Conversion of heme to verdoheme by heme oxygenase-1 is thought to involve α-meso-hydroxylation and elimination of the meso-carbon as CO, a reaction supported by both H2O2 and NADPH-cytochrome P450 reductase/O2. Aerobic reaction of the heme-HO-1 complex with 1 eq of H2O2 produces an enzyme-bound intermediate identified by spectroscopic methods as α-meso-hydroxyheme. This is the first direct evidence for HO-1-catalyzed formation of α-meso-hydroxymethylene. α-meso-Hydroxymethylene exists as a mixture of Fe(III) phenolate, Fe(III) keto anion, and Fe(II) keto π neutral radical resonance structures. EPR shows that complexation with CO enhances the Fe(II) π neutral radical component. Reaction of the α-meso-hydroxymethylene-HO-1 complex with O2 generates Fe(III) verdoheme, which can be reduced in the presence of CO to the Fe(II) verdoheme-CO complex. Thus, conversion of α-meso-hydroxymethylene to Fe(III) verdoheme, in contrast to a previous report (Materia, K. M., Takahashi, S., Fujii, H., Zhou, H., Ishikawa, K., Yoshimura, T., Rousseau, D. L., Yoshida, T., and Ikeda-Saito, M. (1996) J. Biol. Chem. 271, 6618–6624), does not require a reducing equivalent. An electron is only required to reduce ferric to ferrous verdoheme in the first step of its conversion to biliverdin.

Heme oxygenase catalyzes the NADPH- and cytochrome P450 reductase-dependent oxidation of heme (iron protoporphyrin IX regardless of oxidation and ligation state) to biliverdin and CO (1). This enzyme is of physiological interest because of the biological properties of its reaction products: biliverdin and CO. Biliverdin is normally reduced to bilirubin by biliverdin IXa and CO. Biliverdin is normally reduced to bilirubin by biliverdin IXa and CO. Biliverdin IXa is excreted in the urine after conjugation with glucuronic acid (2). Excretion of bilirubin is frequently impaired in newborn children and in individuals with a genetic glucuronyltransferase deficiency (3). Unconjugated bilirubin is neurotoxic, and approaches to the prevention of its accumulation, including the inhibition of heme oxygenase, are of potential clinical utility (4–6). More recently, a potentially important in view of the demonstration that Hia-25 as the proximal heme iron ligand in the HO-1 enzyme-substrate complex (18–23). Recent studies have established that Hia-132, the conserved residue in the distal side of the heme pocket, plays a role in stabilizing the distal water iron ligand and facilitates, but is not absolutely required for, coupled turnover of the enzyme (24).

Heme oxygenase employs heme as both the prosthetic group and substrate. The oxidation of heme by heme oxygenase is thought to involve sequential α-meso-hydroxylation, oxygen-dependent fragmentation of the resulting α-meso-hydroxymethylene product to verdoheme, and oxidative cleavage of verdoheme to biliverdin (Scheme 1). α-meso-Hydroxylation, the first step, requires NADPH-cytochrome P450 reductase-dependent reduction of the iron to the ferrous state, oxygen binding to the reduced iron, and a second one-electron reduction of the oxygen-ferrous complex (25). The resulting ferric peroxide (Fe(III)–OOH) complex reacts as an electrophile with the porphyrin ring (16, 26) and incorporates an oxygen atom from molecular O2 into the α-meso-hydroxymethylene product (27). Although synthetic α-meso-hydroxymethylene has been shown to be converted to biliverdin IXa both by HO-1 (28, 29) and by model systems (30–33), the formation of α-meso-hydroxymethylene has not been directly demonstrated in the enzymatic reaction, possibly because of its high reactivity with oxygen. Intervention of α-meso-hydroxymethylene as a true intermediate in the normal catalytic process therefore remains unproven. This shortcoming is important in view of the demonstration that α-meso-methyl-substituted heme groups are oxidized to the corresponding α-biliverdins without the formation of CO, a reaction that does not involve α-meso-hydroxylation of the heme (34, 35).

