hemoproteins can be divided into two general classes: (a) processes that are abnormal ("pathological") and result in nonphysiological modification and/or degradation of the heme and (b) processes that have evolved as part of the normal function of the proteins in question.

Many examples of the modification of heme groups by radicals or other reactive species generated during the catalytic turnover of hemoproteins are now known, particularly for hemoproteins such as the peroxidases and cytochrome P450 enzymes that catalyze the relatively nonspecific oxidation of xenobiotics. For example, horseradish peroxidase reacts with alkyl and arylhydrazines,[1] 4-alkyldihydropyridines,[2] cyclopropanone hydrate,[3] nitromethane,[4] and azide,[5] to give adducts in which the substrate or a fragment of it is covalently attached to a *meso*-carbon of the heme. In the case of cytochrome P450, reaction with an even more diverse range of compounds, including alkyl and arylhydrazines,[6,7] 4-alkyldihydropyridines,[8,9] 2,2-dialkyldihydroquinolines,[10] terminal olefins and acetylenes,[11−13] 1-aminoaryltriazoles,[14] sydnones,[15] and alkyl aldehydes[16] results in covalent attachment of the xenobiotic or a fragment thereof to the heme iron atom, a pyrrole nitrogen atom of the porphyrin, or a porphyrin *meso*-carbon. Many of these "pathological" autocatalytic reactions of heme groups are covered in other reviews and are not discussed here.[7,17,18]

This review focuses on autocatalytic modifications of the heme group that have been engineered by evolution as part of the normal function of a catalytic protein. These modifications are distinct from, for example, those involved in attachment of the heme to the protein in cytochrome *c*, as that process requires the catalytic participation of additional proteins.[19] The archetype of an autocatalytic system is heme oxygenase, for which the heme group functions as both the prosthetic group and the substrate.[20,21] One of the functions of heme oxygenase is to remove the highly pro-oxidant and therefore toxic heme group from the system, but the free iron, biliverdin, and CO that are produced in the reaction are now known to have critical cellular functions.

A second example of physiological autocatalytic modification is the process that results in covalent cross-linking of the heme group to the protein in the mammalian peroxidases and, as recently discovered, most of the members of the CYP4 family of cytochrome P450 enzymes. The heme is linked to the protein in lactoperoxidase,[22,23] myeloperoxidase,[24] eosinophil peroxidase,[25] and probably thyroid peroxidase[26] by ester bonds between protein carboxylate groups and two of the heme methyl groups. In addition, a third covalent bond is also present in myeloperoxidase between a methionine residue and one of the vinyl groups of the heme.[24] In the CYP4 enzymes, the heme is similarly linked via a single ester bond to the protein.[27,28] The functions of these covalent links remain to be elucidated, as the heme is not covalently bound in the plant or fungal peroxidases, or in most families of cytochrome P450 enzymes. These biosynthetic heme−protein cross-linking processes are to be distinguished from the abnormal, pathological binding of heme or heme fragments to the protein during the reactions of hemoproteins such as myoglobin and cytochrome P450 with peroxides and other reactive species.[29,30]

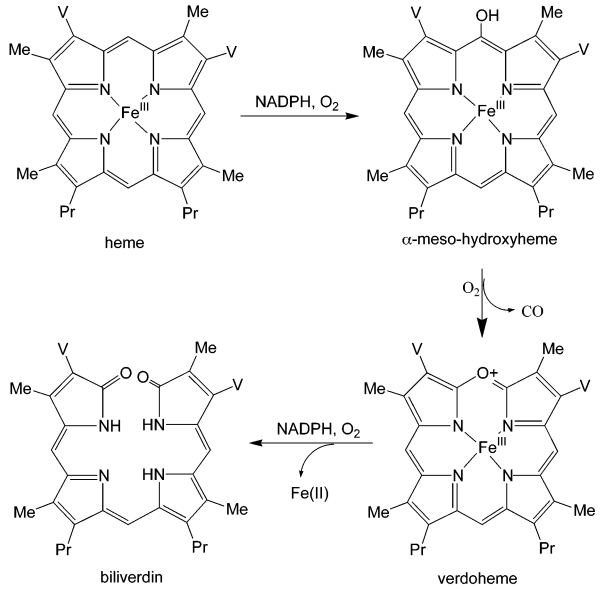
II. Heme Oxygenase

A. Introduction

The oxidation of heme to biliverdin, CO, and free iron is catalyzed by the heme oxygenases. In mammals, the biliverdin is reduced by biliverdin reductase to bilirubin that is subsequently conjugated with glucuronic acid and excreted (Scheme 1).[31] Bilirubin, albeit a potent antioxidant, is toxic at high concentrations and its excessive accumulation is deleterious.[32] Gilbert's syndrome, Crigler−Najjar syndrome, and neonatal hyperbilirubinemia are the result of malfunctions in hepatic uptake or conjugation.[33−36]

The oxidation of heme to biliverdin catalyzed by heme oxygenase proceeds through three distinct stages: oxidation of heme to α-*meso*-hydroxyheme, conversion of α-*meso*-hydroxyheme to verdoheme, and transformation of verdoheme to biliverdin (Scheme 2).[37−39] Heme, which serves as both the prosthetic group and substrate for this enzymatic process, is exclusively cleaved by the mammalian enzymes at the α-meso-position. The overall catalytic process requires three molecules of O_2 and consumes seven electrons, all of which are provided in mammalian systems by NADPH-cytochrome P450 reductase.[37,40−43] The heme oxygenase mechanism has been reviewed.[21,22,44−46]

Scheme 2. The Intermediates in the Heme Oxygenase-Catalyzed Oxidation of Heme to Biliverdin



Three heme oxygenase isoforms have been reported in mammals, but only two of these, HO-1 and HO-2, appear to play important roles in heme catabolism.[47−50] Human HO-1, HO-2, and HO-3 are membrane-bound proteins of 33, 36, and 32 kDa, respectively. Human HO-1 and HO-2 are 42% identical at the protein level, human HO-2 and HO-3 are 90% identical, and the human HO-1 is 88% identical to the corresponding rat isoform.[50,51] The highest sequence conservation is found in the regions that correspond to residues 11−40 and 125−149 in human HO-1.[51,52] Unlike HO-2,[47,53] HO-1 is inducible by agents such as heme, metals, hormones, oxidizing agents, and drugs and has therefore been the most extensively studied.[32,54−58]

In mammals, the heme oxygenases are solely responsible for the physiological (as distinct from pathological) degradation of heme, and all three products of the reaction are physiologically important.[59−62] The iron released from the heme is essential for iron homeostasis, because most of the iron requirements of the body are met by recycling this iron. Less than 3% of the daily iron requirement is obtained from the diet.[63−65] The reaction eliminates the pro-oxidant heme and replaces it with both the pro-oxidant iron and the powerful antioxidant biliverdin (and bilirubin).[59,63,66−69] The balance generally appears to favor an antioxidant effect, as heme oxygenase has been shown to confer protection in situations as diverse as atherosclerosis,[70] psoriasis,[71] vascular constriction,[72] chronic renal inflammation,[73] cellular protection,[74] hyperoxia-induced lung injury,[59,75] and reperfusion injury in transplanted liver.[76]

The proposed role of CO, the third heme oxygenase product, as a messenger in cellular signaling systems remains controversial.[77−80] CO has been proposed, *inter alia*, as a neural messenger in memory and learning,[78,79] a factor in neuroendocrine regulation,[81] an endogenous modulator of vascular tone,[82] and a protective agent in hypoxia and endotoxic shock.[83] One root of the controversy is the widespread use of metalloporphyrins as inhibitors of heme oxygenase, because they can also inhibit other hemoproteins, including guanylyl cyclase, one of the putative receptors for CO.[84] Furthermore, CO is a much weaker activator of guanylyl cyclase than NO, although the identification of molecules that amplify the response to CO complicates this argument, as does the fact that some of the actions of CO do not depend on guanylyl cyclase.[85−88] A further shortcoming of heme oxygenase as a member of signaling pathways is the absence of rapid mechanisms for regulating its activity. However, it has been reported that biliverdin reductase-IXα phosphorylates not only itself but also heme oxygenase.[89] This phosphorylation, if substantiated, could provide a mechanism for short-term regulation of the enzyme. The role of CO in mammalian physiology therefore remains plausible but not established.[90]

Three bacterial heme oxygenases have been partially characterized. HemO from *Neisseria meningitidis* is a soluble 25 kDa protein that converts heme to iron biliverdin-IXα and CO in the presence of NADPH-cytochrome P450 reductase or ascorbate.[91,92] The protein is 21% identical to human HO-1. HmuO from *Corynebacterium diphtheriae* exhibits 33% identity but 70% similarity with human HO-1.[93] PigA, from *Pseudomonas aeruginosa*, is distinct from all the other known heme oxygenases in that its reaction product is biliverdin IXβ rather than IXα.[94] The bacterial heme oxygenases appear to play a vital role in the acquisition of heme iron and in protection of the bacteria from the toxicity of heme.[95,96]

Heme oxygenase is required for the synthesis of the phycobilins and phycochromobilins of photosynthetic organisms such as *Synechocystis sp.* PCC 6803,[97] *Cyanidium caldarium*,[98] *Rhodella violacea*,[99] and *Arabidopsis thaliana*.[100] The plant enzymes, like the bacterial proteins, are soluble and depend on ferredoxin rather than cytochrome P450 reductase for the supply of reducing equivalents.[97,98,101]

B. Heme Oxygenase Protein

Mechanistic studies of the heme oxygenases were hindered prior to recombinant expression of the proteins by the fact that the heme oxygenases are membrane bound and difficult to purify from native sources.[47,102−104] This problem has been solved, at least for HO-1, by expressing the protein in *Escherichia coli* without the 23−25 carboxy terminal amino acids that constitute the membrane binding domain.[105−107] The resulting soluble protein retains high catalytic activity. Unfortunately, the yield and specific activity of the soluble HO-2 obtained after similar truncation is much lower.[108]

The crystal structures of the heme complexes of truncated human HO-1 (Figure 1a) and truncated rat HO-1 have been determined,[109,110] as have the structures of heme-free rat,[111] heme free human,[112] and most recently the azide-coordinated rat HO-1:heme complex.[113] The structure of the bacterial HemO (Figure 1b) has also been reported,[114,115] and bacterial HmuO has been crystallized.[116] No crystallographic data are available for HO-2.

Crystallization of human HO-1 requires truncation of the original 288 residues of the full length protein

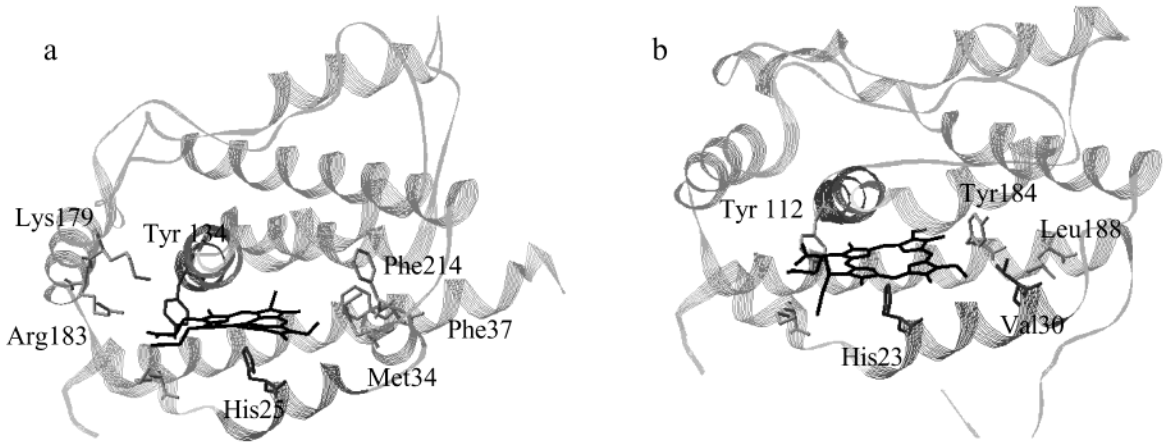


Figure 1. The X-ray crystallographic structure models of truncated human HO-1 (a) and full length bacterial HemO (b). The location of the heme and the proximal histidine iron ligand is shown in each structure. The structures are oriented so that the reader faces the δ-meso edge and pyrrole rings A and D of the heme with the propionic acid substituents pointing to the left. The iron atom has been omitted for clarity.

to a total length of 233 residues, a truncation that removes the 23 residue carboxyl terminal membrane binding domain and a further 32 amino acids. Loss of these 55 amino acids modestly decreases the total catalytic activity but does not alter the high α-meso-regiospecificity of the enzyme.[117,118] In addition, the first nine and last 10 amino acid residues of the human protein are disordered in the crystal and are not seen in the structure. The 55 amino acid truncation does not significantly modify the catalytic properties, but it may alter protein−protein interactions or other facets of heme oxygenase function. Crystallization of rat HO-1 required truncation of the protein to 267 amino acids, and the first nine and last 44 amino acid residues were disordered and are therefore missing from the structure.[110] In contrast, HemO is soluble and was crystallized without truncation, although the first eight N-terminal and last three C-terminal residues are not seen due to crystal disorder.[115]

The heme oxygenase core in both HO-1 and HemO is highly α-helical. The pincer action of two of the α-helixes holds the heme group in place with its carboxyl groups exposed at the protein surface.[109,110,115] Tighter packing of the active site in HemO and rat HO-1 than human HO-1 places the distal helix closer to the heme face. In contrast to the two mammalian HO-1 proteins, which have solvent accessible distal active site volumes of 43.6−59.7 Å^3, the distal pocket of HemO has a solvent accessible volume of only 7.5 Å^3. It is possible that HO-1 appears to be more open than it really is because the C-terminal domain that wraps back around the active site in HemO is missing due to truncation of the protein.

The *δ-meso*-edge of the heme is exposed at the surface in truncated HO-1 (Figure 1), but the other three edges are oriented toward the interior of the protein. The α-*meso*-edge of the heme that is normally oxidized in the mammalian enzymes fits tightly in human HO-1 against the hydrophobic surface presented by Phe214, Met34, and Phe37. The corresponding residues in HemO against which the heme is packed are Val30, Tyr184, and Leu188. In human HO-1, a large internal cavity extends from the heme site over the α-*meso*-heme edge and into the interior of the protein. A similarly large cavity is not present in HemO, in accord with the much smaller volume of its active site. The negatively charged propionic acid groups of the heme interact in human HO-1 with Lys179, Arg183, Lys22, and Tyr134. In HemO, the heme carboxylates interact only with two residues, Lys16 and Tyr112, that correspond to Lys18 and Tyr134 of human HO-1.[109]

In the crystallographic unit cell, human HO-1 is found in two distinct conformations that differ primarily in the position of the distal helix relative to the heme group.[109] The distal helix is kinked above the heme group by approximately 50° due to the flexibility introduced by the three glycines in its Gly^{139}-Asp-Leu-Ser-Gly-Gly^{144} sequence. Gly139 and Gly143 are in direct contact with the heme. The existence of two positions for the helix in the crystal, the fact that the crystallographic thermal factors for the atoms of the distal helix are relatively high, and the presence of three glycine residues in the helix indicate that it is a relatively flexible and mobile feature of the heme oxygenase structure.[109] Indeed, it is likely that binding of the heme and release of biliverdin require dynamic changes in the conformation of the distal helix. In the more closed of the two conformations in the human HO-1 unit cell, the distal helix comes within 4 Å of the heme surface and sterically blocks access to the *β*, *γ*-, and *δ-meso*-carbon atoms.

