Isoporphyrin Intermediate in Heme Oxygenase Catalysis

OXIDATION OF α-MESO-PHENYLMEME

Received for publication, November 27, 2007, and in revised form, May 16, 2008. Published, JBC Papers in Press, May 16, 2008, DOI 10.1074/jbc.M709685200

John P. Evans, Fernando Niemez, Graciela Buldain, and Paul Ortiz de Montellano

From the Department of Pharmaceutical Chemistry, University of California at San Francisco, San Francisco, California 94158 and Departamento de Quimica Orgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (C1113AAD), Buenos Aires, Argentina

Human heme oxygenase-1 (hHO-1) catalyzes the O2- and NADPH-dependent oxidation of heme to biliverdin, CO, and free iron. The first step involves regiospecific insertion of an oxygen atom at the α-meso carbon by a ferric hydroperoxide and is predicted to proceed via an isoporphyrin π-cation intermediate. Here we report spectroscopic detection of a transient intermediate during oxidation by hHO-1 of α-meso-phenylheme-IX, α-meso-(p-methylphenyl)-mesoheme-III, and α-meso-(p-trifluoromethylphenyl)-mesoheme-III. In agreement with previous experiments (Wang, J., Niemez, F., Lad, L., Huang, L., Alvarez, D. E., Buldain, G., Poulos, T. L., and Ortiz de Montellano, P. R. (2004) J. Biol. Chem. 279, 42593–42604), only the α-biliverdin isomer is produced with concomitant formation of the corresponding benzoic acid. The transient intermediate observed in the NADPH-P450 reductase-catalyzed reaction accumulated when the reaction was supported by H2O2 and exhibited the absorption maxima at 435 and 930 nm characteristic of an isoporphyrin. Product analysis by reversed phase high performance liquid chromatography and liquid chromatography electrospray ionization mass spectrometry of the product generated with H2O2 identified it as an isoporphyrin that, on quenching, decayed to benzoylbiliverdin. In the presence of O2, one labeled oxygen atom was incorporated into these products. The hHO-1-isoporphyrin complexes were found to have half-lives of 1.7 and 2.4 h for the p-trifluoromethyl- and p-methyl-substituted phenylhemes, respectively. The addition of NADPH-P450 reductase to the H2O2-generated hHO-1-isoporphyrin complex produced α-biliverdin, confirming its role as a reaction intermediate. Identification of an isoporphyrin intermediate in the catalytic sequence of hHO-1, the first such intermediate observed in hemoprotein catalysis, completes our understanding of the critical first step of heme oxidation.

Heme metabolism by heme oxygenase (HO) is an important process in animals that alleviates the potentially deleterious effects of free heme. The reaction releases iron, which is largely recycled, and produces CO and biliverdin. Recent studies have shown that the CO has important physiological functions, in part as a cell signaling molecule (1), whereas the biliverdin is immediately converted to bilirubin, a powerful antioxidant species (2, 3). A growing body of evidence suggests that these heme oxygenase products have significant protective roles in a number of disease processes, including atherogenesis and carcinogenesis (4, 5). In addition, heme degradation by pathogenic bacterial heme oxygenases, which is essential for their survival because it serves as a primary source of iron, makes these enzymes of potential interest for antibacterial strategies (6).

Human heme oxygenase-1 (hHO-1) utilizes O2 and NADPH in the three-step process shown in Fig. 1 (7–9). The heme acts both as a substrate and a cofactor that sequentially activates a total of three O2 molecules in oxidation of the macrocycle, a process that requires seven electrons provided by NADPH-cytochrome P450 reductase (CPR). In the first step, the two-electron reduction of O2 at the heme iron generates a ferrous hydroperoxide intermediate (Fe(III)-OOH) that inserts the terminal oxygen of the peroxide moiety into the α-meso carbon of the same heme, generating α-meso-hydroxyheme (10, 11). The second step utilizes one molecule of O2 to form verdoheme in a reaction that results in elimination of the α-meso carbon as CO. Finally, in the third step the verdoheme is transformed into biliverdin in a reaction that requires both O2 and five reducing equivalents from CPR.

An analogous heme degradation reaction can occur spontaneously in a coordinating solvent such as pyridine in the presence of an electron donor such as ascorbate. This process of “coupled oxidation” is non-regiospecific so that random oxidation occurs at all four meso carbons to give a mixture of the corresponding IXα, IXβ, IXγ, and IXδ biliverdin isomers (12, 13). In contrast, the hHO1-catalyzed reaction regiospecifically oxidizes the α-meso carbon. Multiple factors contribute to this hHO-1 regiospecificity. Of primary importance are the steric constraints imposed by the distal helix in which two conserved glycine residues at its center, Gly-139 and Gly-143, provide the...
flexibility required to sandwich the heme between two helices (14, 15) while at the same time suppressing conversion of the ferric hydroperoxide intermediate to a ferryl (Fe(IV) = O) species (16). Furthermore, the structure of nitric oxide-bound hHO-1 indicates that in addition to this steric constraint, a hydrogen bonding network involving Asp140-water-NO H-bonds cause a tilt of the NO toward the α-meso carbon (17). Finally, the orientation of the heme relative to an axis through the iron perpendicular to the heme plane is determined by electrostatic interactions between charged residues on the protein and the heme propionate groups (18, 19).