The second step of the catalytic process is elimination of the α-meso-carbon and attached hydroxyl as CO (36) concomitant with replacement of the carbon in the ring structure by an oxygen atom (37, 38). However, the nature of the intermediates and the reaction mechanism for this transformation remain unclear. Several studies of the conversion of α-meso-hydroxymethylene to verdoheme have been carried out with chemical model systems (31, 32, 39–41) as well as systems in which α-meso-hydroxymethylene has been reconstituted into apomyoglobin (31) or HO-1 (29, 42). These studies indicate that a radical species
forms when α-meso-hydroxymyoglobin reacts with O₂ prior to the formation of verdoheme, but the reduction equivalent requirements for the subsequent reaction are a matter of debate. Materia et al. (42) concluded that 1 reducing eq is required for the HO-1-catalyzed reaction, whereas Sano et al. (31) did not find such a requirement in the myoglobin reaction. Our previous demonstration that NADPH-cytochrome P450 reductase finds such a requirement in the myoglobin reaction. Our previous demonstration that NADPH-cytochrome P450 reductase finds such a requirement in the myoglobin reaction.

We report here that α-meso-hydroxymyoglobin is formed as a stable intermediate when the heme-hHO-1 complex reacts anaerobically with H₂O₂ and characterize this intermediate by UV-visible, resonance Raman, and EPR spectroscopy. In the presence of CO, α-meso-hydroxymyoglobin exists predominantly as a CO-complexed Fe(II) π neutral radical. On exposure to O₂, this porphyrin radical reacts with O₂ to yield Fe(III) verdoheme with no requirement for exogenous reducing equivalents. An electron is only required to reduce Fe(III) to Fe(II) verdoheme.

**EXPERIMENTAL PROCEDURES**

**General Methods**—hHO-1 without the 23-amino acid membrane anchor was expressed in E. coli and was purified as previously reported (16, 17). The truncated protein has the same catalytic activity as the full-length form. The catalytic activity was assayed by monitoring the absorbance at 635 nm (ε₆₃₅ = 43.6 M⁻¹ cm⁻¹). The protein solution and H₂O₂ were made anaerobic by flushing the cuvette with oxygen-free argon for at least 40 min. The argon was passed through an oxygen scrubber to remove the trace O₂ contamination from the gas, and a water-filled bubbler was used to prevent drying of the solutions in the cuvette. During the gas exchange, the protein solution was placed in the round bottom of the cuvette to maximize the surface area and the argon exchange rate. After the solutions were fully anaerobic, the reaction was initiated by mixing the protein solution with the H₂O₂ in the side arm at 23 °C and recording the UV-visible spectrum until no further changes were observed. For subsequent reaction with O₂, the sealed septum was removed; the protein solution was flushed with 100% O₂ and the UV-visible spectrum was recorded.

For the experiments performed under an atmosphere of CO, the protein and H₂O₂ solutions were flushed with O₂-free CO. The CO (99.95%) gas was bubbled through a sodium dithionite solution containing reduced methyl viologen (blue) as an indicator to remove oxygen impurities prior to entry into the cuvette. The procedure was otherwise the same as that for the anaerobic experiments.

**Resonance Raman Spectroscopy**—Resonance Raman experiments were performed using the same anaerobic cuvette used for the absorption spectroscopy. The procedures were used to prepare samples for absorption measurements. Each sample was repeated for resonance Raman experiments with higher protein concentrations (≈150 μM). Optical absorption data could be concomitantly obtained using the cuvette with a 0.2-cm path length. Resonance Raman spectra were recorded on a custom McPherson 206/2107 spectrophotometer (0.67-m focal length, 1800 grooves/mm of grating, and 6 cm⁻¹ spectral resolution) using a Kaiser Optical holographic super-notch filter and a Princeton Instruments liquid N₂-cooled CCD detector (LN-1100PBP). The excitation source was provided by an Innova 302 krypton laser (413 nm, 5 mW). Spectra were collected in a 90°-scattering geometry at room temperature with a recording time of a few minutes. Peak frequencies were calibrated relative to an indene standard and are accurate to ±1 cm⁻¹.

**EPR Spectroscopy**—Anaerobic sample handling was as described above except that higher concentrations of the reconstituted heme-hHO-1 complex were used for EPR experiments. The protein solution (300 μM, 250 μl) in a Teflon-sealed stirrer 1-ml reaction vial was made anaerobic by flushing, as required, with either O₂-free argon or CO. The reaction, initiated by anaerobic addition of 1 eq of H₂O₂, was allowed to proceed for 5 min at 23 °C before the protein solution was transferred to an anaerobic EPR tube, in which it was frozen by immersion in liquid nitrogen. For the reaction with O₂, 100% O₂ was used to flush the head space over the protein solution with constant stirring.