Spectroscopic similarities between heme oxygenase and myoglobin first suggested that a histidine is the proximal iron ligand in the heme oxygenases.[119,120] This inference has been confirmed by resonance Raman spectroscopy, which has also shown that the iron is in a six-coordinate, high spin state.[119−121] The ν_{Fe-His} vibration at 216 cm^{-1} in the Fe^{II} deoxy protein indicates that the proximal histidine ligand is not significantly deprotonated, as a correlation exists between the degree of deprotonation and the position of the ν_{Fe-His} band in hemoproteins.[122−126] Identification of the proximal ligand in human and rat HO-1 as His25 by site specific mutagenesis, resonance Raman, and EPR[127,128] has been confirmed by the crystal structures.[109,110] Thus, the ferrous ν_{Fe-His} vibration and the catalytic activity are lost in the

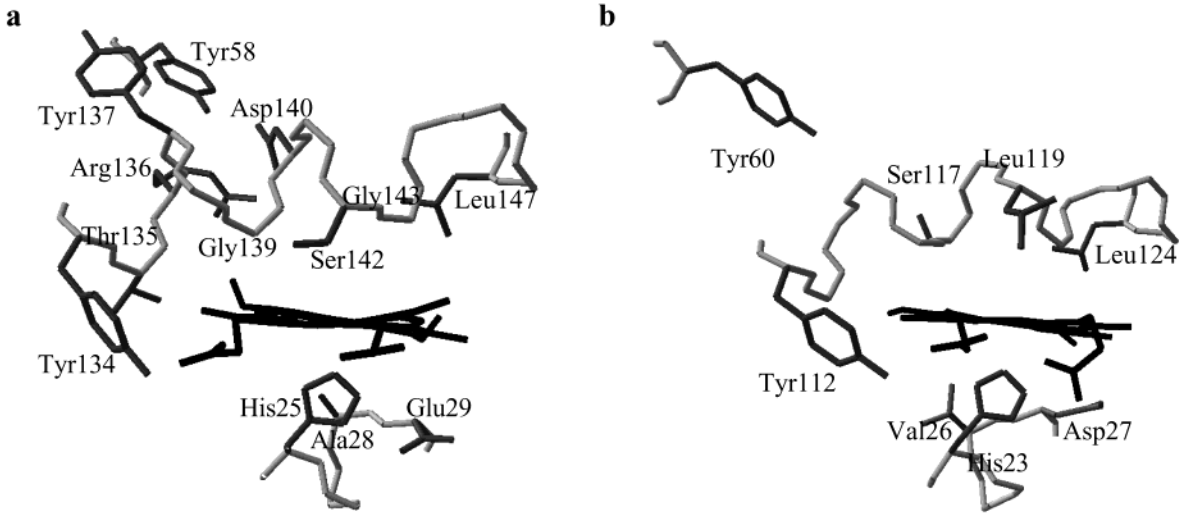


Figure 2. The active sites of human HO-1 (a) and HemO (b) with some of the residues that interact with the heme labeled. The structures are oriented so that the reader faces the γ-meso-position together with pyrrole rings C and D of the heme. The prosthetic group is depicted in black, the peptidic backbone in light gray, and the side chains in dark gray.

His25Ala mutant, in which a water ligand is bound to the iron in place of the proximal histidine nitrogen.[129] Catalytic activity and a $\nu_{Fe-Imidazole}$ resonance Raman band were observed, however, when imidazole was added to the solution due to coordination of the imidazole to the iron.[130] Mutation of His25 to a cysteine or tyrosine yields inactive proteins in which the modified proximal residue is coordinated to the iron in the Fe^{III} but not Fe^{II} state.[131] In consequence, the $Fe^{II}-O_2$ complexes are destabilized and readily autoxidize, giving rise to oxidase but not heme oxygenase activity. Spectroscopic data indicate that a neutral histidine is also the proximal ligand in HO-2.[132]

HmuO forms a 1:1 ferric heme complex with a Soret band at 404 nm and a K_d of 2.5 μM, an $Fe^{II}-CO$ complex with maxima at 421, 568, and 538 nm, and an $Fe^{II}-O_2$ complex with maxima at 410, 570, and 540 nm. Mutagenesis and spectroscopy have identified His20 as the proximal iron ligand in HmuO.[133-135] His23 is shown by the crystal structure to be the proximate ligand in HemO (Figure 2).[109,110,115] The catalytic turnover of HmuO involves formation of the same α-*meso*-hydroxyheme and verdoheme intermediates as in the mammalian proteins.[136] However, in contrast to the human His25Ala HO-1:heme complex, which is inactive,[130] the His20Ala HmuO:heme complex oxidizes heme to Fe^{II} verdoheme in the presence of NADPH-cytochrome P450 reductase.[134] The product has an absorption maximum at 650 nm, similar to that of the Fe^{II} verdoheme−CO complex,[137] but unlike that of the five-coordinate Fe^{III} verdoheme complex at 690 nm.[138] Hydrolysis of the verdoheme establishes that the oxidation occurred specifically at the α-meso-position of the heme. Some disagreement exists concerning this reaction, as Chu et al.[135] have reported that His20 is essential for catalysis by HmuO when ascorbate rather than NADPH-cytochrome P450 reductase is employed as the electron donor.

A water molecule located approximately 1.8 Å from the iron atom is the distal iron ligand in the heme oxygenases.[109,110,115] pH dependent shifts in the absorption, resonance Raman, and EPR spectra indicate that the distal water ligand has a pK_a of ~8 in the rat and human enzymes[119,121] and of ~9 in HmuO.[133] EPR and resonance Raman show that the distal water ligand is present and behaves normally in the proximal His-to-Ala HmuO and HO-1 mutants.[129,131]

A number of distal residues, notably Tyr134, Thr135, Arg136, Ser142, and Leu147 are in contact with the heme in HO-1 (Figure 2). Tyr134 and Leu147 correspond to Tyr112 and Leu124 in HemO, respectively. On the proximal side, the residues that contact the heme in HO-1 include Ala28 and Glu29 (Val26 and Asp27 in HemO). Ser117 appears to be located in HemO at the position occupied in HO-1 by a water molecule hydrogen bonded to Asp140.[112,113] His132, the closest histidine to the iron on the distal side of human HO-1, is too far to interact with iron-bound ligands. The initial observation that mutation of His132 altered the binding of water to the iron atom, and thus interacted with the distal iron ligand, was subsequently shown to be an artifact of the system used to express the protein.[139,140] The crystal structure confirms that His132 does not interact with the distal ligand.[109,110]

2D ^{1}H NMR studies suggest that the active site of human HO-1 is more open than that of the globins or peroxidases.[141] Rapid exchange with the solvent of the proximal His25 peptide NH and ring $N_\delta H$ protons shows that the proximal heme ligand is accessible to the solvent. As found for many hemoproteins, the ^{1}H NMR results show that the heme is bound in truncated HO-1 in two orientations that differ by a 180° rotation about the α,γ-axis, a rotation that does not alter the position of the normally oxidized α-*meso*-carbon (Figure 3).[142] The predominant orientation of the heme indicated by 2D ^{1}H NMR is flipped by 180° relative to its position in the crystal structure of human HO-1.[143] Thus, the NMR data indicate that pyrrole ring A (1-methyl, 2-vinyl) and parts of pyrrole rings B (3-methyl) and D (8-methyl) are bound within the protein, whereas pyrrole ring C (5-methyl, 6-propionate) and parts of pyrrole rings B (4-vinyl) and D (7-propionate) are in

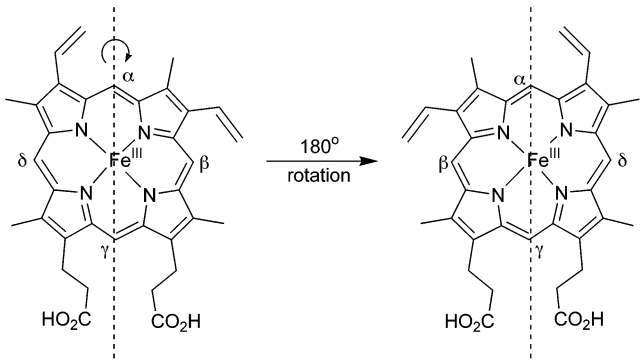


Figure 3. Orientations of the heme in soluble human HO-1 as observed by NMR spectroscopy. The two are related by a 180° flip about the α,γ-*meso*-axis.

contact with the medium.[141] The difference in heme orientation presumably reflects the different lengths of the proteins used in the two types of experiments: the crystal structure was obtained with a protein retaining 233 of the original 288 amino acid residues, whereas the NMR data was obtained with a protein of 265 residues.

NMR studies have identified a cluster of aromatic residues within the substrate binding site of the human heme−HO-1 complex.[141] When the protein is in solution, part of the distal helix and a four-ring aromatic cluster move closer to the heme than they are in the crystal structure.[109] These displacements strengthen the hydrogen bonds that involve Tyr58 and Tyr137 and tighten the active site conformation. The magnetic axis deduced from the NMR spectrum of the cyanide complex indicates that the Fe^{III}−CN bond is tilted by ∼20° toward the α-*meso*-bridge with respect to a vector orthogonal to the heme plane. Tightening of the active site, which decreases access to the *β*-, *γ*-, and *δ*-*meso*-carbons,[142] and tilting of the distal axial ligand toward the α-*meso*-carbon favor the observed α-meso-regiospecificity of the enzyme.

NMR studies of the complex of human heme oxygenase with the symmetric substrate 2,4-dimethyldeuterioheme, which eliminates heme binding heterogeneity, greatly improves the quality of the spectra and has led to the identification of a strong network of hydrogen bonds.[143] The network includes Asp140, which is believed to play a critical role in hydrogen bonding of a water molecule to the iron-bound oxygen molecule and in the delivery of protons during the catalytic process (*vide infra*). The greater clarity of the spectra have also made possible the detection of a series of additional immobilized water molecules associated with the distal hydrogen bonding network.[144]

C. Physiological Ligands

Oxygen is a cosubstrate, CO is a product and potential inhibitor, and the binding of nitric oxide could theoretically couple changes in the activity of heme oxygenase with fluctuations in nitric oxide levels. Ferrous HO-1 and HO-2 have 30−90-fold higher affinities for O_2 than the myoglobins, which increases their ability to bind O_2 in the presence of CO.[117] The mechanism involved in this higher discrimination between O_2 and CO is unclear, but the active site cavity is relatively polar due to the presence of Asp140, Arg136, and Asn210 near pyrrole ring B, and the iron-bound ligands may form hydrogen bonds with the ordered active site water molecules.[109,112,113] The existence of such a hydrogen-bonding interaction is confirmed by the observation of a solvent deuterium isotope effect on the EPR spectrum of the oxy-cobalt−HO-1 complex.[145] Steric effects are probably not critical here for differential binding of CO and O_2, because the Fe−CO bond appears from the resonance Raman data to be at least as linear in heme oxygenase as it is in the myoglobin.[121]

Absorption, resonance Raman, and EPR spectroscopies show that NO binds to Fe^{II} HO.[119,132] The EPR superhyperfine coupling of iron-bound [N^{15}]NO with the nitrogen of the proximal iron ligand helped to identify it as a histidine. Inhibition of HO-1 by NO in endothelial cells has been tentatively correlated by EPR with the formation of the NO complex.[146] However, it has also been reported that NO inhibits rat HO-2 but not rat HO-1 or mutants of HO-2 in which the cysteine residues are mutated to alanines.[147] HO-2, but not HO-1, has two Cys-Pro pairs flanked by a Phe. These sequences have been identified by Zhang and Guarente in various proteins, including HO-2, as a heme regulatory motif (HRM) that binds heme transiently and reversibly, allowing changes in the heme concentration to be detected.[148] Experimental evidence has been reported that the HRM sites in HO-2 do, in fact, bind heme,[147,149] but the role of this secondary heme, if any, in binding of NO or other functions remains to be clearly defined. Thus, although the induction of heme oxygenase by NO has been well-documented,[63] the physiological relevance of direct inhibition of HO-1 by NO has remained obscure. However, recent work has shown that NO binds to ferric HO-1 much more tightly than to metmyoglobin and very tightly to ferrous HO-1.[150] Furthermore, NO and NO donors were shown to directly inhibit the catalytic turnover of the enzyme at concentrations attainable under pathological and possibly physiological conditions. The earlier study apparently did not observe this inhibition because the NO complex is reversible and catalytic assays were carried out after the complex had dissociated.[147]

D. Reduction of the Enzyme and Reductive Reactions

Seven electrons from NADPH, delivered to mammalian heme oxygenases by cytochrome P450 reductase, are required for the oxidation of Fe^{III} heme to biliverdin. A transient complex of heme oxygenase and cytochrome P450 reductase is presumably formed to make possible the transfer of electrons from the flavins of the reductase to the iron of the heme in heme oxygenase. This complex is likely to involve the electropositive surface of the protein around the exposed heme edge,[109] as it complements the predominantly negatively charged surface of P450 reductase and provides a short pathway for electron delivery to the heme.[151] The coupled oxidation of free or protein-bound heme by aerobic incubation with a reductant such as ascorbic acid or hydrazine has been

widely used as a model for the heme oxygenase reaction.[92,152−154] Evidence has been presented that H_2O_2 in a reductive environment is responsible for the first oxidation step in the coupled oxidation reaction in the case of myoglobin.[155] Early coupled oxidation studies with $^{18}O_2$ established that (a) the oxygen atoms incorporated into biliverdin derive from two separate molecules of O_2, (b) verdoheme is converted to biliverdin by an oxidative rather than hydrolytic reaction, and (c) the oxidation of ferric protoporphyrin IX to biliverdin, CO, and ferrous iron requires three O_2 molecules and seven electrons.[156−158]

Anaerobic incubation of the Fe^{II} HO-1:heme complex with $CBrCl_3$ results in covalent attachment of the heme via one of its vinyl groups to His25.[159] On the basis of similar reactions observed with myoglobin,[160] the heme oxygenase reaction is thought to involve reduction of $CBrCl_3$ to the $^{\bullet}CCl_3$ radical, addition of the radical to the terminal carbon of one of the vinyl groups to generate an adduct with a radical adjacent to the porphyrin ring, transfer of the unpaired electron to the iron to give the corresponding cation, and trapping of the cation by the His25 imidazole nitrogen. This reaction is an example of a degradative rather than biosynthetic attachment of the heme to the protein.

E. α-*meso*-Hydroxylation

1. Formation of the Activated Oxygen Species

The oxidation of heme by heme oxygenase is initiated by the reduction of the iron from the Fe^{III} to the Fe^{II} state by NADPH-cytochrome P450 reductase. This reduction in the presence of CO forms the $Fe^{II}-CO$ complex with an absorption maximum at 418 nm.[103,154] Normally, however, the ferrous iron binds molecular oxygen to give the $Fe^{II}-O_2$ complex with an absorption maximum at 410 nm.[21] The spectroscopic properties of the $Fe^{II}-O_2$ complex are similar to those of oxymyoglobin, except for an abnormal oxygen isotope shift in the resonance Raman spectrum. This abnormal resonance Raman signal suggests that the iron−oxygen bond in the HO-1 complex is tilted, presumably to bring the terminal oxygen of the $Fe^{II}-O_2$ complex closer to the α-meso edge that is to be oxidized.[161] Furthermore, differences in the EPR spectrum of the oxy-cobalt−HO-1 complex in D_2O and H_2O suggest that the terminal oxygen in the $Fe^{II}-O_2$ complex is hydrogen-bonded.[145] Likewise, ^1H NMR analysis of rat HO-1 with a cyano group coordinated to the heme iron suggests that the cyanide ligand is also tilted toward the heme edge.[142,143] Tilting of the axial ligands therefore may involve a hydrogen-bonding interaction with a proton donor in the active site, most probably a water molecule that is itself hydrogen bonded to Asp140.[109,112,113]

2. Surrogate Reaction with H_2O_2

Anaerobic reaction of the HO-1:heme complex with 1 equiv of H_2O_2, as shown by a decreased Soret absorption and increased absorption at ∼680 nm, is oxidized to the Fe^{III} HO-1:verdoheme complex.[105] This complex can be converted in good yield to biliverdin IXα by the addition of NADPH and cytochrome P450 reductase under aerobic conditions.[105] H_2O_2 can therefore promote the heme oxygenase-mediated α-*meso*-hydroxylation of heme but does not effectively promote the subsequent oxidation of verdoheme to biliverdin. H_2O_2 has similarly been shown to be a suitable cosubstrate for the hydroxylation of heme by HO-2 and HmuO, although the reaction with HmuO is less efficient.[132,136] Anaerobic reaction of the HO-1:heme complex with 1 equiv of H_2O_2 yields an intermediate that has absorption and resonance Raman spectroscopic properties similar to those of the authentic HO-1−α-*meso*-hydroxyheme complex.[138,162]

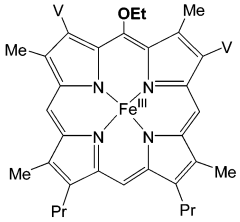
Radiolytic reduction of the HO-1 $Fe^{II}-O_2$ complex at 77 K yields the Fe^{III}−hydroperoxy ($Fe^{III}-OOH$) complex characterized by rhombic EPR signals at g = 2.37, 2.18, and 1.93 and an exchangeable hydroperoxide proton with a hyperfine coupling (g_1) of approximately 10.5 MHz.[163,164] These spectroscopic properties are similar to those of the corresponding hemoglobin β-chain complexes. Raising the temperature to 200 K converts this $Fe^{III}-OOH$ complex into Fe^{III} α-*meso*-hydroxyheme. More recent experiments using the same technique have shown that the Asp140Ala mutation allows the formation of the $Fe^{III}-OOH$ intermediate, but this does not lead on to α-*meso*-hydroxyheme due to heterolytic cleavage of the peroxide O−O bond to presumably give the ferryl species.[165] This result is consistent with the earlier findings that mutation of Asp140 to alternative residues results in the formation of a ferryl species and the acquisition of peroxidase activity at the expense of *meso*-hydroxylation of the heme group.[166,167]

3. Reaction with Peracids and Alkyl Hydroperoxides

The catalytic action of the HO-1:heme complex supported by peracids has provided useful mechanistic information.[105] A species that resembles the compound II ferryl ($Fe^{IV}=O$) species of a classical peroxidase is produced in the reaction with *meta*-chloroperbenzoic acid.[168] The two oxidation equivalents provided by the peracid are used to raise the ferric iron one oxidation state higher and to oxidize the protein to a short-lived but EPR-detectable protein radical.[105] Electron donors such as ascorbic acid, phenol, or guaiacol (*ortho*-methoxyphenol) can reduce the ferryl species back to the Fe^{III} state.[105] The oxidation of guaiacol produces the characteristic color due to peroxidative polymerization of the phenol, confirming the one-electron nature of the oxidation. Furthermore, no verdoheme is formed when 1 equiv of H_2O_2 is added to the intermediate formed by prior addition of 1 equiv of *meta*-chloroperbenzoic acid. It is therefore evident that the ferryl moiety is not the normal reactive species in the heme oxygenase reaction.