Heme oxygenase can utilize a number of alternate Fe(III)porphyrins as substrates with the only clear prerequisite being the presence of the two vicinal propionate groups (20). Previous studies have investigated the oxidation of hemes in which the α-meso carbon bears a methyl, formyl, or phenyl substituent (21–23). Surprisingly, phenyl-substituted hemes readily bind to the hHO-1 active site due to the presence of a hydrophobic cavity, formed by residues Met-34, Phe-37, and Phe-214, that is postulated to function as a CO trapping site (24). The hHO-1 oxidation of α-meso-methyl and -phenyl-substituted hemes produces α-biliverdin, whereas the oxidation of α-meso-formyl heme does not. The product containing the α-meso carbon in the oxidation of α-meso-methylheme was not identified but was inferred to be acetic acid. More recently, benzoic acid has been identified as a product of the hHO-1-catalyzed oxidation of α-meso-phenylheme. An additional intermediate is required to account for benzoic acid formation (23).

To further characterize the oxidation mechanism of hHO-1, we report here additional studies of the hHO-1-catalyzed oxidation of α-meso-phenylheme as well as the symmetrical meso-heme III (MH), α-meso-(p-methylphenyl)-meso-heme-III (CH$_2$PMH), and α-meso-(p-trifluoromethylphenyl)-meso-heme-III (CF$_3$PMH). We anticipated that the addition of substituents with varying electron donating/withdrawing properties would modulate the reactivity in a way that would facilitate the detection of intermediates. A unique absorbance appeared transiently during the catalytic reaction with NADPH-CPR in the near IR region that is characteristic of an isoporphyrin. The formation of a long-lived isoporphyrin was confirmed by mass spectrometry in the reaction supported by H$_2$O$_2$. This is the first actual evidence for formation of this unique tetrahedral intermediate during HO catalytic turnover.
Isoporphyrin in Heme Oxygenase Catalysis

anaerobic cuvette with a side arm, where \( \text{H}_2\text{O}_2 \) was contained in the side-arm until gas exchange was complete, as described previously (34). To monitor the stability of the isoporphyrin-hHO-1 complex, 10 \( \mu \text{g} \)/ml catalase was added to the reaction of 5 \( \mu \text{M} \) arylheme-hHO-1 and 40 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) after 20 min, and the decay was monitored over 6 h.

**CO Formation Assay**—Horse myoglobin (Sigma) was used as a CO trap according to the protocol previously described (23). The reactions (1 ml) consisted of 10 \( \mu \text{M} \) arylmesoheme-hHO-1 complex, 60 nm CPR, and 100 \( \mu \text{M} \) NADPH in standard buffer and were allowed to proceed for 20 min in a sealed container before the addition of myoglobin via syringe to a final concentration of 4 \( \mu \text{M} \).

**Biliverdin Isomer Analysis**—A solution (300 \( \mu \text{l} \)) of 100 \( \mu \text{M} \) heme-hHO-1 complex, 2.7 \( \mu \text{M} \) CPR, 5 nm NADPH, 10 \( \mu \text{g} \)/ml catalase, and 17 units/ml superoxide dismutase in standard buffer was incubated for 1.5 h at room temperature. The reaction was quenched with two drops of hydrochloric acid (37%) plus a few drops of acetic acid and was then extracted with 700 \( \mu \text{l} \) of \( \text{CH}_2\text{Cl}_2 \). The work-up, esterification, and HPLC analysis were carried out as described previously (19).

**Product Analysis by HPLC and LC-ESI-MS**—Reactions (500 \( \mu \text{l} \)) containing 10 \( \mu \text{M} \) heme-hHO-1 and either 80 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) or 10 \( \mu \text{g} \)/ml catalase, 160 nm CPR, and 100 \( \mu \text{M} \) NADPH in standard buffer were quenched after 20 min with 100 \( \mu \text{l} \) of 1 \( \text{M} \) \( \text{HCl} \). Additional reactions were performed by first incubating with 80 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 20 min followed by the addition of 10 \( \mu \text{M} \)/ml of catalase, 160 nm CPR, and 100 \( \mu \text{M} \) NADPH for an additional 20 min before the acid quench. The quenched reactions were immediately extracted with 700 \( \mu \text{l} \) of \( \text{CH}_2\text{Cl}_2 \), and the organic phase was evaporated under a stream of air. The resulting residue was dissolved in 100% acetonitrile and was then analyzed by either an Agilent 1200 Series HPLC equipped with a G1315B diode array detector using a YMC ODS-AQ column (S-5, 120 Å, 4.5 \( \times \) 250 mm) or by LC-ESI-MS performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system using a Symmetry ODS column (Waters, 3.5 \( \mu \text{m} \), 2.1 \( \times \) 150 mm). Solvents were water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The running conditions were identical for each case differing only in flow rate, 1.0 ml/min for the 4.5-mm inner diameter column or 0.2 ml/min for the 2.1-mm inner diameter column, and were as follows: 30% B for 5 min, 30–80% B in 35 min, 80–95% B in 5 min, 95% B for 5 min, 95–30% B in 5 min, and finally 30% B for 5 min. The settings for the mass spectrometer were as follows: mode, ESI+; capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 300 °C; source temperature, 120 °C.