Sodium dithionite was prepared in anaerobic buffered solutions and was quantitated by titration with potassium ferriyaindane (ε₄₃₀ = 1.03 mM⁻¹ cm⁻¹). X-band EPR spectra were recorded using a Varian E-109 spectrometer equipped with an Oxford Instruments ESR-910 liquid helium cryostat, a Hewlett-Packard 436A power meter, and a Hewlett-Packard 5350B microwave frequency counter. Temperature, g value calibrations, data acquisition, subtraction, and integration procedures were as described previously (44–46).

**RESULTS**

UV-visible and Resonance Raman Analyses of the Formation of α-meso-Hydroxymyoglobin with H₂O₂.—When the heme-hHO-1 complex (Fig. 1, ———) reacts anaerobically (argon atmosphere) with 1 eq of H₂O₂ at pH 7.4, the Soret band (−−−) becomes broader, decreases in intensity, and crosses at 425 nm that of the original heme-hHO-1 complex. In the visible region, the α and β bands at 574 and 536 nm are greatly attenuated; the absorbance at 635 nm increases; and a new band appears at 425 nm that of the original heme-hHO-1 complex. In the visible region, the α and β bands at 574 and 536 nm are greatly attenuated; the absorbance at 635 nm increases; and a new band appears around 820 nm. These spectroscopic changes differ dramatically from those observed when verdoheme is formed by aerobic addition of 1 eq of H₂O₂ to the heme-hHO-1 complex (16). The new species that is formed anaerobically is stable at 23 °C for at least 30 min if anaerobicity is not lost. The band at 405 nm and the relatively featureless visible region of the anaerobic intermediate are similar to those of the reconstituted α-meso-hydroxymyoglobin-HO-1 complex reported by Materia et al. (42) and the α-meso-hydroxymyoglobin-myoglobin complex reported by Sano et al. (31). When the anaerobically generated intermediate is exposed to O₂, the Soret intensity undergoes a
further slight decrease; the broadening of the Soret band and the band at 820 nm disappear; and the absorption at 660–690 nm increases markedly (Fig. 1, –––). This spectrum, which is the same as that obtained upon aerobic addition of H$_2$O$_2$ (16), suggests that verdoheme is formed by reaction of the anaerobically generated intermediate with O$_2$. The formation of verdoheme was confirmed by the addition of 20% pyridine to the protein solution, which produced the typical spectrum of the verdoheme-pyridine complex (Fig. 1, ––). In panel III is shown the product of spectrum c after the addition of 20% pyridine (—–).

Additional evidence for the formation of $\alpha$-meso-hydroxyheme in the anaerobic reaction of H$_2$O$_2$ with the heme-hHO-1 complex is provided by comparison of the resonance Raman spectrum of the product with that reported for HO-1 reconstituted with synthetic $\alpha$-meso-hydroxyheme (42). The resonance Raman spectrum of the ferric heme-hHO-1 complex (Fig. 2, trace A), as previously reported (18, 22), is characteristic of a mixture of high and low spin hexacoordinate heme dominated by a $v_4$ porphyrin skeletal mode at 1375 cm$^{-1}$. Anaerobic addition of 1 eq of H$_2$O$_2$ leads to a decrease of the contributions from the ferric heme and the appearance of new resonance Raman features that signal the formation of a new species (Fig. 2, trace B), although the residual band at 1375 cm$^{-1}$ indicates that some unreacted ferric heme is still present. Reaction with higher concentrations of H$_2$O$_2$ further decreases the ferric heme bands, but no increase is observed in the bands attributed to $\alpha$-meso-hydroxyheme. This is probably due to side reactions of $\alpha$-meso-hydroxyheme with H$_2$O$_2$.$^2$ The difference spectrum (Fig. 2, trace C) obtained by subtracting the spectrum of the residual starting material from trace $B$ exhibits major resonance Raman bands at 889, 1125, 1226, 1334, 1354, 1401, 1581, and 1616 cm$^{-1}$. Allowing for an experimental error of 2 cm$^{-1}$, the difference spectrum reproduces the features of the previously reported spectrum of the $\alpha$-meso-hydroxyheme complex (42). Although previous studies have shown that chemotherapy synthesized $\alpha$-meso-hydroxyheme can be converted to biliverdin (29, 31, 42), this is the first demonstration of the heme oxygenase-catalyzed formation of $\alpha$-meso-hydroxyheme.