Reaction of the HO-1:heme complex of *tert*-butyl or cumyl hydroperoxide, like the reaction with *meta*-chloroperbenzoic acid, results in the formation of a ferryl species as well as partial degradation of the heme to nonbiliverdin products.[105,107] However, the reaction with ethyl hydroperoxide, which is smaller

Chart 1. Structure of α-*meso*-Ethoxyheme, a Product Formed in Reaction of the Fe^{III} HO-1:Heme Complex with Ethyl Hydroperoxide



than *tert*-butyl or cumyl hydroperoxide, is more informative. Reduction with ascorbate of the ferryl species that is partially formed, followed by HPLC analysis of the prosthetic group, shows that a substantial fraction of the heme is converted into Fe^{III} α-*meso*-ethoxyprotoporphyrin IXα (Chart 1).[169] The reaction with ethyl hydroperoxide is thus a remarkable analogue of the normal reaction with either H_2O_2 or O_2 and NADPH-cytochrome P450 reductase in that the oxygen of the peroxide not bound to the iron adds to the α-meso-position to give α-*meso*-ethoxyheme. In contrast to α-*meso*-hydroxyheme, the α-*meso*-ethoxy product is relatively stable to oxygen, because the subsequent reaction requires deprotonation of the α-*meso*-oxygen (*vide infra*). Formation of the α-*meso*-ethoxy adduct with ethyl hydroperoxide virtually rules out a mechanism involving nucleophilic attack of the Fe^{III}−peroxo anion (Fe^{III}−OO^-) on the porphyrin ring. Formation of a ferryl species or an electrophilic addition product in the reactions of the HO-1:heme complex with alkyl and acyl peroxides strongly suggests that the ring oxidation involves electrophilic attack of the Fe^{III}−OOH intermediate on the aromatic ring (Scheme 3) but does not completely exclude a mechanism in which addition to the ring is preceded by homolytic scission of the O−O bond to give a ferryl species and a hydroxy (or alkoxy) radical.

No polar side chains are present in the HO-1 and HemO structures that can directly stabilize an iron-coordinated aquo, dioxy, or peroxo ligand through hydrogen-bonding interactions.[109,110,115] The only functions positioned for such interactions are the carbonyl oxygen of Gly139, the amide nitrogen of Gly143, and, through the relay action of a water molecule, the carboxylic acid group of Asp140. Indeed, these residues have significant functions because replacement of any one of them by site specific mutagenesis results in partial formation of a ferryl species and the acquisition of peroxidase activity.[166,167,170] As demonstrated earlier with alkyl and acyl peroxides that produce a ferryl species,[105] a protein radical is also likely to be formed in the process. In the absence of a peroxidase substrate, formation of a ferryl species and protein radical leads to gradual inactivation of the enzyme. Inclusion of guaiacol in the heme oxygenase assay, which quenches any ferryl species and/or radicals formed in the parallel peroxidase reaction, protects the enzyme from inactivation and greatly increases the production of biliverdin by these mutant enzymes. The absence of obvious catalytic residues,[109,110,115] and the shift from heme oxygenase to peroxidase activity upon mutation of any of the three residues that possibly form hydrogen bonds with iron ligands, suggests that the principal role of the heme oxygenase active site may be to suppress the formation of a ferryl species.[166,167,170] Any perturbation of the active site, including mutations or the introduction of large alkyl peroxides, apparently promotes formation of the ferryl species at the expense of the normal heme oxygenase reaction.

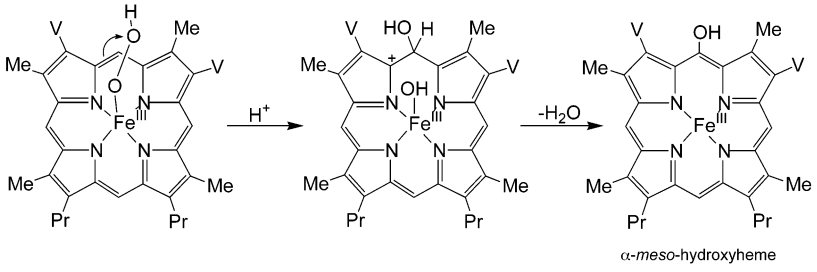
4. Oxidation of α-meso-Methyl Heme

The oxidation of α-*meso*-methylmesoheme (Scheme 4) and the α-*meso*-methyl derivative of a symmetric 2,4-dimethyl rather than 2,4-divinyl heme was examined with the expectation that the normal reaction would be blocked by the α-methyl group.[171,172] However, contrary to expectation, the α-*meso*-methyl hemes were oxidized to the normal biliverdin products. These products were formed by a reaction that eliminated the α-*meso*-carbon and the attached methyl group but did not form CO. The product containing the α-*meso*-carbon and methyl group has not been identified.[173] The mechanism of this unusual heme cleavage reaction therefore remains unclear but cannot involve a two-step sequence in which the unsubstituted heme is first generated by removal of the α-*meso*-methyl group.

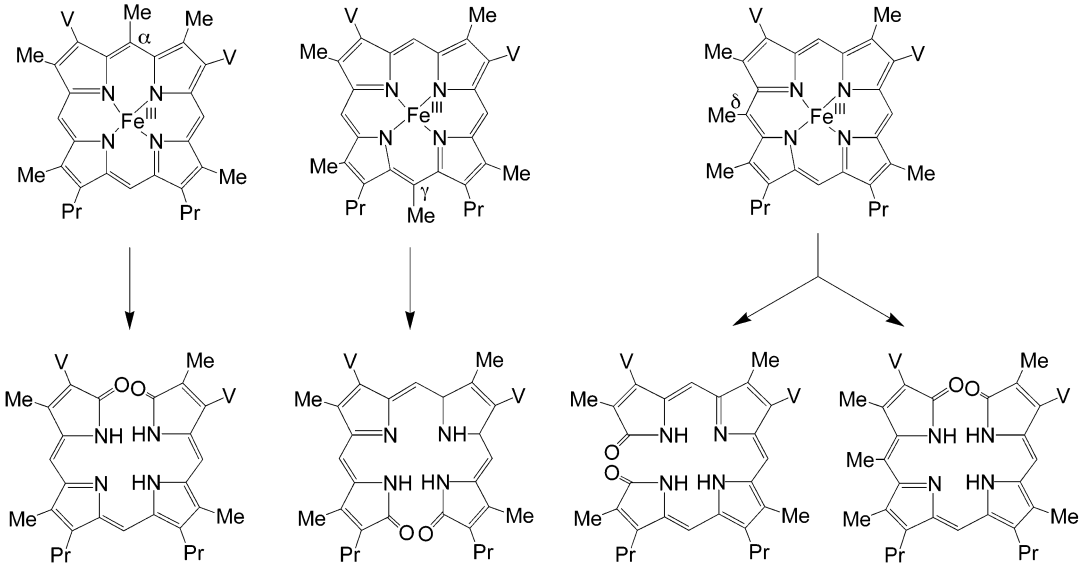
5. Heme Oxidation Regiochemistry

The coupled oxidation of free heme occurs at all four meso-positions, although the ratio of the isomeric products is sensitive to the solvent.[152,153,174] In contrast, the coupled oxidation of myoglobin gives biliverdin IXα, whereas that of hemoglobin gives the α- and β-biliverdin isomers.[41,175−177] The coupled oxidation of cytochrome b_{562} and a b_5 mutant with one histidine iron ligand replaced by a methionine occurs exclusively at the α-meso-position,[178,179] and the oxidation of heme by the bacterial heme oxygenase PigA occurs exclusively at the β-position.[94] Analysis of the myoglobin and hemoglobin active site structures initially

Scheme 3. Electrophilic Addition of the Fe^{III}−OOH Intermediate to the Porphyrin Ring during the Oxidation of Heme to α-*meso*-Hydroxyheme by Heme Oxygenase



Scheme 4. Structure of the α-, *γ*-, and *δ*-*meso*-Methyl Hemes and Their Oxidation to Biliverdin Products[a]



[a] The incubations contained human HO-1, cytochrome P450 reductase, and NADPH.

suggested that the observed regiospecificities could be rationalized by steric orientation of the iron-bound dioxygen ligand toward the appropriate meso-position(s). However, NMR studies of the binding of *meso*-nitroetioheme-1, a symmetric *meso*-nitroporphyrin, to myoglobin have recently shown that the bulky nitro group can equally well be located at the α- or *δ*-meso-positions.[180] This finding conflicts with the postulate that the heme cleavage regiospecificity in coupled oxidation of myoglobin is exclusively determined by steric steering of the dioxygen ligand. Furthermore, the steric effects observed in the crystal structure of cytochrome b_{562} apparently do not explain the regiochemistry of the coupled oxidation of its heme *c* prosthetic group.[178]

Relocation of the distal histidine in sperm whale myoglobin by Leu29His/His64Leu, Phe43His/His64Leu, and His64Leu/Ile107His mutations alters the coupled oxidation product distribution, resulting in 97%, 44%, and 22% *γ*-meso-oxidation, respectively.[181] An alternative set of myoglobin mutations (Val67Ala/Val68Ser) increased the efficiency of the reaction without altering its regiospecificity.[182] The authors of the first study proposed that the regiospecificity of the reaction was controlled by hydrogen-bonding interactions and the polarity of the heme crevice. Electronic factors can contribute to control of the reaction regiochemistry, as demonstrated by the finding that the coupled oxidation of an iron porphyrin with a CF_3 substituent occurs at the meso-position with the highest electron density.[183] The regiospecificity of heme oxidation thus may be largely controlled by steric factors, but electronic and other factors can also be important.

The oxidation by HO-1 of the *β*-, *γ*-, and *δ*-*meso*-methylmesohemes (Scheme 4) was compared with that of the α-*meso*-methyl isomer, with the expectation that the *β*-, *γ*-, and *δ*-*meso*-methylmesohemes would either not be substrates or would be cleaved at the usual α-meso-position.[172,173,184] However, *γ*-*meso*-methylmesoheme was exclusively oxidized by HO-1 to mesobiliverdin IX*γ* from which the *γ*-*meso*-carbon and its methyl substituent had been eliminated. The *δ*-*meso*-methyl isomer was oxidized to mesobiliverdin IX*δ*, from which both the *δ*-*meso*-carbon and its methyl substituent were eliminated, and to a methyl-substituted mesobiliverdin IX isomer. In contrast, the *β*-*meso*-methyl derivative was a poor substrate for the enzyme. A *meso*-methyl substituent thus causes the heme oxygenase reaction to shift from the α-meso-position to the methyl-substituted *meso*-carbon. Of course, when the methyl substituent is on the α-*meso*-carbon, it simply reinforces the normal specificity of the enzyme.

The drastic effect of *meso*-methyl substitution on the reaction course suggests that factors other than steric interactions of the iron-bound dioxygen molecule with the active site residues may help to determine the reaction regioselectivity.[109] The powerful directing effect of a methyl substituent is most clearly illustrated by the finding that a *γ*-*meso*-methyl quantitatively inverts the regiochemistry of heme oxidation, even though the methyl cannot interact directly with the iron-bound dioxygen molecule. In any case, if a direct steric interaction were involved, it would be expected to disfavor reaction at the methyl-substituted carbon. Indeed, the sensitivity of the heme oxidation regiochemistry to methyl substitution is consistent with a role for electronic effects in determining the course of the reaction.[172] As already mentioned, HO-1-mediated heme oxidation appears to involve electrophilic attack of the distal oxygen of the ferric peroxo ($Fe^{III-}OOH$) complex on the *meso*-carbon of the porphyrin, a reaction that would be favored by an electron-donating methyl substituent. The electron-donating effect of the methyl would thus reinforce the normal specificity when placed at the α-meso-position but would favor *δ*-meso over α-meso oxidation when placed at the *δ*-meso-position. It is surprising, however, that the effect of a *γ*-*meso*-methyl is large enough to overwhelm whatever steric and other factors normally channel the reaction to the α-meso-position.

To test the hypothesis that the electron-donating properties of a methyl substituent are responsible for its pronounced effect on the regiochemistry of heme oxidation by HO-1, the oxidation of the four possible *meso*-formyl mesoheme regioisomers by HO-1 has been examined.[185] The formyl substituent, in contrast to a methyl, is electron-withdrawing and would be expected, if electronic factors are paramount, to direct the oxidation to the unsubstituted rather than substituted positions. In agreement with this prediction, α-*meso*-formylmesoheme was oxidized at a position other than the formyl-substituted α-meso-carbon, as demonstrated by the concomitant formation of CO and the retention of the formyl group in the biliverdin product. In fact, CO was released in the oxidation of all of the *meso*-formyl hemes, whereas no CO was formed with the α- or δ-*meso*-methyl hemes. However, the formation of a formyl-substituted biliverdin was only confirmed by mass spectrometry for the α-*meso*-formyl heme. These results again are consistent with an electronic effect in control of the reaction regiochemistry. It must be kept in mind, however, that alternative mechanisms could mediate the effects of the meso substituents.

The formation of all four biliverdin regioisomers when free heme is oxidized by coupled oxidation establishes that there is no inherent electronic or steric preference for oxidation of the α-*meso*-carbon.[180,186] The electronic effects of substituents and/or of iron ligands must therefore alter the intrinsic electronic properties of the heme. One mechanism for altering the electronic distribution is provided by the ruffling of the porphyrin structure caused by the steric interaction of a meso substituent with the adjacent porphyrin substituents. Ruffling has been shown to alter the electronic distribution in the highest occupied molecular orbitals of the porphyrin, favoring accumulation of electronic density at the *meso*-carbons and placing them closer to the reactive oxygen atom.[187] This increased electron density would be expected to facilitate the meso-hydroxylation reaction. It is also possible that a meso substituent could perturb the active site steric barriers that help control the reaction regiochemistry. However, the differential effects of the methyl and formyl substituents are not consistent with control of the regiochemistry by porphyrin ruffling, as the two substituents would be expected, at least qualitatively, to influence the reaction in the same manner.[187−189]

NMR analysis of the heme:HO-1 complex indicates that there are major differences in the electron density on the two β-carbons within a given pyrrole ring of the heme. This pattern is distinct from the more commonly observed pattern in which the electronic densities at the β-carbons of a given pyrrole ring are similar but different from those of the β-carbons of the adjacent pyrrole rings.[142] Iron porphyrins with electron-donating or -withdrawing meso-substituents have been shown to have an electronic density pattern analogous to that of the heme:HO-1 complex. These electronic density patterns have been rationalized by theoretical studies.[190,191]

It has been suggested that the heme orientation in the active site is determined by interactions between the propionate carboxyl groups and the positively charged Arg183.[192] This conclusion is based on the finding that mutation of Arg183 to a glutamic or aspartic acid in rat HO-1 changes the regioselectivity of the enzyme. Under coupled oxidation conditions, the Arg183Glu mutant reportedly gives a 60:40 mixture of α- and δ-biliverdin, respectively. The authors proposed that this might represent a rotation of the heme group in the active site in the absence of the carboxyl−arginine interactions to present a different meso-position for oxidation at the normal site. Lightning and Ortiz de Montellano have also independently demonstrated that the Arg183Glu mutant of human HO-1 also gives a mixture of biliverdin isomers under coupled oxidation conditions, but they found that under normal turnover with NADPH-cytochrome P450 reductase the only product was the normal α-meso cleavage product (unpublished results). These results show that coupled oxidation differs from normal turnover with cytochrome P450 reductase and specifically show that a loss of regiospecificity is only observed with the Arg183Glu mutant under coupled oxidation conditions. Although the Arg183Glu mutation does not alter the regiochemistry of HO-1 under normal cytochrome P450-dependent conditions, the Asp140Lys and Asp140His mutants have been shown by Lightning and Ortiz de Montellano to produce the α-, β-, and γ-biliverdin isomers (unpublished results). However, other recent work has shown that the heme oxygenase from *Pseudomonas aeruginosa* normally produces a mixture of β- and δ-biliverdin isomers.[193] This unusual oxidation regiochemistry has been traced by NMR studies to a rotational displacement of the heme within the active side due to the absence of the stabilizing interactions between the heme carboxyl groups and cationic protein residues that apparently fix the heme orientation in other heme oxygenases.