**Origin of the Oxygen Atoms in the Products**—Oxidation of arylmesohemes by hHO-1 was carried out in the presence of either \( ^{18}\text{O}_2 \), \( ^{18}\text{O}_2\text{H}_2 \), or \( ^{18}\text{O}_2\text{H}_2\text{O} \). Reactions in the presence of \( ^{18}\text{O}_2 \) and \( ^{18}\text{O}_2\text{H}_2\text{O} \) were performed as described previously (23). All reactions in the presence of \( ^{18}\text{O}_2\text{H}_2\text{O} \) were performed as described above but with \( ^{18}\text{O}_2\text{H}_2\text{O} \) in place of \( \text{H}_2\text{O}_2 \).

**RESULTS**

**UV-visible Spectra of meso-Phenylheme-reconstituted hHO-1**—The symmetrical MH, \( \text{CH}_3\text{PMH} \), and \( \text{CF}_3\text{PMH} \) were combined with hHO-1 followed by hydroxypatite chromatography to remove excess and non-specifically bound heme. The UV spectra of the ferric, ferrous, and ferrous-CO forms at pH 7.4 (supplemental Fig. S1) reveal that all three heme analogues bind to the active site in a similar mode as heme and, most importantly, that the heme-iron can undergo redox cycling. In the spectrum of MH, replacement of the vinyl groups of heme by ethyl groups places greater electron density on the iron and causes a blue shift of the Soret band from the normal 404 to 394 nm. Upon reduction to the ferrous state, the Soret maximum shifts to 421 nm and, upon further exposure to CO, to 410 nm, consistent with formation of the ferrous-CO complex. In comparison to the MH-hHO-1 complex, the \( \text{CH}_3\text{PMH} \) and \( \text{CF}_3\text{PMH} \) complexes exhibit a red-shifted Soret maximum at 400 nm that shifts to 426–428 nm in the ferrous state and to ~416 nm in the ferrous-CO complex. This red-shift is consistent with the presence of an electron-withdrawing substituent (i.e. aryl group) on the heme \( \alpha\text{-meso} \) carbon that removes electron density from the iron.

**Formation of CO during the NADPH/CPR-supported Reaction**—The normal degradation of heme by hHO-1, supported by electrons from NADPH via CPR, produces biliverdin IXα and CO. An assay based on the binding of CO to ferrous deoxymyoglobin was used to investigate its formation in the NADPH/CPR-supported reactions of MH, \( \text{CH}_3\text{PMH} \), and \( \text{CF}_3\text{PMH} \). In the presence of CO, the Soret maximum of Fe(II) Mb at \( \lambda_{\text{max}} = 434 \text{ nm} \) shifts to \( \lambda_{\text{max}} = 422 \text{ nm} \) (Fe(II)-CO Mb) with \( \alpha/\beta \) bands at 540 and 570 nm, respectively. In the reaction of MH with hHO-1, degradation of the heme clearly generates CO, as shown by the resulting peak at 422 nm. The reactions of \( \text{CH}_3\text{PMH} \) and \( \text{CF}_3\text{PMH} \) do not show the clear shift in the Soret maximum of Fe(II) Mb, instead giving peaks at 431 and 428 nm, respectively, without \( \alpha/\beta \) bands. The absence of the expected peak shifts indicates that no CO was formed.

**Biliverdin Isomer Analysis by HPLC**—To facilitate the isolation and identification of the biliverdin isomers produced from the normal HO reaction, the products were dimethyl-esterified before HPLC analysis. Chemical oxidation in the absence of hHO-1 using ascorbic acid under coupled oxidation conditions gives a mixture of the isomers from reaction at the non-substituted \( \text{meso} \) positions of each heme analogue (Fig. 2) (28). However, the hHO-1-catalyzed oxidation of each heme analogue in the presence of NADPH/CPR produces only the \( \alpha\text{-meso} \) biliverdin isomer. Even using sodium ascorbate as the electron donor, hHO-1 enforces the specificity for the \( \alpha\text{-meso} \) position, producing only the \( \alpha\text{-meso} \) biliverdin derivative (not shown).