Kinetics of the Formation of $\alpha$-meso-Hydroxyheme—Rapid scanning spectroscopy at 23°C shows that formation of the $\alpha$-meso-hydroxyheme intermediate in the anaerobic reaction of the heme-hHO-1 complex with H$_2$O$_2$ is complete within 4 min (Fig. 3A). As before, completion of the reaction is associated with a decrease in and broadening of the Soret peak with an isosbestic point at 425 nm and a slight increase in the absorbance at 635 nm. Aerobic reaction of the heme-hHO-1 complex with H$_2$O$_2$ also reaches completion within 4 min at 23°C (Fig. 3B). However, the isosbestic point at 425 nm and the broadening of the Soret band are not observed, and the 660–690 nm absorption characteristic of verdoheme increases in parallel with the decrease in the Soret band. In the aerobic reaction, the heme appears to be directly transformed into verdoheme without the accumulation of $\alpha$-meso-hydroxyheme. When preformed $\alpha$-meso-hydroxyheme reacts with oxygen, there is a slight loss of Soret absorption and an increase in the 660–690 nm absorption (Fig. 3C). These changes are complete within 5 s at 23°C, indicating that the conversion of $\alpha$-meso-hydroxyheme to verdoheme is very rapid. The kinetics of these aerobic and anaerobic reactions of the heme-hHO-1 complex with H$_2$O$_2$ indicate that (a) a new stable intermediate with a different UV-visible spectrum forms anaerobically with H$_2$O$_2$; (b) the new intermediate is a precursor of verdoheme and is converted rapidly to verdoheme upon exposure to O$_2$; and (c) the rate of the H$_2$O$_2$-dependent oxidation of heme to verdoheme appears to be lim-

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$^2$ When $>1$ eq of H$_2$O$_2$ is used, anaerobic reaction of the ferric heme-hHO-1 complex with H$_2$O$_2$ yields products in addition to $\alpha$-meso-hydroxyheme. The anaerobically generated $\alpha$-meso-hydroxyheme possibly reacts with H$_2$O$_2$ to give secondary products.
H2O2. In 20 s for 4 min at 23°C after initiation of the reaction by the addition of radical resonance forms (32). The EPR data in Fig. 4 (a) at geq of H2O2, as slight loss of the trace A heme-hHO-1 complex with H2O2. hydroxyheme does not accumulate in the aerobic reaction of the ferric state (42), the ferric phenolate, ferric keto anion, and ferrous keto p neutral radical resonance forms (32). The EPR data in Fig. 4 (trace B) are consistent with this interpretation: the p neutral radical species accounts for the g = 2.008 signal, and the EPR-silent ferrous iron accounts for the decrease in the g = 6 signal. The g = 6 signal is broader (Fig. 4, trace B) than in the parent ferric state (trace A). The broadening of the signal may be due to either the generation of a rhombic signal contributed by the ferric α-meso-hydroxyheme or the presence of a more heterogeneous electronic structure in the keto-enol iron complex. It is difficult to differentiate the signal type due to interference by the g = 6 axial signal from unreacted ferric heme, the presence of which is confirmed by the resonance Raman experiments. When the EPR signal from unreacted ferric heme is subtracted, the remaining spectrum shows a ferric α-meso-hydroxyheme rhombic signal (Fig. 4, trace C) consistent with that reported by Matera et al. (42). The rhombic signal suggests that the ferric keto-enol form of α-meso-hydroxyheme has a high spin iron (S = 5/2). However, the ferrous p neutral radical species at g = 2.008 was not observed previously in either the reconstituted α-meso-hydroxyheme-HO-1 (42) or α-meso-hydroxyheme-myoglobin (31) system. Strong evidence for the ferrous p neutral radical character of α-meso-hydroxyheme is provided by the change in the EPR spectrum observed upon complexation of the iron with CO (Fig. 4, trace D). The EPR spectrum of the α-meso-hydroxyheme generated with 1 eq of H2O2 under an atmosphere of CO shows that the g = 6 signal is virtually eliminated, whereas the g = 2.008 signal is greatly enhanced (Fig. 4, trace D). The dramatic increase in the g = 2.008 radical species in the presence of CO suggests that CO forms a complex with the ferrous form of the intermediate and thereby stabilizes the Fe(II) p neutral radical structure relative to the enol and
keto anion resonance forms. This inference is consistent with the UV-visible spectrum in Fig. 5, which shows that when \( \alpha \)-meso-hydroxyheme is formed with 1 eq of \( \text{H}_2\text{O}_2 \) under an atmosphere of CO, the Soret maximum is red-shifted (from 405 to 408 nm) relative to its position in the absence of CO. The Soret shift argues for the formation of a CO complex of the Fe(II) \( \pi \) neutral radical species.