F. α-*meso*-Hydroxyheme to Verdoheme

The HO-1 complex with α-*meso*-hydroxyheme is stable in the absence of O_2.[138,162] Under anaerobic conditions, the HO-1:α-*meso*-hydroxyheme complex gives rise to an EPR spectrum with rhombic signals at $g = 6.07$ and 5.71 due an Fe^{III} and a signal at $g = 2.008$ attributable to an organic radical (Figure 4). Addition of CO in the absence of oxygen enhances the signal at $g = 2.008$ at the expense of the signals at $g = 5-6$.[138] These results reveal the existence of an equilibrium between an Fe^{III} heme species and an Fe^{II} species associated with an organic radical. The binding of CO stabilizes the Fe^{II} with respect to the Fe^{III} form, increasing the proportion of the organic radical and decreasing that of the paramagnetic Fe^{III} component. Formation of the HO-1:α-*meso*-hydroxyheme Fe^{II}−CO complex is supported by the observation of shift from 405 to 408 nm in the Soret maximum in the presence of CO. Independent evidence for the proposed equilibrium is provided by the finding with resonance Raman that an oxophlorin-like rather than a porphyrin-like structure is obtained upon deprotonation of the Fe^{III} α-*meso*-hydroxyheme complex.[162] It has also been reported that an EPR signal at $g = 2.004$ is observed when

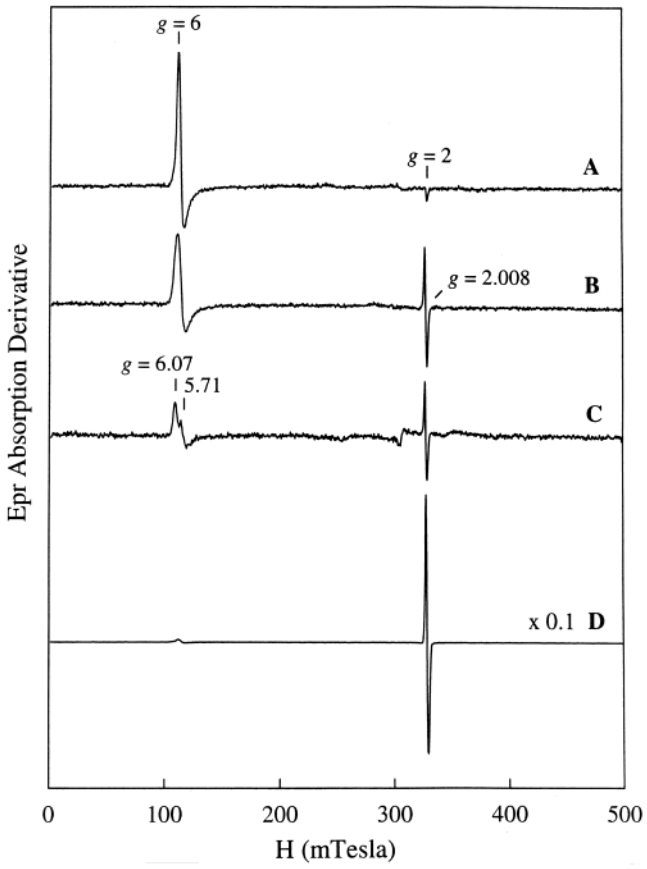
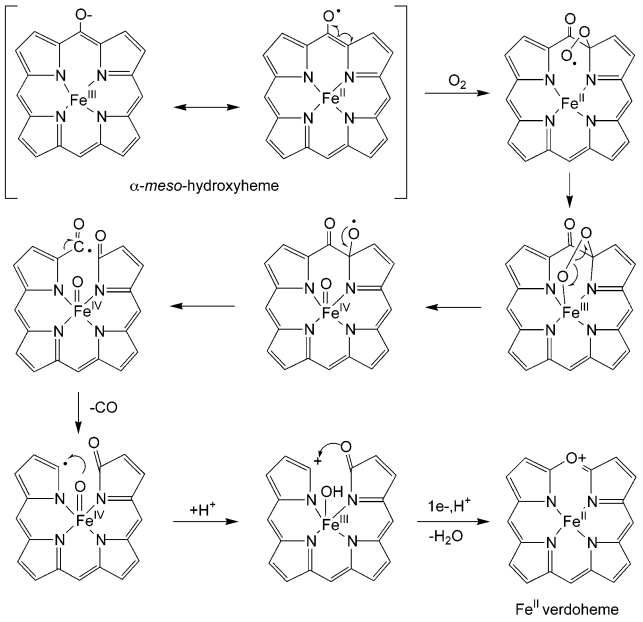


Figure 4. EPR of the α-*meso*-hydroxyheme-HO-1 complex: (A) Fe^{III} heme:HO-1 complex, (B) α-*meso*-hydroxyheme-HO-1 complex formed anaerobically with 1 equiv of H_2O_2, (C) α-*meso*-hydroxyheme complex after subtraction of the spectrum of unreacted heme from trace B, and (D) spectrum in B after the addition of CO to form the Fe^{II}–CO complex. The spectra are from Liu et al.[138] (reprinted with permission. copyright 1997 XXX).

O_2 is added to the HO-1:α-*meso*-hydroxyheme complex generated under anaerobic conditions.[162] This EPR signal was tentatively attributed to the peroxy radical complex expected from addition of O_2 to the Fe^{II} porphyrin radical, but this assignment requires confirmation. It is nevertheless likely that a peroxy radical species is formed by addition of molecular oxygen to a deprotonated, free radical form of α-*meso*-hydroxyheme (Scheme 5).

The HO-1:α-*meso*-hydroxyheme complex produced by reaction of the HO-1:heme complex with 1 equiv of H_2O_2 under an atmosphere of nitrogen is rapidly converted by molecular oxygen to the HO-1:Fe^{III} verdoheme complex.[138] This Fe^{III} complex is reduced by dithionite to Fe^{II} verdoheme identical to that obtained when the reaction is carried out in the presence of NADPH-cytochrome P450 reductase. Similar results have been obtained upon exposure to oxygen of the complex obtained by reconstitution of apo-HO-1 with synthetic Fe^{III} α-*meso*-hydroxyheme, although the authors reported formation of the Fe^{II} rather than Fe^{III} verdoheme product.[194,195] The source of the electron required to reduce the iron in these experiments remains unclear. However, it is clear that the only requirement for conversion of Fe^{III} HO-1:α-*meso*-hydroxyheme to Fe^{III} verdoheme is one molecule of O_2.[138,194] In agreement with these results,

Scheme 5. Hypothetical Mechanism for the Oxidation of Fe^{III} α-*meso*-Hydroxyheme (shown deprotonated) to Fe^{II} Verdoheme Catalyzed by Heme Oxygenase[a]



[a] It is also possible that the O_2 binds to the Fe^{II} first and only then binds to the carbon to form the peroxo bridge. For the sake of clarity, the porphyrin substituents are not shown.

molecular oxygen is the only requirement for conversion of the Fe^{III} apo-myoglobin:α-*meso*-hydroxyheme complex to the verdoheme complex.[196] Together, these results contradict the report that the conversion of Fe^{III} α-*meso*-hydroxyheme to verdoheme requires an exogenous electron in addition to a molecule of oxygen.[162,197] In that instance, the product appeared to be Fe^{II} verdoheme, as would be expected in the presence of an external electron donor. Nevertheless, the demonstration in three laboratories that verdoheme is formed in good yield from α-*meso*-hydroxyheme in the absence of reducing equivalents precludes a mandatory requirement for an electron for this reaction to occur. It is nevertheless possible that in the presence of the normal electron donor components, the reaction follows one or both of two possible pathways: (a) conversion of Fe^{III} α-*meso*-hydroxyheme to Fe^{III} verdoheme, followed by reduction of the iron to the Fe^{II} state, and (b) reduction of the Fe^{III} α-*meso*-hydroxyheme to the Fe^{II} state, followed by direct conversion to Fe^{II} verdoheme.

In a relevant model system, anaerobic apomyoglobin reconstituted with α-*meso*-hydroxyheme has absorption maxima at 410, 590, and 640 nm and a high-spin Fe^{III} EPR signal at $g = 6.3$.[196] Upon reaction with 1 equiv of O_2 a molecule of CO is eliminated and an intermediate is formed with absorption maxima at 408, ~540, 660, and 704 nm and an EPR signal at $g = 2.0012$. These spectra were attributed to either Fe^{III} verdoheme or a π-neutral radical derived from it. As found for human HO-1, no reducing agent was required for the conversion of α-*meso*-hydroxyheme to verdoheme, but O_2 could not be replaced in the reaction by H_2O_2.

No experimental evidence is available on the mechanism that leads from α-*meso*-hydroxyheme to

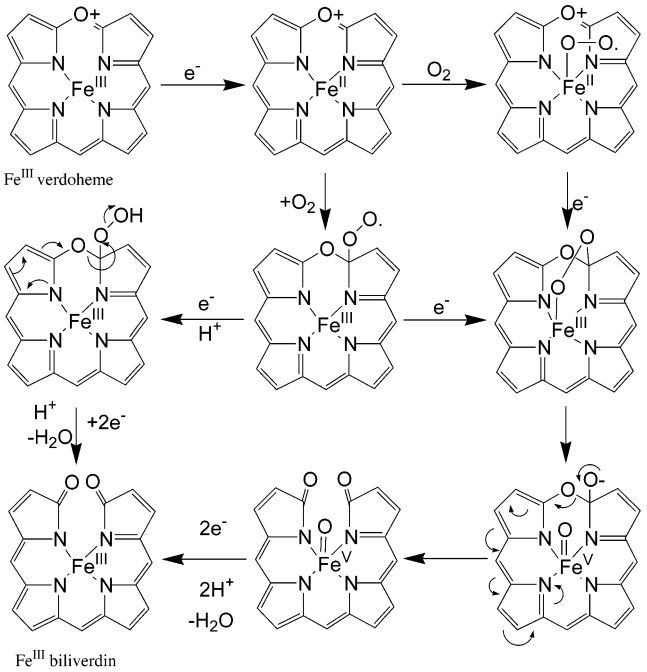
biliverdin after formation of the Fe^{II} radical intermediate that presumably binds oxygen to give the Fe^{II} peroxy radical. It is possible, however, to postulate a plausible mechanism for the reaction. In the most probable mechanism (Scheme 5), the porphyrin peroxy radical collapses with the Fe^{II} to produce a peroxy-bridged species. Formation of the iron−oxygen bond could be preceded by an internal electron transfer from the iron that oxidizes it to the Fe^{III} state and simultaneously generates the porphyrin peroxy anion. The alkoxy radical intermediate formed by homolysis of the oxygen−oxygen bond would readily account for the elimination of the α-*meso*-carbon as CO. Elimination of the CO by this mechanism would leave an unpaired electron on the α-carbon of the pyrrole ring that could be oxidized to a cation by intramolecular electron transfer to the ferryl species that would be concomitantly formed in the dioxygen bond scission. The final verdoheme structure would result from trapping of this cationic center by the carbonyl group on the vicinal pyrrole ring.

G. Verdoheme to Biliverdin

The mechanism that results in the conversion of verdoheme to biliverdin remains obscure. The individual verdoheme isomers obtained by coupled oxidation of heme have been assigned by NMR, and their conversion of the corresponding biliverdin isomers has been demonstrated.[198] The EPR spectrum of apo-HO-1 reconstituted with iron α-verdoheme is consistent with the presence of a hexacoordinated iron with a hydroxyl as the sixth ligand.[198] It is also known from very early work that the biliverdin produced by HO-1 under an atmosphere of $^{16}O-^{16}O$ and $^{18}O-^{18}O$ includes products that incorporate not only two ^{16}O atoms or two ^{18}O atoms but also the product with one atom each of ^{16}O and ^{18}O.[39] These results show that the two oxygen atoms incorporated into biliverdin have their origin in different molecules of O_2.[38] It has also been demonstrated that an atom from molecular oxygen is incorporated into the biliverdin product formed with verdoheme as the starting material.[158] The physiological conversion of verdoheme to biliverdin therefore proceeds via a redox rather than hydrolytic pathway, in accord with the requirement for reducing equivalents and O_2.[105,158,161] ^{18}O-Labeling studies have demonstrated that the biosynthesis of phycocyanobilin involves a two-oxygen-molecule heme cleavage mechanism[199,200] similar to that for the mammalian heme oxygenases.[39]

The conversion of verdoheme to biliverdin (Scheme 6) can be envisioned to involve reduction of Fe^{III} verdoheme to the Fe^{II} state, binding of molecular oxygen, and reduction of the resulting ferrous dioxy complex to a ferric hydroperoxy ($Fe^{III}-OOH$) intermediate. A number of alternative sequences can be postulated for the conversion of this hydroperoxy intermediate to Fe^{III} biliverdin, all of which eventually involve a sequential ring cleavage and two-electron reduction. Fe^{III} biliverdin is then reduced to the Fe^{II} state prior to sequential, ordered release of ferrous iron and biliverdin. Experimental evidence has confirmed that the iron is reduced and is released

Scheme 6. Hypothetical Mechanism(s) for the Heme Oxygenase-Catalyzed Conversion of Fe^{III} Verdoheme to Fe^{III} Biliverdin[a]



[a] The final steps involving reduction of the Fe^{III} biliverdin to the Fe^{II} state, followed by release of the iron and biliverdin, are not shown. Two alternative mechanisms are depicted that differ in the order of electron addition and the initial site of oxygen binding. For the sake of clarity, the porphyrin substituents are not shown.

prior to the organic moiety. An early study showed that coupled oxidation produces an Fe^{III} biliverdin complex that is not a ready substrate for biliverdin reductase. However, the biliverdin produced by the normal NADPH-cytochrome P450 reductase-supported reaction is readily reduced to bilirubin, presumably because iron reduction and dissociation make the biliverdin accessible to biliverdin reductase.[154] The requirement for reduction of Fe^{III} to Fe^{II} biliverdin has been confirmed by single turnover kinetic studies.[201]

Coupled oxidation of the prosthetic group of the cytochrome b_5 His63Met mutant proceeds as far as verdoheme but not biliverdin.[202] Arrest of the reaction at an intermediate state was proposed to result from formation of a six-coordinate $His-Fe^{II}-Met$ complex at the verdoheme stage. The binding of high-affinity ligands to both the fifth and sixth coordination sites is known to inhibit the coupled oxidation conversion of verdoheme to biliverdin.[203] In a sense, inhibition of the conversion of Fe^{II} verdoheme to biliverdin by CO is an illustration of this phenomenon.[196] It has also been shown that coupled oxidation of the heme in a His63Val mutant of cytochrome b_5 produces verdoheme, but a His39Val mutant yields biliverdin.[202] It was argued that the two mutants differentially affect the ability of the protein to stabilize the ferrous dioxyheme complex.