**Single Turnover Reaction Supported by NADPH-CPR**—We examined the reactivity of each of the modified heme-hHO-1 complexes with NADPH/CPR (Fig. 3). Upon the addition of NADPH (50 \( \mu \text{M} \), 10 eq relative to heme-hHO-1 complex), CPR (0.04 \( \mu \text{M} \)), and catalase, the heme-hHO-1 complex changed immediately to the ferrous-oxy form, as indicated by a shift of the Soret maximum from 405 to 408 nm and the appearance of 530- and 566-nm peaks. Under these conditions this intermediate decays over the course of 20 min with a corresponding increase in the visible region at 680 nm due to the formation of verdoheme and at 640 nm due to CO-bound ferrous verdoheme. Ultimately biliverdin is formed with a broad peak cen-
Isoporphyrin in Heme Oxygenase Catalysis

Oxidation of the MH-hHO-1 complex is noticeably slower than the oxidation of heme but proceeds through the obligatory verdoheme intermediate to mesobiliverdin with an absorption maximum at 650 nm (Fig. 3C). The intensity of the verdoheme intermediate and mesobiliverdin product are significantly lower than those of the equivalent species in the oxidation of heme itself. The CH₃PMH-hHO-1 (Fig. 3D) and CF₃PMH-hHO-1 (not shown) complexes underwent a similar Soret shift, going from 400 to 409 nm on conversion to the ferrousoxy form, and a transient increase in absorbance at 440 and 900 nm. In the arylheme reactions there was a transient increase in absorbance at both 440 and 900 nm that was not observed in the oxidation of heme or MH. These absorbances are characteristic of both isoporphyrins (35) and benzoylbiliverdins (36), but it was first necessary to determine whether this species was a side product or an intermediate in the pathway of biliverdin formation.

**Single Turnover Reaction Supported by H₂O₂**—Fe(III)-verdoheme can be generated by reaction of the heme-hHO-1 complex with H₂O₂ (30, 34). The single turnover reaction of MH with 4 eq of H₂O₂ (20 μM) resulted in the accumulation of verdoheme, as indicated by a decrease in the Soret band and a concomitant increase at 650 nm (Fig. 4A). In contrast, when the α-meso position is blocked with a phenyl group, as in CH₃PMH (Fig. 4B), CF₃PMH, and PH (not shown), the reaction exclusively generates what looks like an isoporphyrin intermediate (Fig. 4B), as shown by the decrease in the Soret band with concomitant increases in absorbance at 440 and 930 nm.

The spectral features of the proposed isoporphyrin-hHO-1 complexes formed in the reactions of the CH₃PMH and CF₃PMH hHO-1 complexes with H₂O₂ are very similar (Fig. 5A). Both intermediates exhibit peaks at ∼360 and 435 nm in the UV region and a strong peak with a maximum at ∼935 nm in the near IR region with a side band at 840 nm. This species can be removed from the protein by mild quenching with acid and extraction with dichloromethane. The optical absorption spectra of the extracted isoporphyrins of CH₃PMH and CF₃PMH dissolved in CH₂Cl₂ (Fig. 5B) are very similar to those of ferric and zinc isoporphyrins (37, 38) but not to Ni(II) and Cu(II) benzoylbiliverdins (36), which have very broad and featureless peaks in the near-IR. Although both CH₃PMH and CF₃PMH look very similar in CH₂Cl₂, there is a significant difference between their maxima in the near-IR. Both show a shift of the 435 peak to 428 nm, but CH₃PMH has a peak at 949 nm and a side band at 855, whereas CF₃PMH has a peak at 927 nm with a side band at 842 nm.

To confirm that the intermediate observed spectroscopically was an isoporphyrin rather than the more oxidized benzoylbiliverdin, we performed the peroxide reaction under anaerobic conditions. A reaction analogous to that seen under aerobic conditions, with the same rate of Soret disappearance and intermediate formation, was observed. This evidence is consistent with formation of an isoporphyrin rather than a more oxidized benzoylbiliverdin species, although it is possible that peroxide could facilitate further oxidation of the isoporphyrin.