H. Heme Oxygenase Kinetics

Four distinct catalytic events separated by three relatively well-established intermediates, α-*meso*-

hydroxyheme, verdoheme, and iron biliverdin, intervene in the heme oxygenase-mediated conversion of Fe^{III} heme to biliverdin, CO, and Fe^{II}. Three molecules of oxygen and seven electrons are consumed in the process.[37] In addition to this inherent complexity, the low solubility of heme at neutral pH and ambiguities concerning its physical state in vivo make kinetic analysis of this reaction sequence difficult. The problem is made more difficult by purely technical issues, such as the dependence on an assay that measures biliverdin formation through its coupled reduction to bilirubin by biliverdin reductase.[103] Nevertheless, useful mechanistic information has been obtained by single turnover kinetic studies.[201]

The K_m of 3 μM for heme using human HO-1 truncated at the C-terminus by 23 amino acids is similar to the K_m values for native rat liver and bovine spleen HO-1 (K_m = 3 μM), all of which bind heme less tightly than HO-2 (K_m = 0.2 μM).[49,103,104,107,132,204,205] The V_{max} for the recombinant truncated protein is 40 nmol h^{-1} $nmol^{-1}$.[107]

Single turnover studies indicate that the conversion of Fe^{III} heme to Fe^{III} verdoheme is biphasic.[201] The Fe^{III} heme−HO-1 complex was reduced to the Fe^{II} state at a rate of 0.11 s^{-1} at 4 °C and 0.49 s^{-1} at 25 °C with a cytochrome P450 reductase:HO-1 ratio of 0.1:1.0. The reduction reaction was actually followed by spectroscopically monitoring formation of the $Fe^{II}-O_2$, because oxygen binding occurred much more rapidly than reduction of the iron. Formation of the Fe^{III} verdoheme−HO-1 complex via slow electron transfer to the $Fe^{II}-O_2$ complex, formation of α-*meso*-hydroxyheme, and CO elimination occurred at 25 °C at a rate of 0.21 s^{-1}.[201] The Fe^{III} verdoheme complex was then reduced to the Fe^{II} state at a rate of 0.55 s^{-1}. Rapid oxygen binding was followed by a second electron transfer at a rate of 0.10 s^{-1} to give the Fe^{III} biliverdin product.[197] In sequential order, the Fe^{III} biliverdin was then reduced to the ferrous state at rates of 0.15 s^{-1}, the ferrous iron was released at rates of 0.39 s^{-1}, and the biliverdin was finally released at rates of 0.03 s^{-1}. The rate-limiting step in the single turnover studies was the final release of the biliverdin product, but biliverdin release was accelerated when biliverdin reductase was present and the rate-limiting step became the transformation of Fe^{II} verdoheme to Fe^{III} biliverdin. Acceleration of biliverdin release by biliverdin reductase suggests that an interaction of the two proteins facilitates the dissociation of biliverdin from heme oxygenase.[109]

III. Mammalian Peroxidases

A. Introduction

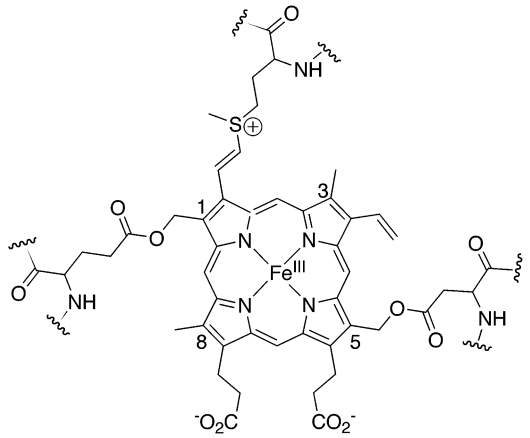
Heme peroxidases constitute a very important group of enzymes both from a biological and historical point of view. Their general properties have been the subject of a recent and very thorough review.[206] On the basis of sequence comparisons, they can be divided into three superfamilies: catalases, plant peroxidases, and mammalian peroxidases. The latter can be subdivided into the cyclooxygenase and myeloperoxidase families, the second of which includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase (TPO). Alternate subdivisions of the mammalian peroxidase superfamily based on extended and striking sequence similarities have been suggested[207−210] that imply the inclusion of invertebrate representatives such as the *Drosophila* peroxidasin,[211] *Xenopus* ribosomal ribonuclease-1,[212] and even plant representatives in the family.[211] Regardless of the phylogenic tree that is selected, the crystallographic data, which is only available for myeloperoxidase and cyclooxygenase-2, demonstrates that extensive three-dimensional similarities exist among these members.[213]

MPO, an enzyme of 150 kDa consisting of two heavy and two light chains obtained by post-transcriptional processing of a homodimeric protein, is found within the endocytotic vacuoles of blood neutrophils and monocytes and is responsible for the microbial killing that occurs therein. EPO, a homodimer of 70 kDa subunits, is the related enzyme found in eosinophils. LPO, a 78 kDa monomer with bactericidal and bacteriostatic properties, is produced by the leukocytes in milk, tears, and saliva, whereas TPO, a somewhat heavier membrane-bound monomer of 105 kDa, is localized in the thyroid gland and is responsible for production of the thyroid hormones T_3 and T_4.

These four mammalian peroxidases *stricto sensu* share very similar structural features. Their prosthetic group is a ferric protoporphyrin IX that is covalently linked to the protein. The fifth (i.e. proximal) iron ligand is provided by the N_ϵ of a histidine, and the sixth position, as indicated by the crystal structure of MPO and resonance Raman studies on LPO,[24,214] is occupied by a water molecule. Catalytic histidine and arginine residues are present in the distal cavity of all of the mammalian peroxidases. The proteins also contain a calcium binding site and are about 10% glycosylated at sites that have been shown by 3D modeling to be at least partially conserved.[215] Another important feature is the high degree of sequence similarity among the four members of the group. For instance, human MPO has 72%, 58%, and 49% identity with the amino acid sequences of human EPO, SPO, and TPO, respectively.[209] SPO, the salivary peroxidase, differs by a single amino acid from LPO and the two are usually considered to be equivalent.

Perhaps the most striking feature of the mammalian peroxidases is the existence of two to three covalent bonds between the prosthetic group and the protein in the functional, mature enzymes. The patterns of these bonds, postulated early on,[216,217] have been clearly demonstrated for MPO by crystallography,[218] for LPO by NMR and mass spectrometry,[21,22] and for EPO/LPO by mass spectrometry and peptide sequencing.[24] The presence of ester links in all three enzymes has furthermore been corroborated by difference FTIR.[219] Compelling evidence, based on sequence similarity and biochemical analysis, suggests the presence of similar heme−protein cross-links in TPO.[220] The bonds between the heme and the protein are of two types (Chart 2): (i) two ester bonds, observed in all four members, one linking a

Chart 2. Covalent Binding Pattern in Mammalian Peroxidases[a]



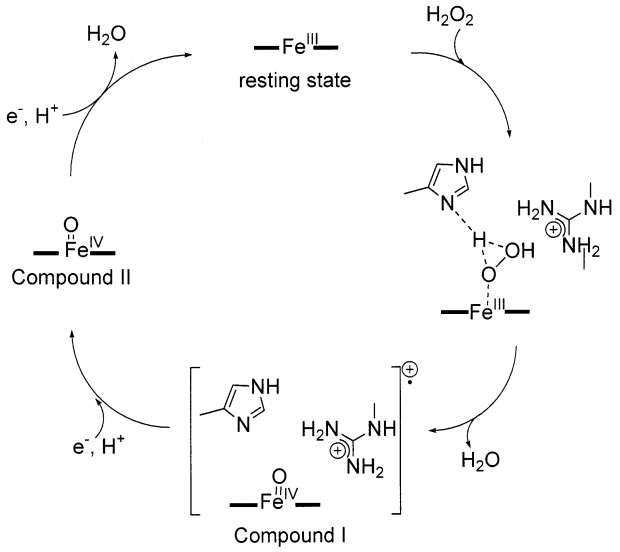
[a] Both ester bonds are present in all four members, but only MPO also features the vinyl−methionyl sulfonium bond.

hydroxyl group on the 1-methyl substituent to a glutamic acid carboxylic group and the other a hydroxyl group on the 5-methyl substituent to the carboxyl group of an aspartyl residue, and (ii) a vinyl−sulfonium bond, only present in MPO, linking the β-carbon of the 2-vinyl substituent and the sulfur of Met243.[23] This third link is responsible for the noticeable red shift of the Soret band of ferric MPO (λ_{max} = 430 nm) relative to its position in the other peroxidases (λ_{max} = 412 nm).[219] The prosthetic group in these enzymes is therefore derived from Fe^{III} protoporphyrin IX by the formal replacement of one of the hydrogen atoms on the 1- and 5-methyls by an ester oxygen and one of the vinyl β hydrogens by a methionine sulfur. The unique sulfonium link in MPO is thought to be directly linked to the higher oxidizing potential of this enzyme as compared to the other three (*vide infra*).

B. General Properties

Upon reaction with H_2O_2, their natural cosubstrate, the mammalian peroxidases are transformed into a short-lived intermediate, known as compound I, in which one of the oxidizing equivalents of H_2O_2 is stored on the iron as an Fe^{IV} oxyferryl moiety and the other on the porphyrin ring as a π radical cation. The latter can be transferred to a protein side chain, as has been shown for MPO[221] and LPO.[222−224] Transfer of one electron from a substrate reduces the enzyme to the so-called compound II, in which the single remaining oxidizing equivalent is almost invariably present as the Fe^{IV} oxyferryl group. The transfer of a second electron reduces the enzyme back to its Fe^{III} resting state (Scheme 7). The transit through compound II can be by-passed in some situations through a direct two-electron transfer from the substrate. The demonstrated ability, at least for MPO and LPO, to transfer one oxidizing equivalent from the porphyrin to a protein residue situates these enzymes between the two well-known extremes represented by cytochrome *c* peroxidase (C*c*P), in which the iron IV porphyrin π-radical cation is virtually undetectable, and HRP, in which this species is surprisingly stable.[207]

Scheme 7. General Enzymatic Cycle of Peroxidases. The Heme Plane Is Schematized by the Two Solid Black Bars on Both Sides of the Iron[a]



[a] The ubiquitous catalytic diad, composed of a histidine and an arginine, has been depicted only for compound I and the transition state leading to it.

All four peroxidases are capable of directly oxidizing small aromatic substrates such as phenols, anilines, and phenothiazines through two successive single electron-transfer reactions. In the case of phenols, the oxidation generates aryl radicals that can react further with each other to form dimeric or higher oligomeric species. Indeed, this latter reactivity is the basis of the physiological action of TPO, whose primary function is the synthesis of the iodotyrosine dimers present in the hormone thyroxin.[225,226] The generation of free organic radicals is also responsible, in part, for the cytotoxic properties of LPO.[227] However, these enzymes are also characterized by their ability to oxidize I^-, SCN^-, and Br^-, albeit with different efficiencies,[228] and even Cl^- in the sole case of MPO at pH = 7, a property probably linked to the presence in that enzyme of a vinyl−methionyl sulfonium bond. The oxidation of these small inorganic ions is achieved through a direct two-electron-transfer process that generates the corresponding hypo(pseudo)halides. The oxidation of diverse biological targets, including cysteines, methionines, amines, and aromatic rings by these reactive species contributes to the high bactericidal or, in the case of TPO mediated tyrosyl iodinations, biosynthetic potential of the mammalian peroxidases.[229] Although the ability to oxidize iodide and thiocyanate is found equally well among the plant peroxidases (e.g. HRP), there are fewer examples of other heme peroxidases that readily oxidize bromide, e.g., manganese peroxidase[230] and soybean peroxidase,[231] and only two others, chloroperoxidase[232] and the *Synechocystis* catalase-peroxidase,[233] are known to be capable of oxidizing chloride.

C. Active Site Structure

As already mentioned, the only members of the mammalian peroxidase family for which crystal

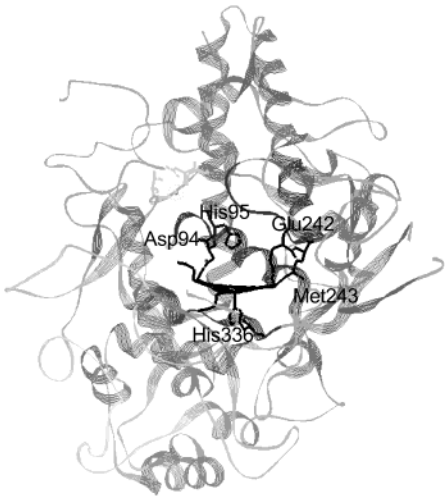


Figure 5. Structural elements of MPO around its active site as seen from ring D. Helices 2, 5, 8, and 12 are stressed in dark gray. Heme, His94, Arg239, and His336 and both residues implicated in covalent binding are represented in black.

structures are available are canine and human MPO.[23,218] The already high overall sequence similarity among the four enzymes is even higher for the residues in close proximity to the heme in MPO.[234] Hence, the three-dimensional structure of this enzyme is likely, as has been reported,[215] to be a good template for the structures of the other three enzymes.

The major features of the MPO heme binding pocket are summarized in Figure 5. Four main secondary structures line the active site. On the proximal side, two helices, H8 which runs along the B−C edge of the heme, and H12, which is roughly parallel to H8, lies underneath pyrrole ring D. On the distal side, helix H2 is placed above pyrrole rings B and C in a relationship to the heme that somewhat mirrors that of the H8 on the proximal side. In addition, a strand-turn-helix (H5) structure is pointing toward pyrrole ring A. The links between the heme and protein are all on the distal side: Glu242 just before H5 is linked to the ring A methyl, Met243 to the ring A vinyl, and Asp94 from helix H2 to the ring C methyl. The heme−protein bonds cause a considerable deformation of the heme tetrapyrrole framework from planarity, forcing it to adopt a bowed shape with rings A and C tilted toward the distal side.[218]

The catalytically important residues are detailed in Figure 6 and arranged as follows. On the proximal side, His336 interacts at a distance of 3 Å with the iron atom through its N_ϵ atom. The δ nitrogen of the same residue is hydrogen bonded to Asn421, a situation reminiscent of the presence in a geometrically equivalent location of a carboxylate residue in the class I (e.g., Asp235 in C*c*P),[235] class II (e.g., Asp238 in lignin peroxidase),[236] and class III (e.g., Asp247 in HRP) peroxidases.[237] The interaction of a hydrogen-bond acceptor with the imidazole is believed to confer partial imidazolate character to the fifth iron ligand, thus increasing the stability of the high valent iron intermediate and enhancing the oxidative power of the enzyme. In the plant/fungal/bacterial peroxidases, an aromatic residue lies parallel to, and closely interacts with, the proximal histidine. As a case in point, Trp191 in C*c*P is the residue that bears one of the oxidizing equivalents in the compound I intermediate. In MPO, there is no equivalent to Trp191 close to His336, but a tryptophan conserved in all four mammalian enzymes, Trp477, is located slightly further away. No role has yet been assigned to this tryptophan, however.

On the distal side of the heme lie the three major catalytic residues: Gln91 (∼4.5 Å from the iron), His95 (∼5.5 Å from the iron), and Arg239 (∼7 Å from the iron). The two latter residues are the counterparts of the well-established His and Arg catalytic couple in the fungal and plant peroxidases.[237,238] The histidine plays a key role in a proton relay associated with the binding of H_2O_2 and the transfer of a proton from the iron-bound O_α to the leaving O_β. The charged arginine side chain helps to polarize the O−O bond, thus promoting its heterolytic scission.

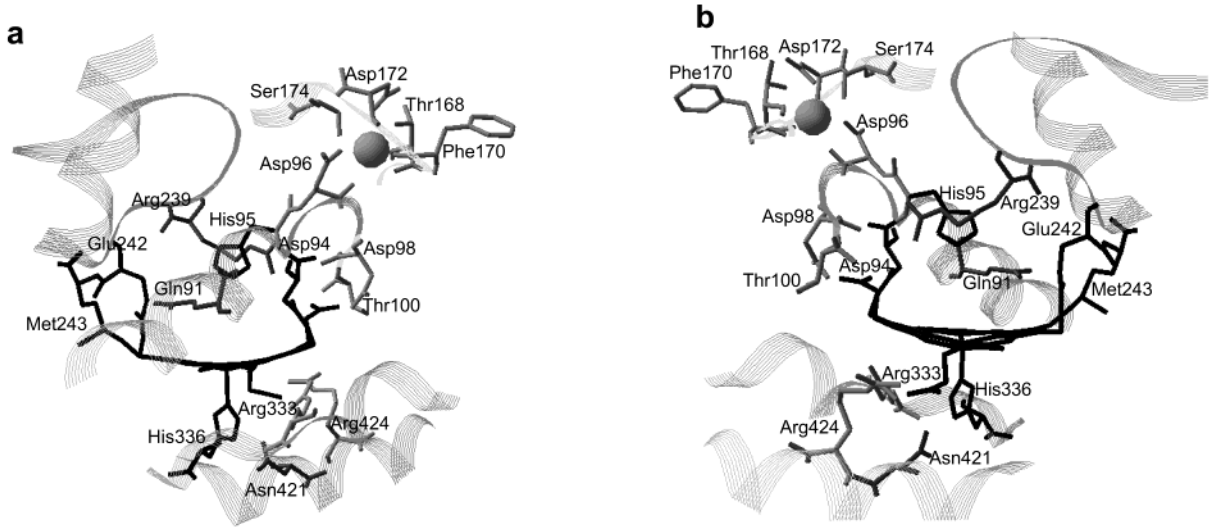


Figure 6. Detailed view of the active site residues implied in catalysis that interact with the heme or are part of the distal calcium binding site: (a) view from ring B, (b) view from ring D. The iron has been omitted for the sake of clarity, and the calcium is represented as a light gray sphere.

Table 1. Sequence Comparisons of the Four Secondary Structural Elements That Line the Active Sites of the Four Mammalian Peroxidases[a]

		Helix H2														
hMPO	85	M	F	M	Q	W	G	**Q**	L	L	**D**	**H**	**D**	L	**D**	F	**T**
hEPO	211	M	F	M	Q	W	G	**Q**	F	I	**D**	**H**	**D**	L	**D**	F	**S**
bLPO	216	L	F	M	Q	W	G	**Q**	I	V	**D**	**H**	**D**	L	**D**	F	**A**
hTPO	229	L	L	M	A	W	G	**Q**	Y	I	**D**	**H**	**D**	I	A	F	**T**
		Turn-helix H5														
hMPO	236	G	**D**	T	**R**	S	S	**E**	**M**	P	E	L	T	S	M	**H**	T
hEPO	362	G	**D**	T	**R**	S	T	**E**	T	P	K	L	A	A	M	**H**	T
bLPO	369	G	**D**	F	**R**	A	S	**E**	Q	I	L	L	A	T	A	**H**	T
hTPO	393	G	**D**	G	**R**	A	S	**E**	V	P	S	L	T	A	L	**H**	T
		Helix H8														
hMPO	324	**I**	**A**	**N**	**V**	F	T	N	A	F	**R**	Y	G	**H**	T	L	I
hEPO	450	**V**	**A**	**N**	**V**	F	T	L	A	F	**R**	F	G	**H**	T	M	L
bLPO	456	**I**	**S**	**N**	**V**	F	T	F	A	F	**R**	F	G	**H**	M	E	V
hTPO	481	**V**	**S**	**N**	**V**	F	S	T	A	A	F	**R**	F	G	**H**	A	T	I
		Helix H12														
hMPO	418	P	A	L	**N**	M	Q	R	S	**R**							
hEPO	544	A	A	L	**N**	M	Q	R	S	**R**							
bLPO	551	A	A	I	**N**	L	Q	R	C	**R**							
hTPO	576	A	S	I	**N**	L	Q	R	G	**R**							

[a] The human MPO, EPO, and TPO sequences are compared with the bovine LPO sequence. Important residues (see the text) are in bold, embossed residues are in direct contact with the iron and/or Fe═O moiety, and underlined residues participate in covalent attachment of the heme.

There does not seem to be an equivalent of Gln91 in the plant and fungal enzymes, but its location and putative role in hydrogen bonding within the active site hint at a probable role in catalysis.[239] Noncovalent polar heme−protein interactions primarily involve Thr100, which interacts through its O_γ and NH, and Asp98, which interacts through its O_δ, with the propionate side chain of ring C. The binding of the propionic acid group of ring D is stabilized by the guanidinium moieties of Arg333 and Arg424.[24]

Two halide binding sites have been identified: one is located on the proximal side of the heme at the N-terminus of helix H8, whereas the other, roughly located above the δ-methylidenyl bridge embedded in a network of hydrogen bonds formed by three water molecules, is the putative site for the halide that is oxidized. It has been proposed that a less restricted access to this second site in MPO than in other peroxidases (based on sequence comparisons) may partially explain why only MPO oxidizes chloride ions.[24,239] The binding site for small aromatic molecules is located just off the 8-methyl substituent on the distal side of the heme,[215,240,241] directly adjacent to the distal halide binding site. Finally, a calcium binding site is composed of residues Asp96, Thr168, Phe170, Asp172, and Ser174, confirming earlier suggestions.[242]

This analysis indicates that the set of four secondary structural elements surrounding the active site encompasses no less than 16 residues that are directly implicated in proper functioning of the enzyme (Table 1). Indeed, the structural elements that bear the catalytic residues also incorporate nearby residues that interact directly with the heme, the substrate, or the calcium. It is likely that these residues will be conformationally interdependent.

The sequence comparison clarifies why the third link present in MPO is not found in LPO, EPO, and TPO: in none of the latter proteins is the requisite methionine located adjacent to the conserved glutamate. Indeed, the three residues following this glutamate, located at the turn in the polypeptide chain just before helix H5, represent one of the highest regions of sequence variability within the active sites of the four peroxidases. Similar analysis of the sequence homology among related proteins[209] shows that while a proline is a frequent residue at the position equivalent to Pro244 in MPO, a carboxylate at the equivalent of position 245 is only found in MPO. These relationships suggest that the ability

to form the third bond may also depend on the presence of selected residues around the Met243 position (see below).

D. Mutagenesis Studies of Covalent Heme Binding

An earlier study demonstrated that the protein−heme ester bonds are formed through an autocatalytic, H_2O_2-dependent process.[22] In this process the methyl groups of the heme are formally oxidized into hydroxymethyl moieties. All four peroxidases have been cloned and successfully expressed: MPO and LPO in CHO cells;[243,244] MPO,[245] LPO,[22,245,246] and TPO[247] in HighFive insect cells; and EPO in K-562 cells.[248] Site specific mutagenesis studies directed at understanding the molecular basis for formation of the ester bonds and the properties conveyed by their presence have been conducted with both MPO and LPO. The analysis of bond formation in MPO is complicated by the presence of the third vinyl−sulfonium bond. The ester bonds are suspected of contributing to the ~10 nm bathochromic shift of the Soret band in LPO, EPO, and TPO relative to those of the classical peroxidases such as HRP, whereas the link between Met243 and the 2-vinyl in MPO induces the additional shift of ~18 nm characteristic of this protein.

1. The Ester Bond

Studies of the carboxylate ester links have centered on two approaches: (a) substitution of Asp by Glu and vice versa to investigate the influence of factors such as chain length, basicity, and nucleophilicity and (b) substitution by isosteric aliphatic chains to prevent bond formation and thereby explore the properties associated with ester bond formation. Substitution of a glutamine for Glu242 in MPO produced an enzyme with a ferric Soret band at 417 nm similar to that of LPO at 413 nm.[249−251] Furthermore, resonance Raman spectroscopy showed a large increase in the ν_4 band (~1370 cm^{-1}), indicative of a more symmetrical heme. The high- and low-spin EPR spectra revealed an increase in heme flexibility, and the oxidized versus reduced FITR difference spectrum showed that no bond had been formed between the amide side chain and the heme group. The peroxidatic and chlorinating activities of this mutant were decreased by more than 90% relative to the wild-type activities. Clearly, replacement of the glutamic acid by an isosteric, uncharged amino acid prevented formation of a bond to the 1-methyl group. The observed ipsochromic shift could equally well be explained by simultaneous loss of the link between the heme and Met243, but an increase in the intensity of the resonance Raman vinyl stretching bands make this unlikely.[250] It was not possible to distinguish in these experiments whether the decrease in activity simply reflected loss of the ester (and/or sulfonium) bond or also involved local conformational changes within the active site.

Replacement of Glu375 in LPO, the residue that corresponds to Glu242 in MPO, by a glutamine yielded a partially heme deficient enzyme with approximately 3% of the wild-type activity.[252] Here again, loss of the nucleophilic and/or basic character of the carboxylic acid group seems to bring about drastic changes. Mutation of Glu375 into an alanine yields an inactive enzyme (Kuo, J. M.; Ortiz de Montellano, P. R., unpublished results). The replacement of Glu375 by an Asp, in which the charge and properties of the carboxylic acid group are retained with a shorter tether to the backbone, has proved to be more informative.[253] In this study, HPLC analysis of the prosthetic group released from the protein made possible the determination of the extent and precise pattern of covalent heme binding in the mutant protein. The Glu375Asp mutant bound the heme in a 1:1 ratio with the protein, but with a much lower degree of covalent bonding. Approximately two-thirds of the heme was reversibly docked in the active site, and the other third was covalently bound to the protein through a single link with Asp225, the LPO equivalent of Asp94 in MPO. The HPLC analysis, which found virtually no prosthetic group with two additional hydroxyl groups, demonstrated the nearly complete absence of the doubly cross-linked heme group. Interestingly, the enzyme only had approximately one-third of the activity of wild-type LPO. However, as suggested by previous studies,[22] careful incubation with H_2O_2 resulted in essentially complete covalent attachment of the heme group to the protein. Analysis of the reaction course established that the bond to Asp225 was formed first, followed by bond formation to the mutated residue at position 375. However, the peroxidative activity of the enzyme with the singly bound heme group was very close to that of the wild-type enzyme with two covalent bonds. Thus, shortening the side chain at position 375 while retaining its nucleophilic and/or basic properties impairs formation of the ester bond at that site but still yields a functional enzyme with an unimpaired Asp225 to 5-methyl link. Formation of the second link to the 1-methyl in the mutant enzyme is still possible, but the resulting ester link does not improve the activity.[253]

The link between the aspartate and the 5-methyl has also been investigated. In MPO, Asp94 has been replaced by an isosteric analogue, either an apolar alanine or a polar asparagine.[219,250,251] The UV/visible spectra, the high- and low-spin EPR data, and the resonance Raman bands of these mutants revealed the presence of two enzymatic species, a nativelike species in which the Met243−vinyl link was preserved and another in which it was lost. The asparagine substitution generated an enzyme with one-third of the wild-type activity, whereas the valine mutant retained only a few percent of the chlorinating and peroxidatic activities. Reduced/oxidized difference FTIR analysis confirms that no ester bond is formed with the Asp94Val mutant.[219] The similarity of the results obtained with the Asp94Val and Asp94Asn mutants suggests that also no ester bond was formed in the latter case. Hence, formation of a covalent bond in MPO requires a carboxylic acid residue at position 94, but formation of such a bond may not be a compulsory condition for activity. In LPO, the equivalent Asp225 has been replaced by either a glutamate[253] or a valine.[252] The valine-

containing mutant reportedly retained 10-20% of wild-type activity at acidic pH but had unchanged UV/visible spectral properties. On the other hand, the Asp225Glu mutation caused a 60-fold decrease in activity and gave rise to a slight red shift of the Soret band and a clear 50% decrease in the RZ value. The RZ or "rheinheitzahl" value is the ratio of the 403 nm (heme) to the 275 nm (protein) absorbance. Analysis of the enzyme prosthetic group showed that only the bond between the unmutated Glu375 and the 1-methyl was formed. No evidence was found for formation of an ester bond between the 5-methyl and the Glu225 side chain. Contrary to what was observed with the Glu375Asp mutant, incubation with limited amounts of H_2O_2 did not restore the activity of the enzyme, nor did it trigger further covalent attachment of the cofactor to the enzyme. A similar incubation with H_2O_2 in the presence of added heme did not increase the extent of covalently bound heme. Thus, mutations of Asp225 impaired formation of the bond to the 5-methyl group and, in the case of the glutamate substitution, apparently had deleterious effects on the active site architecture (see above).

The collective results indicate that formation of the ester bonds is both essential for catalytic activity and is governed by stringent conformational/electronic factors, as an isosteric amide substitution does not lead to covalent bond formation and the Glu/Asp mutation impairs autocatalytic heme attachment. The results further suggest that the two ester bonds can be formed independently but may not be completely equivalent with respect to function of the protein.

2. The Vinyl–Sulfonium Bond

Met243 has been mutated to the amino acids found in the other members of the mammalian peroxidase family: the glutamine of bovine LPO,[251,254,255] threonine of human EPO,[254] and valine of human TPO.[254] In addition, a cysteine was also introduced at position 243 to explore a possible relationship with cytochrome *c*.[254] In each instance, a 14−18 nm blue shift of the Soret band relative to that of the wild-type was observed, confirming the role of the vinyl sulfonium band in the unusual spectroscopic properties of MPO. A more flexible heme environment was suggested by the increased rhombicity of the EPR signals, a D_{4h}-like chromophore symmetry similar to that of LPO revealed by resonance Raman, and the marked strengthening of the vinyl stretching bands. However, the peroxidatic activity of the mutants toward SCN^- and ABTS and the taurine chlorinating activity were reduced to <6% of the wild type activities. The chloride dissociation constants decreased 100−2000-fold, depending on the mutant and the pH. In each case, the Met243Thr mutant was the most active. Conversely, introducing a methionine in LPO at position 376 (equivalent to position 243 in MPO) leads to an enzyme with only ∼20% of the ABTS activity of wild-type LPO and no chlorinating activity at all.[244] On the other hand, when the whole Gln-Ile-Leu sequence of LPO was replaced by its Met-Pro-Glu MPO counterpart, one-third of the original ABTS activity of LPO was obtained; H_2O_2 treatment neither increased heme binding nor the specific activity, and no methionyl−vinyl sulfonium bond could be detected (Kuo, J.; Ortiz de Montellano, P. R., unpublished results). In sum, the vinyl sulfonium bond confers on MPO its unique red-shifted Soret band, lowers the chromophore symmetry, and reduces its flexibility.

E. Proposed Mechanisms for Covalent Bond Formation

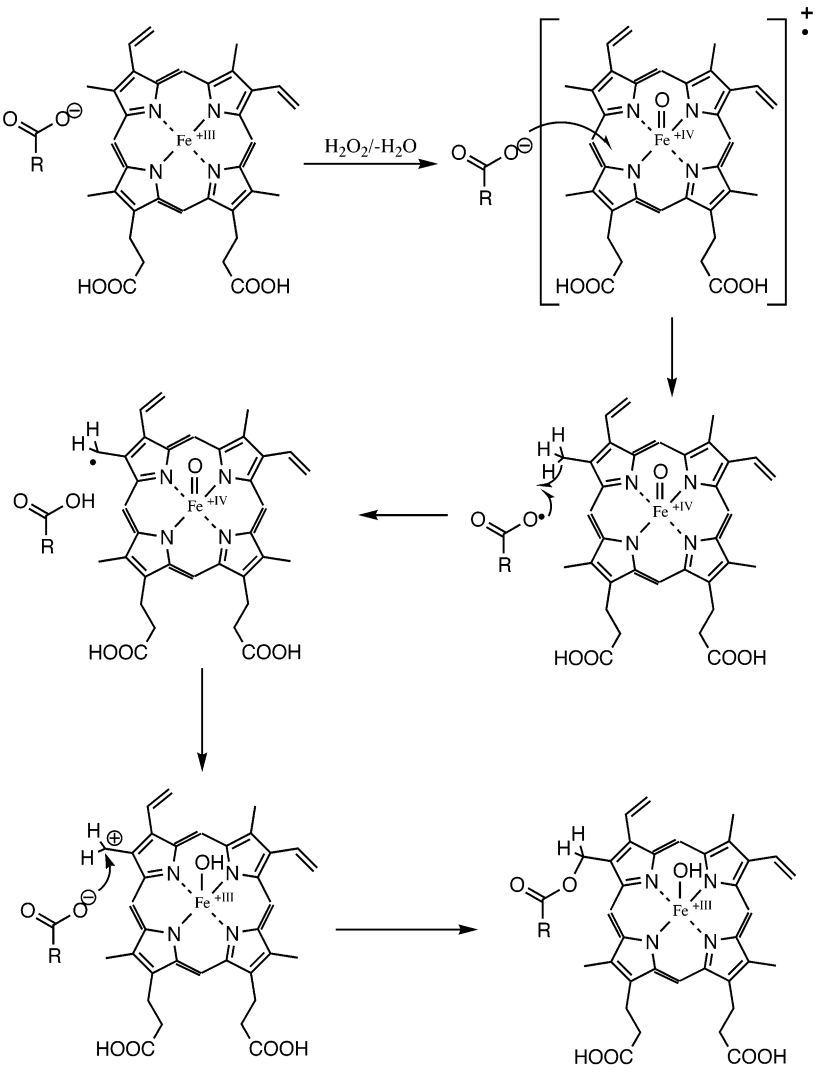
Despite the demonstration that the ester links in the mammalian peroxidases can be formed through an autocatalytic process,[22] and the subsequent extensions of this original finding (*vide supra*), little is known about the details of covalent bond formation or about the mechanism that forms the vinyl sulfone cross-link in MPO. It has been reported, however, that formation of mature MPO requires interaction with two chaperones, calnexin and calreticulin.[256] The organic chemistry literature provides little guidance on methyl group functionalization of intact tetrapyrrole ring systems, as this reaction apparently has not been investigated.