Next we asked whether the isoporphyrin complex formed with H₂O₂ was a true intermediate in the conversion of the heme to a biliverdin analogue or a dead end side product. The verdoheme intermediate formed from the reaction of MH-hHO-1 with peroxide, when exposed to additional reducing equivalents in the form of NADPH/CPR, rapidly converts to mesobiliverdin (Fig. 6A). The addition of NADPH/CPR to the CH₃PMH-hHO-1 isoporphyrin complex resulted in a shift of the 935-nm peak to 900 nm, a broadening with loss of the sideband, and conversion to mesobiliverdin over 10 min (Fig. 6B). A very similar but more rapid decay was seen with the PH and CF₃PMH-hHO-1 isoporphyrin complexes (supplemental Fig. S2). The increase in absorbance at 650 nm is indicative of the formation of biliverdin as the primary reaction product.
Product Analysis by HPLC and LC-ESI-MS—We analyzed the products formed in the reactions of the CH₃PMH-hHO-1 (Fig. 7), CF₃PMH-hHO-1, and PH-hHO-1 (not shown) complexes in the presence of either NADPH/CPR or H₂O₂ by quenching and extracting the reaction mixtures. The reaction of CH₃PMH-hHO-1 with NADPH/CPR in the presence of catalase generates primarily /H₉₂₅₁-mesobiliverdin (Fig. 7A, top). The reaction with H₂O₂ shows two products eluting on HPLC at 20.1 and 25.5 min. The major product eluting first has an absorbance spectrum (Fig. 7B, middle) similar to that of the observed spectroscopic product (Fig. 5). ESI-MS analysis obtained in the ES⁺ mode identified this major product as the isoporphyrin that is detected as the formate conjugate with m/z 772 and as its fragment at m/z 608 due to the loss of the α-meso
carbon (Fig. 7, middle, and Fig. 8). The second HPLC peak has a unique absorbance spectrum with peaks at 310 and 408 nm as well as a smaller peak at 750 nm (Fig. 7B, bottom). ESI-MS analysis identified this product as the iron-benzoylbiliverdin with a molecular ion at $m/z$ 742 (Fig. 7C, bottom, and Fig. 8). Therefore, it appears that the iron-benzoylbiliverdin is formed by a non enzymatic process when the isoporphyrin is exposed to aqueous conditions. This process confounded our early attempts to detect the intermediate as described in more detail in the supplemental data (supplemental Fig. S3).

The major product of the reactions of the CH$_3$PMH-hHO-1 and CF$_3$PMH-hHO-1 complexes incubated for 20 min with H$_2$O$_2$ (8 eq) and then with catalase (10 μg/ml) and NADPH (100 μM)/CPR (160 nM) (Fig. 7A, bottom) was α-mesobiliverdin, confirming that the isoporphyrin is a true intermediate in biliverdin formation. The amount of mesobiliverdin produced was quantitated by integrating the area under the peak at 360 nm for the NADPH/CPR-catalyzed oxidation and comparing it to the corresponding area from the peroxide oxidation followed by NADPH/CPR, for CH$_3$PMH-hHO-1, CF$_3$PMH-hHO-1, and MH-hHO-1 (Fig. 9). The relative amounts of mesobiliverdin produced in the separate reaction pathways are similar, further evidence that the isoporphyrin is a true intermediate in the oxidation of phenylheme to biliverdin. Notable is the relative decrease in mesobiliverdin produced from the oxidation of CH$_3$PMH versus that of MH (59%), suggesting that oxidation of unnatural substrates like phenyl hemes can lead to the formation of uncharacterized side products.

In the absence of an electron donor, the isoporphyrin-hHO-1 complexes are surprisingly long-lived with half-lives of 2.4 and 1.7 h at pH 7.4 for CH$_3$PMH and CF$_3$PMH, respectively. The primary product of uncatalyzed isoporphyrin decay over the course of 6 h is iron-benzoylbiliverdin (Fig. 8). An additional product detected, but to a lesser extent, was biladienone (Fig. 10), in which an additional oxidation has occurred at one of the previously unreacted meso carbons. This appears to be analogous to the primary product from the nonenzymatic ascorbate-catalyzed coupled oxidation of tetraarylporphyrin iron complexes which generates a biladienone product (39, 40). However, the source of electrons is unknown, as whatever extra

---

**FIGURE 5.** Characterization of the intermediate. A, spectra of the hHO-1-isoporphyrin complex in standard buffer for CH$_3$PMH (---) and CF$_3$PMH (---). B, spectra of the extracted isoporphyrin in CH$_2$Cl$_2$ for CH$_3$PMH (---) and CF$_3$PMH (---).

**FIGURE 6.** Further reaction of H$_2$O$_2$-generated intermediate. Shown are the addition of reducing equivalents in the form of CPR/NADPH to the ferric mesoverdoheme-hHO-1 complex (A) and to the CH$_3$PMH hHO-1-isoporphyrin complex (B). The spectra were recorded before (thin solid line) and after the addition of NADPH (---), 2 (---), 4 (---), 6 (---), and 10 min (thick solid line).
pH peroxide was present was consumed by catalase, but it may be formed through redox reactions involving protein residues. The free isoporphyrin is remarkably stable in organic solvents; however, in the presence of water it readily decays. Spectroscopic monitoring of the decomposition of the extracted isoporphyrin dissolved in the HPLC running buffer shows a time-dependent increase in the absorbance at 578 nm (Fig. 11), characteristic of the iron-free benzoylbiliverdin (36). Isoporphyrin formation is uniquely catalyzed by HO, as there was no such reaction in control experiments in which CH₃PMH and CF₃PMH were incubated with NADPH/CPR or peroxide but in the absence of hHO-1 under otherwise identical reaction conditions (not shown).