1. The Ester Bond

The mutagenesis studies indicate that a properly positioned carboxylic acid group is strictly required for covalent attachment of the heme group. The demonstration that formation of the ester bonds in LPO can be achieved simply by reaction of the heme: LPO complex with H_2O_2 [22] suggests that formation of a compound I-like intermediate is followed by a process that removes a hydrogen from each of the two methyl groups involved in cross-link formation. Removal of a hydrogen atom would generate a carbon radical that would be converted to a carbocation by transfer of one electron to the Fe^{IV} of the ferryl species. In this mechanism, the ester bond is then formed by trapping of the carbocation by the protein carboxylic acid group (Scheme 8).

A key question with regard to this mechanism is the identity of the species that abstracts the hydrogen atom from the methyl group. As it is sterically impossible for the ferryl species of compound I to directly remove the hydrogen atom, this mechanism requires that compound I first oxidize a protein side chain to a radical species that, in turn, removes the hydrogen from the methyl group. As two spatially distant methyls must be activated, two distinct, sequentially formed protein radicals must be invoked for formation of the mature protein. Two possibilities can be envisioned: in one, the protein radical is located on residues distinct from the carboxylic acid groups that trap the two final carbocation intermediates; in the other, the carboxylic acid groups themselves could be oxidized to radical species that abstract the hydrogen from the methyl groups and then, in a subsequent step, also trap the carbocation intermediates. The latter mechanism appears more probable as no easily oxidizable side chains are evident near Glu375 or Asp225 in the three-dimensional homology model of LPO.[215] For this mechanism to be valid, it must be possible (i) to abstract a hydrogen atom from the heme methyls in hemoproteins and (ii) to promote the one-electron oxidation

Scheme 8. Proposed Mechanism for the H_2O_2-Dependent Autocatalytic Formation of the Ester Bonds in Mammalian Peroxidases[a]



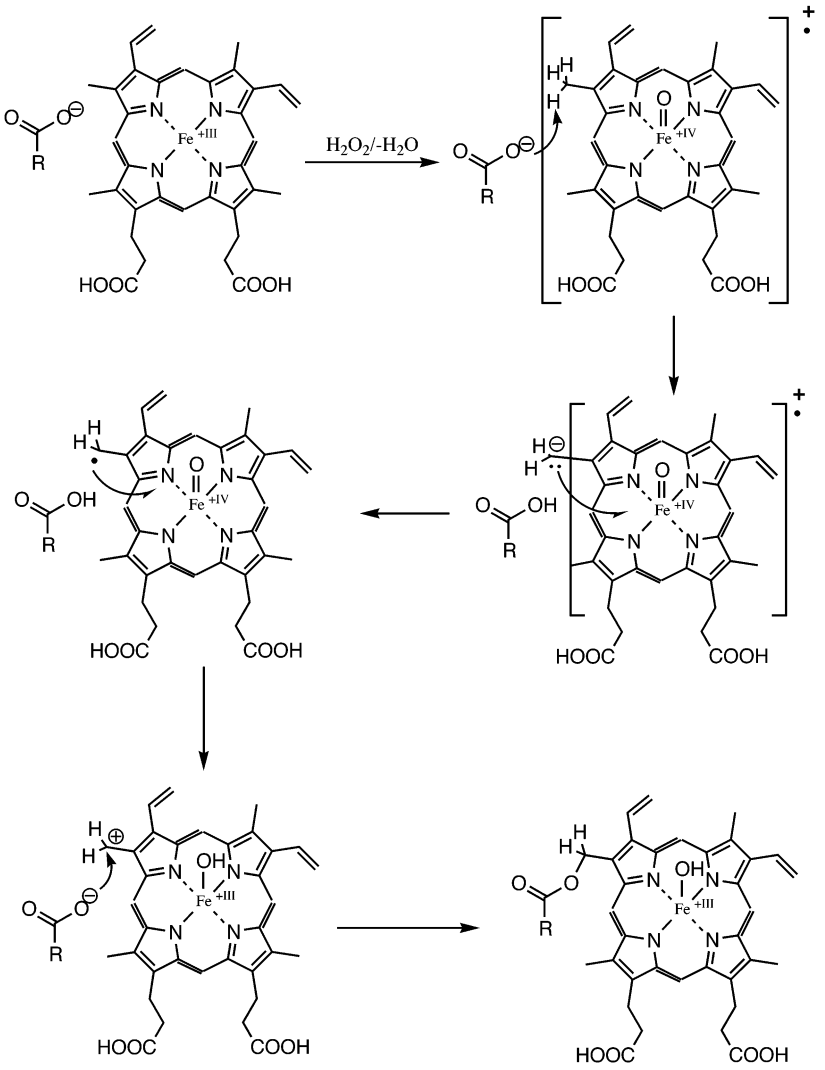
[a] The carboxylate plays the role of a redox catalyst before quenching the carbocation.

of carboxylic acids or carboxylate anions by hypervalent porphyrin intermediates.

It has already been demonstrated that a hydrogen can be abstracted from a heme methyl by a radical species and that this abstraction gives rise, if the iron is in the ferryl state, to a methyl carbocation. Specifically, it was found in the H_2O_2-mediated reaction of HRP with phenylhydrazine that the phenyl radical formed in the reaction can abstract a hydrogen atom from the 8-methyl to give, after transfer of an electron to the iron IV, a carbocation that is quenched by water. The heme group is converted in the reaction to the eventually isolated Fe^{III} 8-hydroxymethyl protoporphyrin IX.[1] Trapping of the carbocation by water was confirmed by the incorporation of labeled oxygen into this product when the reaction was run in $[^{18}O]H_2O$. When reconstituted with this latter compound instead of the natural Fe^{III} protoporphyrin IX, HRP shows spectral and kinetic properties very similar to those of the native enzyme, indicating that the introduction of an oxygen atom on the 8-methyl position does not in itself profoundly alter the chromophore properties of the hemoprotein.[257] This result suggests that the ester links to the methyl groups should not greatly alter the electronic properties of the heme in the mammalian peroxidases. An analogous study with the class II *C. cinereus* peroxidase also produced the Fe^{III} 8-hydroxymethyl protoporphyrin IX, but the reaction of phenylhydrazine with lignin peroxidase, another class II heme peroxidase, did not.[258] Subtle steric and/or electronic factors related to chromophore accessibility and reactivity thus intervene in this reaction.

Evidence relevant to the second point, the ability of hypervalent hemoprotein intermediates to oxidize carboxylic acids to free radical species, is also available in the literature. The oxidative decarboxylation of α-aryl-substituted carboxylic acids by porphyrins and cytochrome P450 enzymes, a reaction involving oxidation of the carboxylate group to a carboxylic radical, has been reported.[259,260] Indeed, it is relevant that the carboxylate group, the most probable physiologically relevant ionization state in the protein, is 600 times more reactive toward single electron abstraction than the carboxylic acid.[261,262] However, these results also point to a critical requirement if the carboxyl groups are to be involved in the hydrogen abstraction step: hydrogen abstraction from the

Scheme 9. Alternate Mechanism for the H_2O_2-Dependent Autocatalytic Formation of the Ester Bonds in Mammalian Peroxidases[a]



[a] In this instance, the carboxylate plays the role of an acid/base catalyst before quenching the carbocation.

methyl group by the carboxylate radical must be fast enough to outcompete the decarboxylation reaction, which itself is known to be fairly rapid.[262]

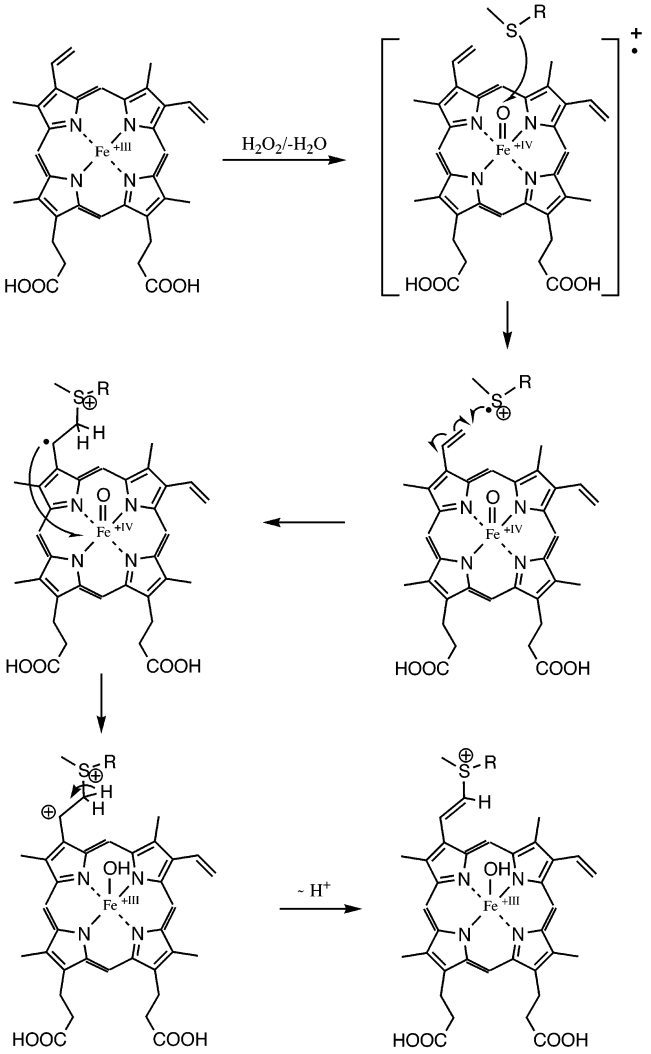
Two alternative mechanisms can be readily envisaged for formation of the ester bonds. In one of these, trapping of the carbocation by water would give the hydroxymethyl-heme that would, in a second step, react with the carboxylic acid to form the ester link. All that can be said about this mechanism, apart from the fact that is circuitous, is that noncovalently bound singly or doubly hydroxylated heme groups are not observed in the Glu/Asp mutants, although such products have been observed in the studies of cytochrome P450 to be discussed later. The ^{18}O studies required to definitively test this mechanism have not yet been carried out. In the second alternative mechanism (Scheme 9), the protein carboxylate anion functions as a base and removes a proton from the methyl group. Clearly, the porphyrin radical cation will cause some acidification of the methyl group, although it is not known whether this acidification would be sufficient for deprotonation by a carboxylate to occur. The carbanion thus formed would then transfer *two* electrons to quench the porphyrin radical cation and reduce the iron from the IV to the III state. The resulting carbocation would then be trapped as already discussed for the radical mechanism.

2. The Vinyl–Sulfonium Bond

A mechanism for formation of the vinyl−sulfonium bond in MPO can be proposed that is based on the autocatalytic mechanism invoked for formation of the ester links. Thus, oxidation of the methionine sulfur by compound I would generate a thioether radical cation that attacks the β-carbon of the 2-vinyl substituent. Electron transfer to the ferryl iron followed by deprotonation would produce the desired vinyl sulfonium link (Scheme 10). The β regiochemistry of the bond between the sulfur and the vinyl group is consistent with such an electrophilic attack of the sulfur on the vinyl group.

The reactions of radicals with the vinyl groups of porphyrins have been reviewed.[17] For instance, the $CCl_3^{\bullet}$ radical has been shown to add to the β-carbon of the 2-vinyl substituent of metmyoglobin, resulting in the formation of γ-trichloro methyl derivatives of protoporphyrin IX.[263] The nitrogen dioxide radical $NO_2^{\bullet}$ has also been reported to react following a

Scheme 10. Suggested Mechanism for Autocatalytic Formation of the Methionyl−Vinyl Sulfonium Link in MPO



similar reaction pathway with equine myoglobin[264] and with a model oxyferryl synthetic porphyrin.[265]

Inspection of the side chains in the vicinity of Met243 in MPO shows that Glu245, even if not in optimal geometry, is close enough to interact with the methionine sulfur atom.[24] Although not required, the arrangement of three carbonyl groups within less than 4 Å of the sulfonium moiety might explain the stereoselectivity and specificity of the methionine/vinyl adduct (Figure 7).

F. Function of Covalent Heme Binding

Conservation of covalent bonds to the heme in all the mammalian peroxidases, together with the requirement for at least one such bond for catalytic activity, persuasively argues that covalent heme attachment conveys a functional and/or structural advantage. However, the structural, mechanistic, or physiological advantage of a cross-linked heme remains elusive. No relationship appears to exist between the presence of the ester links and the substrate specificities of the enzymes. All of the peroxidases, including the plant and fungal enzymes without heme covalent bonds, readily oxidize phenols,

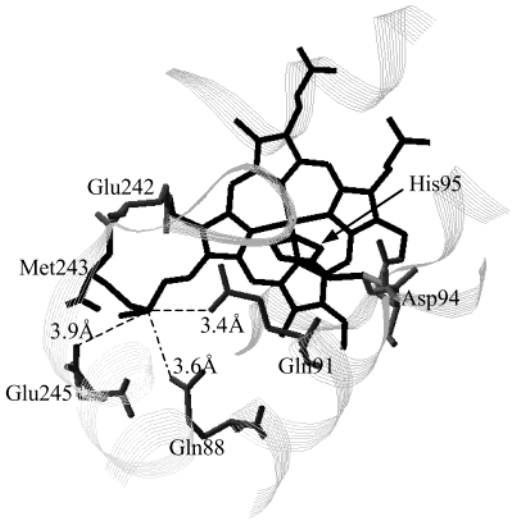


Figure 7. Carbonyl groups that possibly interact with the positive charge of the methionyl−vinyl sulfonium. The active side around porphyrin is viewed from the distal side. The proximal residues have been omitted for the sake of clarity. The carbonyl group of Glu242, 4.1 Å away, has not been taken into account.

iodide ion, and thiocyanate. The more difficult oxidation of bromide is catalyzed by the mammalian peroxidases but, albeit less efficiently, also by peroxidases without heme covalent bonds.[231,233] However, a clearer relationship may exist between the presence of the vinyl sulfone link in MPO and its exceptional ability to oxidize Cl^-. The electron-withdrawing effect of the positively charged vinyl−methionyl sulfonium link, which demonstrably shifts the Soret maximum, could also increase the redox potential of the ferryl species or, as postulated earlier,[239] decrease the electron density at the δ-methine bridge in a manner that increases electron transfer to that position from a vicinal Cl^- ion. However, the redox potentials of MPO and EPO appear to differ by only 0.06 V at pH 7, despite the presence of this additional methionine sulfonium bond in MPO.[266] Chloride is generally not oxidized by the other mammalian peroxidases, although EPO has a chlorine-oxidizing activity at acidic pH[267] or, with possible exceptions,[233] by most other classical peroxidases. Chloroperoxidase, which readily oxidizes chloride, cannot be compared with the classical peroxidases, because it has a cysteine thiolate rather than imidazole proximal iron ligand.[268]

Covalent heme attachment could contribute to binding of the water molecule that is produced by heterolytic scission of H_2O_2 in the formation of compound I. The presence of this specific H_2O in the active site has been postulated to play a key role in differentiating peroxidase and catalase activities.[269,270] Interestingly, Asp94 is the spatial equivalent of Phe41 in HRP, a residue that restricts access to the active side in that enzyme.[271,272] Covalent heme attachment could also distort the porphyrin framework, as has been postulated for the two cysteine−vinyl links in cytochrome *c*.[273−275] Heme deformation can facilitate spin−spin coupling between the compound I porphyrin π radical cation and oxoferryl moiety.[276] However, this cannot be critical, as the mutagenesis experiments show that LPO with a

single covalent link retains full catalytic activity and the electronic influence of a hydroxymethyl versus methyl group is unlikely to be significant. Thus, it is not known why covalent attachment of the heme is sufficiently important for the ester links to be consistently preserved through the evolution of the mammalian peroxidases.