Oxygen-18 Incorporation into the Products—Oxygen-18 experiments were used to look for oxygen incorporation into either the benzoic acid, mesobiliverdin, or isoporphyrin products generated from the HO-catalyzed oxidation of CH₃PMH and CF₃PMH and, thus, to aid in determining the origin of the products. We used three sets of incubation conditions, one in which the reaction was carried out in H₂¹⁸O, another in which the reaction contained ¹⁸O₂ in normal water, and a third in which H₂¹⁸O₂ was employed in normal water followed by incubation with NADPH/CPR. The results showed that one atom of labeled oxygen was incorporated from water and one from the peroxide or dioxygen into the corresponding para-substituted benzoic acid product, as previously reported for the oxidation of /H₉₂₅₁⁻meso⁻phenylheme (supplemental Fig. S4) (23). No oxygen-18 was incorporated into biliverdin from H₂¹⁸O₂, in accord with the fact that the two lactam oxygens arise from two different molecules of O₂ (supplemental Fig. S5) (23, 41). Reaction in the presence of H₂¹⁸O₂ confirmed that one molecule of oxygen is incorporated into the isoporphyrin from the peroxide (m/z 774) and is lost by fragmentation to the product at m/z 608 (Fig. 7C).

DISCUSSION

The first step in the mechanism of heme oxidation catalyzed by heme oxygenase has been well characterized, but important details remain obscure. We previously showed that /H₉₂₅₁⁻meso⁻phenylheme is a substrate of HO that is regiospecifically cleaved at the /α⁻meso position and identified the fragment lost from the oxidation of /α⁻meso⁻phenylheme as benzoic acid (23).
From this we suggested a mechanism that postulated the existence of at least one additional intermediate in the oxidation of α-meso-substituted hemes by HO (23). We set out to determine whether we could detect or isolate any of these intermediates utilizing alternate substrates with decreased reactivity. Here we demonstrate that the reaction of α-meso-substituted phenylhemes proceeds through an isoporphyrin intermediate. Its formation, indicated by absorbances at 440 and 900 nm in the electronic absorption spectra, has been unambiguously confirmed by HPLC and LC-MS analysis of the intermediate after extraction from the enzyme. This is the first time that an isoporphyrin has been detected as a productive intermediate in hemoprotein catalysis, although they have previously been detected as intermediates in the inactivation of various hemoproteins.

Earlier work demonstrated that the cytochrome P450BM3-F87G mutant is inactivated by aromatic aldehydes and H₂O₂, generating meso-substituted covalent heme adducts via a proposed isoporphyrin intermediate (42). An isoporphyrin intermediate was detected earlier in the heme alkylating reactions of horseradish peroxidase with both cyclopropanone hydrate and alkyl hydrazines (35, 43). These additions occur at the δ-meso position of the heme, whereas reactions of alkylhydrazines with myoglobin produce γ-meso-alkylated hemes (44). Particularly relevant is the observation that the autoinactivation of horseradish peroxidase in the presence of excess oxidizing agent involves hydroxylation at a meso position, a reaction in which a transient (presumably isoporphyrin) species with an absorption at 940 nm has been detected (45, 46).

In the proposed mechanism of HO catalysis, formation of Fe(III)-OOH precedes reaction with the α-meso carbon via an electrophilic mechanism or, more likely, via a homolytic “tethered” mechanism (11) to give an isoporphyrin intermediate. This isoporphyrin has a saturated α-meso carbon and a disrupted π-electron system. In the normal oxidation of heme, the proton at the α-meso position is rapidly lost, resulting in rapid rearomatization of the porphyrin ring. This is consistent with the early observation that replacement of the α-meso proton by a deuterium did not alter the rate of the heme oxygenase reaction (47). The observed inverse secondary kinetic isotope effect indirectly characterized this transformation, as it suggests a change in hybridization state from sp² to sp³ at the meso carbon during the rate-limiting step of the transformation from Fe(III)-OOH to α-meso-hydroxylheme (47). However, no proton loss is possible when the α-meso-hydrogen is replaced by a carbon substituent, resulting in the formation of a relatively long-lived
isoporphyrin complex, thus unmasking a transient, unobserved intermediate in normal catalysis.

A possible mechanism (Fig. 12; peripheral substituents are not shown) for oxidation of the isoporphyrin 1 circumvents formation of the \( \alpha\)-meso-hydroxyheme intermediate, but incorporates elements similar to those proposed to explain its degradation to verdoheme (8). Two-electron reduction concurrent with \( \text{O}_2 \) binding produces a bridged peroxo species 2 that undergoes iron-catalyzed homolytic cleavage. Elimination of the phenyl-substituted \( \alpha\)-meso carbon as a carbene followed by intramolecular electron transfer generates verdoheme 5. The steps beyond verdoheme are the same as those for oxidation of heme itself. An ambiguity exists concerning the fate of the carboxylate, as the addition of water would generate the acetal and, therefore, the aldehyde. However, the only product we have detected is the carboxylic acid, not the aldehyde. It is possible that the aldehyde is rapidly oxidized to the carboxylic acid.