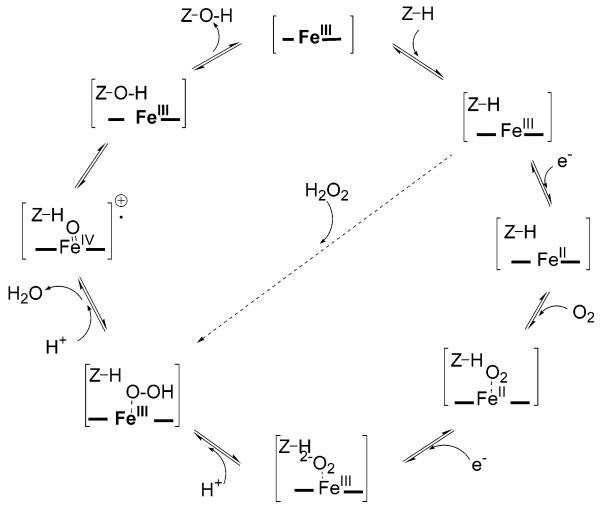
IV. CYP4A Family of Cytochrome P450 Enzymes

A. Introduction

The cytochromes P450 (CYP) represent a ubiquitous and very important class of heme-based monooxygenases. In mammals, these enzymes are not only critical for the metabolism and clearance of xenobiotic agents but are also essential for the biosynthesis of cholesterol and all sterol hormones,[277] the formation of physiologically active eicosanoids from arachidonic acid,[278] and, through metabolic control of the physiological levels of endobiotics such as retinoic acid,[279] in diverse aspects of cell biology. Genomic studies have shown that the complement of cytochrome P450 genes in different organisms ranges from one in *Mycobacterium leprae* to as many as ~450 in rice, ~270 in *Arabidopsis*, and ~57 in humans.[280] The present discussion, however, focuses exclusively on the CYP4 family of P450 enzymes that catalyze the ω- and (ω − 1)-hydroxylation of fatty acids. Despite an active search among the other families of P450 enzymes, the CYP4 family is the only one that has so far been found to undergo self-catalyzed covalent attachment of the prosthetic heme group to the protein.

The P450 enzymes are distinguished from the heme oxygenases and peroxidases (other than chloroperoxidase) by the fact that a cysteine thiolate rather than a histidine nitrogen functions as the proximal heme iron ligand.[281] The catalytic cycle of cytochrome P450 is also more complex than that of the peroxidases in that the activated oxidant is generated by a catalytic cycle that involves (a) reduction of the iron from the ferric to the ferrous state ($PFe^{III} \rightarrow PFe^{II}$), (b) reversible binding of molecular oxygen to give a dioxy complex ($PFe^{II}-O_2$), (c) reduction of the ferrous dioxy complex by a second electron to give a putative ferric peroxy anion complex ($PFe^{III}-O_2^-$), (d) diprotonation of the distal oxygen in the complex with concomitant extrusion of a molecule of water to give a ferryl species coupled to a porphyrin or protein radical ($P^{+\bullet}Fe^{IV}=O$), (e) oxygen transfer to the substrate, and finally (f) product dissociation.[282,283] The P in the parenthetical formulas refers to the porphyrin and/or protein. The final activated ferryl species can transfer the oxygen to even unactivated C−H bonds via either a quasi-concerted or stepwise mechanism (Scheme 11).[284,285] The two electrons required for oxygen activation derive from NADPH or NADH and are transmitted to the P450 enzyme by ancillary proteins, either NADPH-cytochrome P450 reductase or a flavoprotein and iron−sulfur protein pair, although in some instances the electron donor protein is directly fused to the P450 enzyme.[286] In some mammalian systems, including the CYP4 family of enzymes, catalytic

Scheme 11. General Cytochrome P450 Catalytic Cycle Illustrated for Hydroxylation of a Hypothetical Substrate Z[a]



[a] The H_2O_2-dependent shunt cycle is also shown.

turnover is facilitated by the additional presence of cytochrome b_5, which appears to function by more rapidly delivering the second electron.[287] In many, but not all, instances, it is possible to short circuit the oxygen activation step by providing the P450 enzyme with H_2O_2 or a comparable peroxide rather than with oxygen and electrons.[288,289] This shunt mechanism usually results in more rapid degradation of the heme group and protein, does not always give the same substrate oxidation regiospecificities, and in some instances simply does not work. Nevertheless, it shows that the mechanisms of the peroxidases and P450 enzymes are not entirely distinct. Indeed, the ferric peroxo complex ($PFe^{III}-OOH$) obtained after protonation of the ferric peroxo anion is thought to be similar to the intermediate obtained in the shunt reaction with H_2O_2, although the additional protons brought into the P450 active site by the H_2O_2 (as compared to O_2) may contribute to whatever catalytic differences exist between the two systems. In essence, the equivalent of a peroxo complex appears to be generated in situ in the normal NADPH and O_2-dependent turnover of these enzymes.

B. Covalent Heme Binding

The recent finding that many members of the CYP4 family of P450 enzymes covalently bind their prosthetic heme group is completely unexpected. The presence of a covalent bond between the heme and the apoprotein was first demonstrated for five members of the CYP4A family, specifically rat CYP4A1, CYP4A2, CYP4A3, and CYP4A8, and human CYP4A11, by HPLC, gel electrophoresis, and mass spectrometry.[27] Proteolytic digestion of the enzymes showed that the modification of the prosthetic group shifted its mass by 16 amu, consistent with the introduction of a single oxygen atom. Subsequent comparison of the modified heme with the products obtained in the mammalian peroxidase studies has established that the modified prosthetic group proteolytically released from the protein is

5-hydroxymethylheme.[290] Analysis of the sequences of the heme-bound peptides, all of which involved the same alkylated residue, furthermore established that the porphyrin is probably linked to the protein backbone through a specific glutamic acid residue (Glu318 in CYP4A3). The covalent link is therefore almost certainly an ester bond between the glutamic acid and a hydroxyl group on the 5-methyl of the heme.[290] A strong analogy is thus apparent between the heme link in the CYP4 enzymes and the heme links in the mammalian peroxidases. These studies were independently extended by evidence that members of the CYP4B and CYP4F families also covalently bound their prosthetic heme group.[28] In all cases, covalent binding correlated with the presence in the protein sequence of the equivalent of Glu318 in CYP4A3.

Site-directed mutagenesis has clarified the process of covalent bond formation. Replacement of the glutamate implied heme covalent binding in the CYP4A family by an alanine suppressed covalent binding.[290] In the case of CYP4A3, a Glu318Gln substitution also suppressed covalent bond formation, which suggests that the negative charge or oxidizability of the carboxyl group, in addition to its nucleophilicity, contributes to covalent bond formation, as the glutamine side chain only retains the nucleophilicity of the glutamic acid. Even more informative is the finding that replacement of the glutamic acid by an aspartate both attenuates covalent binding of the heme group and results in the appearance of a new, noncovalently bound heme derivative.[291] This modified heme has been identified as the same 5-hydroxymethylheme as is obtained when the covalently bound heme is released from the wild-type proteins. Thus, the carboxyl group is the single requirement for both formation of the ester bond and oxidation of the methyl to a hydroxymethyl group. Mechanisms proposed to explain methyl hydroxylation and covalent bond formation must therefore satisfactorily explain the role of the carboxylic acid group.

A key additional finding is that formation of the covalent bond, as found earlier for the mammalian peroxidases,[22] occurs through a self-catalyzed process. Thus, the CYP4 proteins obtained from an *E. coli* expression system commonly contain both covalently and noncovalently bound heme. Aerobic incubation of these proteins with cytochrome P450 reductase, cytochrome b_5, lauric acid, and NADPH results in an increased extent of covalent heme binding.[290,291] Furthermore, some of the CYP4F isoforms do not have the glutamic acid required for covalent binding and do not covalently bind their heme. However, if the glutamic acid is introduced by mutagenesis, as shown with CYP4F5, which has a glycine at the position in question, some covalent heme binding is observed, as is the formation of noncovalently bound 5-hydroxyheme.[291] Both of these parameters increase when the complex of heme with the CYP4F5 Ala→Glu mutant is incubated with the components required for catalytic turnover of the protein.[291] These results confirm the earlier findings that (a) the carboxylic acid is required for both heme methyl oxidation and covalent link formation, (b) precise positioning of the heme is more critical for formation of the protein ester link than for oxidation of the heme methyl group, and (c) heme oxidation and covalent heme formation are autocatalytic processes.

No crystal structure is available for a CYP4 enzyme, or for any other closely related protein, but a homology model of CYP4A11 based on the crystal structure of CYP102 has been reported.[292] The CYP4A11 model has been used to obtain an approximate evaluation of the position of the critical glutamic acid with respect to the heme. The analysis indicates that Glu318 in CYP4A3, and the corresponding residues in CYP4A11 and the other CYP4 enzymes, is located on a highly conserved stretch of amino acids within the I-helix that spans the distal face of the active site.[290] Although the position of the glutamic acid cannot be defined very accurately in such homology models, it is interesting that the side chain of the glutamic acid is close to the heme 5-methyl group in the CYP4A11 model.

The above results suggest that the mechanism proposed for covalent heme binding in the mammalian peroxidases applies in essential detail to covalent heme binding in the CYP4 enzymes (see Scheme 8). The cofactor requirements and the autocatalytic nature of the process indicate that an oxidative species identical or similar to that formed in normal turnover of the P450 enzymes is required for heme modification. The critical role of the glutamate carboxyl group in oxidation of the methyl to a hydroxymethyl group can be rationalized, as in the case of the peroxidases, by postulating a single electron abstraction by the ferryl species from the carboxyl group to give a protein carboxyl radical. Abstraction of a hydrogen atom from the 5-methyl of the activated heme by the carboxyl radical then generates a benzylic-type radical that is converted to a carbocation by transfer of an electron to the iron atom, reducing it from the Fe^{IV} to the Fe^{III} state. Covalent heme attachment results from trapping of the carbocation by the carboxylate group. However, when the carboxyl group is not optimally placed relative to the 5-methyl group, the carbocation can be competitively trapped by a water molecule to give 5-hydroxyheme without a covalent link to the protein, as observed when the glutamic acid is replaced by a aspartate in CYP4A3 and when a glutamic acid is introduced into CYP4F5.[291]

C. Function of Covalent Heme Attachment

Why is the heme covalently bound in some but not all CYP4 enzymes? The fact that some of the CYP4 enzymes do not covalently bind their heme and the observation that most P450 enzymes do not have such a covalent link indicate that covalent heme attachment satisfies a requirement that is specific for the CYP4 enzymes and, indeed, to only some CYP4 enzymes. The possibility that covalent heme binding is accidental must be considered, although the relatively high conservation of this active site feature in the CYP4 family would seem to make this unlikely. An extensive investigation of the properties of the CYP4 enzymes with and without a covalently

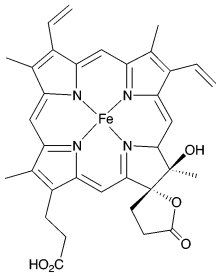
bound heme will be required to convincingly establish the reason for covalent heme binding. However, preliminary data suggest at least one key role for covalent heme binding (Lebrun, L.; Ortiz de Montellano, P. R., unpublished results). Thus, whereas CYP4A1 oxidizes lauric acid with a 20:1 $\omega/(\omega - 1)$ regiospecificity ratio, the regiospecificity ratio for the E320A mutant that does not bind the heme covalently drops to 5:1. Similarly, CYP4A11 favors ω-over $(\omega - 1)$-hydroxylation of lauric acid by a 17:1 ratio, while the ratio for the E321A mutant without a covalent bond drops to only 2.8:1. A comparable decrease in ω-hydroxylation specificity is not observed, however, with a P450 enzyme that has a lower intrinsic ω-regiospecificity. Thus, the $\omega/(\omega - 1)$ hydroxylation ratio for CYP4A3 is 3:1, and an E318Q mutation that suppresses covalent heme binding in this protein has little effect on the ratio (3.6:1). The results suggest that covalent heme binding is required to obtain a high $\omega/(\omega - 1)$ hydroxylation ratio, but is not critical for lower ratios. ω-Hydroxylation produces vasoconstrictor eicosanoids and is required for the formation of fatty acid dicarboxylic acids, so a high preference for this reaction may be important in specific physiological situations.

A role for covalent heme binding in favoring higher ω- to $(\omega - 1)$-regiospecificities is readily envisioned. As argued earlier,[293,294] ω-hydroxylation of the fatty acid chain is more difficult on thermodynamic grounds than oxidation of one of the chain methylenes, because of the higher bond strength of a primary (methyl) C−H bond relative to that of a secondary (methylene) C−H bond, all other things being equal. This inherent reactivity difference underlies the observation that P450 enzymes not specifically evolved for fatty acid ω-oxidation, such as CYP2B1 or CYP2E1, heavily favor $(\omega - 1)$- over ω-hydroxylation. In mammals, only the CYP4 enzymes exhibit a preference for ω-hydroxylation. To achieve this preference, the CYP4 family of enzymes must suppress $(\omega - 1)$-hydroxylation, presumably by sterically restricting access to the ferryl species, as the ferryl species will react with the weaker methylene C−H bond if allowed to do so. It is therefore likely that the active site of CYP4 enzymes is highly constrained to only allow exposure of the terminal fatty acid methyl group to the reactive oxygen species. Covalent binding of the heme to the protein, by anchoring the heme to the protein, may allow a finer discrimination against exposure of the $(\omega - 1)$-methylene group to the ferryl species and thus enhance the ω-regiospecificity of the enzyme.

V. Conclusions

The physiological, autocatalytic modification of heme has been illustrated here in the context of (a) the conversion of heme to biliverdin, ferrous iron, and CO by heme oxygenase, (b) double or triple cross-linking of the heme to the protein in the mammalian peroxidases, and (c) single cross-linking of the heme to the protein in most members of the CYP4 family of P450 enzymes. In each instance, a normal catalytic process is employed to produce either mature hemoproteins or heme metabolites with important physiological functions. The "biosynthetic" nature of these transformations distinguishes them from the many modifications of heme groups commonly mediated by interaction with xenobiotics that result in abnormal heme degradation or modification and enzyme inactivation.[17]

Chart 3. Structure of the Heme *d* Prosthetic Group of *E. coli* Catalase-Peroxidase HPII



The incidence of autocatalytic heme modification is not limited to the examples discussed in this review. Autocatalytic processing of the prosthetic heme group has been clearly demonstrated, or at least shown to be conceivable, in other hemoprotein systems in which a modified heme is present. A case in point is the autocatalytic modification of the heme group in the catalase peroxidase HPII of *Escherichia coli*. The prosthetic heme group in this protein is heme *d* (Chart 3), in which one of the propionate carboxylic acid groups cyclizes to form a lactone ring with the porphyrin carbon to which the propionate is attached and a hydroxyl is added at the adjacent carbon, resulting in a partially saturated pyrrole ring.[295] The structure of the modified prosthetic heme group is confirmed by the high-resolution crystal structure of the protein, which also shows that the proximal tyrosine (Tyr415) that is coordinated to the iron has acquired a covalent bond from its benzylic C^{β} carbon to the N^{δ} nitrogen of a proximal histidine.[296,297] To determine whether the modified heme arose by an autocatalytic process, the authors made use of the fact that a fraction of the protein obtained from *E. coli* grown under low oxygen conditions contains unmodified iron protoporphyrin IX.[298] Incubation of this protein with H_2O_2 was shown to convert the heme group into heme *d*, a clear demonstration of autocatalytic processing. It is likely that the unusual tyrosine−histidine bond in the protein is also the result of autocatalytic processing. Interestingly, mutation of a valine proximal to the distal catalytic histidine residue to a cysteine results in inactivation of the protein concomitant with the formation of a covalent bond between the cysteine and the histidine.[299]

Although not unambiguously established, free radical reactions appear to be involved in some, and possibly all, of the autocatalytic heme modification reactions. Free radical intermediates have been specifically identified in the case of heme oxygenase and are likely intermediates in formation of the ester bonds that cross-link the heme to the mammalian peroxidase and CYP4 proteins. Free radical reactions are consistent with the unique features of the heme group, which contains an iron atom that readily

undergoes one-electron interconversions between the Fe^{II}, Fe^{III}, and Fe^{IV} states.

VI. Acknowledgment

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