The iron-benzyobiliverdin species 6, also shown in Fig. 8, was previously proposed as a possible intermediate (23) but can be ruled out as such in the reaction pathway for the \( \alpha\)-meso-substituted hemes that cannot simply lose CO. Although this product with and without iron is obtained when the isoporphyrin is directly quenched with acid, our results indicate that it is not a precursor of the final biliverdin product but a side product resulting from nonenzymatic decomposition of the isoporphyrin. Nevertheless, the \(^{18}\text{O}\)-labeling experiments establish that the ketone oxygen in the benzoyl group of this product derives initially from \( \text{H}_2\text{O}_2 \).

This mechanism suggests that the \( \text{H}_2\text{O}_2 \)-supported oxidation of \( \alpha\)-meso-methylheme by hHO-1 should be similarly arrested at the isoporphyrin stage. It was previously concluded that no reaction occurred between peroxide and \( \alpha\)-meso-methylheme; however, re-examination of the spectra appears to show a slight increase in absorbance at 440 nm, whereas changes in absorbance at wavelengths >800 nm were not investigated at the time (48). This suggests that the isoporphyrin intermediate is stabilized to a greater extent by interactions of the substituted phenyl groups with the protein that are not available when the \( \alpha\)-substituent is only a methyl. In addition to interactions with the \( \alpha\)-meso-aryl substituent, the isoporphyrin may be stabilized by electrostatic interactions with negatively charged active site residues because isoporphyrins have one more positive charge than the parent heme group. This decreased electron density is delocalized over the porphyrin nitrogens, conjugated ring system, and the iron atom. Another consequence of this is that the Fe(III)-isoporphyrin has redox properties that differ from those of heme, although the fact that NADPH/CPR can convert the isoporphyrin to \( \alpha\)-meso-biliverdin indicates that the redox potential for the isoporphyrin is still compatible with electron transfer from CPR.

In summary, these results firmly establish the isoporphyrin as an intermediate in phenylheme metabolism. HO catalytic activity is uniquely tailored to excise the \( \alpha\)-meso carbon as it provides the sole source of endogenous carbon monoxide, a molecule essential for cell signaling in mammals. Surprisingly, substitutions at this position do not hinder this mechanism. However, the nature of the mechanism after isoporphyrin degradation remains unclear. At least 5 electrons and 2 molecules of \( \text{O}_2 \) would be necessary to fully oxidize the isoporphyrin to biliverdin, whereas no additional intermediates apart from verdoheme were detected.

REFERENCES
1. Kim, H. P., Ryter, S. W., and Choi, A. M. (2006) Annu. Rev. Pharmacol. Toxicol. 46, 411–449
2. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., and Ames, B. N. (1987) Science 235, 1043–1046
3. Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16993–16998
4. Stocker, R., and Perrella, M. A. (2006) Circulation 114, 2178–2189
5. Berberat, P. O., Dambrauskas, Z., Gullubinas, A., Giese, T., Giese, N., Kunzli, B., Autschbach, F., Meuer, S., Buchler, M. W., and Friess, H. (2005) Clin. Cancer Res. 11, 3790–3798
6. Furci, L. M., Lopes, P., Eakanunkul, S., Zhong, S., MacKerell, A. D., Jr., and Wilks, A. (2007) J. Med. Chem. 50, 3804–3813
7. Unno, M., Matsui, T., and Ikeda-Saito, M. (2007) Nat. Prod. Rep. 24, 553–570
8. Ortiz de Montellano, P. R. (2000) Curr. Opin. Chem. Biol. 4, 221–227
9. Colas, C., and Ortiz de Montellano, P. R. (2003) Chem. Rev. 103, 2305–2322
10. Wilks, A., Torpey, J., and Ortiz de Montellano, P. R. (1994) J. Biol. Chem. 269, 29553–29556
11. Kumar, D., de Visser, S. P., and Shaik, S. (2005) J. Am. Chem. Soc. 127, 8204–8213
12. Bonnett, R., and McDonagh, A. F. (1973) J. Chem. Soc. Perkin Trans. 1, 9, 881–888
13. Sano, S., Sano, T., Morishima, I., Shiro, Y., and Maeda, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 531–535
14. Takahashi, S., Ishikawa, K., Takeuchi, N., Ikedasaito, M., Yoshida, T., and Rousseau, D. L. (1995) J. Am. Chem. Soc. 117, 6002–6006
15. Schuller, D. L., Wilks, A., Ortiz de Montellano, P. R., and Poulos, T. L. (1999) Nat. Struct. Biol. 6, 860–867
16. Liu, Y., Lightning, L. K., Huang, H. W., Moenen-Loccoz, P., Schuller, D. L., Poulos, T. L., Loehr, T. M., and Ortiz de Montellano, P. R. (2000) J. Biol. Chem. 275, 34501–34507
17. Lad, L., Wang, J., Li, H., Friedman, J., Bhaskar, B., Ortiz de Montellano, P. R., and Poulos, T. L. (2003) J. Mol. Biol. 330, 527–538
18. Zhou, H., Migita, C. T., Sato, M., Sun, D. Y., Zhang, X. H., Ikeda-Saito, M., Fujii, H., and Yoshida, T. (2000) J. Am. Chem. Soc. 122, 8311–8312
19. Wang, J., Evans, J. P., Ogura, H., La Mar, G. N., and Ortiz de Montellano, P. R. (2006) Biochemistry 45, 61–73
20. Frydman, R. B., Tomaro, M. L., Buldain, G., Awruch, J., Diaz, L., and
Frydman, B. (1981) *Biochemistry* **20**, 5177–5182
21. Torpey, J., Lee, D. A., Smith, K. M., and Ortiz de Montellano, P. R. (1996) *J. Am. Chem. Soc.* **118**, 9172–9173
22. Torpey, J., and Ortiz de Montellano, P. R. (1997) *J. Biol. Chem.* **272**, 22008–22014
23. Wang, J., Niemez, F., Lad, L., Huang, L., Alvarez, D. E., Buldain, G., Poulos, T. L., and Ortiz de Montellano, P. R. (2004) *J. Biol. Chem.* **279**, 42593–42604
24. Sugishima, M., Sakamoto, H., Noguchi, M., and Fukuyama, K. (2004) *J. Mol. Biol.* **341**, 7–13
25. Niemez, F., Vazquez, M. S., and Buldain, G. Y. (2008) *Synthesis* **6**, 875–882
26. Robinsohn, A. E., Maier, M. S., and Buldain, G. Y. (2000) *Heterocycles* **53**, 2127–2142
27. Niemez, F., Alvarez, D. E., and Buldain, G. Y. (2002) *Heterocycles* **57**, 697–704
28. Niemez, F., and Buldain, G. Y. (2004) *J. Porphyrins Phthalocyanines* **8**, 989–995
29. Hildebrandt, A. G., Roots, I., Tjoe, M., and Heinemeyer, G. (1978) *Methods Enzymol.* **52**, 342–350
30. Wilks, A., and Ortiz de Montellano, P. R. (1993) *J. Biol. Chem.* **268**, 22357–22362
31. Wilks, A., Black, S. M., Miller, W. L., and Ortiz de Montellano, P. R. (1995) *Biochemistry* **34**, 4421–4427
32. Diers, E. A., Davis, S. C., and Ortiz de Montellano, P. R. (1998) *Biochemistry* **37**, 1839–1847
33. Lightning, L. K., Huang, H., Moenne-Loccoz, P., Loehr, T. M., Schuller, D. J., Poulos, T. L., and Ortiz de Montellano, P. R. (2001) *J. Biol. Chem.* **276**, 10612–10619
34. Liu, Y., Moenne-Loccoz, P., Loehr, T. M., and Ortiz de Montellano, P. R. (1997) *J. Biol. Chem.* **272**, 6909–6917
35. Ator, M. A., David, S. K., and Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* **264**, 9250–9257
36. Ongayi, O., Fronczek, F. R., and Vicente, M. G. H. (2003) *Chem. Commun.* **2298–2299
37. Gold, A., Ivey, W., Toney, G. E., and Sangaih, R. (1984) *Inorg. Chem.* **23**, 2932–2935
38. Dolphin, D., Felton, R. H., Borg, D. C., and Fajer, J. (1970) *J. Am. Chem. Soc.* **92**, 743–745
39. Yamauchi, T., Mizutani, T., Wada, K., Horii, S., Furukawa, H., Masaoka, S., Chang, H. C., and Kitagawa, S. (2005) *Chem. Commun.* 1309–1311
40. Asano, N., Uemura, S., Kinugawa, T., Akasaka, H., and Mizutani, T. (2007) *J. Org. Chem.* **72**, 5320–5326
41. Docherty, J. C., Schacter, B. A., Firneisz, G. D., and Brown, S. B. (1984) *J. Biol. Chem.* **259**, 13066–13069
42. Raner, G. M., Hatchell, A. J., Morton, P. E., Ballou, D. P., and Coon, M. J. (2000) *J. Inorg. Biochem.* **81**, 153–160
43. Wiseman, J. S., Nichols, J. S., and Kolpak, M. X. (1982) *J. Biol. Chem.* **257**, 6328–6332
44. Choe, Y. S., and Ortiz de Montellano, P. R. (1991) *J. Biol. Chem.* **266**, 8523–8530
45. Bagger, S., and Williams, R. J. P. (1971) *Acta Chem. Scand.* **25**, 976–982
46. Nakajima, R., and Yamazaki, I. (1980) *J. Biol. Chem.* **255**, 2067–2071
47. Davydov, R., Matsui, T., Fujii, H., Ikeda-Saito, M., and Hoffman, B. M. (2003) *J. Am. Chem. Soc.* **125**, 16208–16209
48. Torpey, J., and Ortiz de Montellano, P. R. (1996) *J. Biol. Chem.* **271**, 26067–26073