No significant changes are observed in the high frequency region of the resonance Raman spectrum of the \( \alpha \)-meso-hydroxyheme-hHO-1 complex when CO is added (data not shown). Several explanations are possible for the absence of a change in the resonance Raman spectrum on CO complexation. Although the EPR changes measured at 6 K are dramatic, they may be tempered at high temperature. For example, the shift in the Soret absorption at room temperature is relatively minor. Thus, temperature may affect the population ratio of the different resonance states of the \( \alpha \)-meso-hydroxyheme-hHO-1 complex. In addition, the formation of radical states on the porphyrin ring is known to greatly decrease their resonance Raman intensity [47]. Therefore, the signal for the ferrous \( \pi \) neutral radical species may be difficult to detect under the present conditions, where several other species are known to coexist. Moreover, the photolability of heme-bound CO is well known; hence, CO may be dissociated by the laser beam during data collection. The same results were observed when a power of \(-1\) mW was used, but the light sensitivity of this particular complex is unknown. Similarly, the extent of the resonance Raman changes of the ferrous \( \alpha \)-meso-hydroxyheme upon CO binding are not known.

**Reaction of \( \alpha \)-meso-Hydroxyheme with \( \text{O}_2 \) Observed by Resonance Raman and EPR Spectroscopy**—As already discussed, the 660–690 nm absorption maximum indicative of verdoheme formation increases when \( \text{O}_2 \) is added to the \( \alpha \)-meso-hydroxyheme generated anaerobically by reaction of the heme-hHO-1 complex with \( \text{H}_2\text{O}_2 \) (Fig. 1, ––). Identical experiments were carried out to determine the resonance Raman spectroscopic changes caused by the reaction. The presence of the unreacted ferric heme (\( v_2 \) mode at 1375 cm\(^{-1}\)) from the anaerobically generated \( \alpha \)-meso-hydroxyheme (shown in Fig. 2, trace B) remains unchanged upon exposure to \( \text{O}_2 \). Once the resonance Raman contributions from the unreacted heme have been subtracted (Fig. 6, trace A), the spectrum clearly shows that the bands characteristic of \( \alpha \)-meso-hydroxyheme (e.g. 889, 1226, 1354, and 1581 cm\(^{-1}\); Fig. 2, trace C) are replaced by a new set of vibrations with intense peaks at 1258, 1463, and 1612 cm\(^{-1}\) (Fig. 6, trace A). We have also recorded the resonance Raman spectrum of the verdoheme-hHO-1 complex generated by aerobic reaction of the heme-hHO-1 complex with \( \text{H}_2\text{O}_2 \) (Fig. 6, trace B). In this reaction, a higher \( \text{H}_2\text{O}_2 \) concentration was used, and the conversion of heme to verdoheme is nearly quantitative, as indicated by the smaller unreacted heme peak at 1375 cm\(^{-1}\). A perfect match in frequencies is observed between the two spectra (Fig. 6, traces A and B), clearly establishing that verdoheme is formed when the \( \alpha \)-meso-hydroxyheme complex reacts with \( \text{O}_2 \). An identical resonance Raman spectrum is also obtained when CO-complexed \( \alpha \)-meso-hydroxyheme reacts with \( \text{O}_2 \), showing that the same verdoheme product is formed in the presence of CO (data not shown). These results demonstrate that oxygen alone is required for the conversion of ferric \( \alpha \)-meso-hydroxyheme to verdoheme and specifically show that an exogenous electron is not required for this transformation. This finding contradicts the report by Matera *et al.* [42] that no verdoheme is formed from \( \alpha \)-meso-hydroxyheme unless an exogenous electron is provided.

*Trace B* in Fig. 6 is not identical to that reported by Matera *et al.* [42] for the Fe(II) verdoheme complex. Major peaks are found at higher frequencies, 1612 (+3) and 1258 (+4) cm\(^{-1}\), and the single broad band at 1463 cm\(^{-1}\) is shown as a pair of bands at 1446 and 1486 cm\(^{-1}\). To determine whether these differences are due to the presence of Fe(III) rather than Fe(II) verdoheme, the resonance Raman spectrum of Fe(II) verdoheme generated by anaerobically adding a stoichiometric amount of dithionite to *trace B* in Fig 6 was recorded. A direct comparison of the resonance Raman spectra of the Fe(III) and Fe(II) verdohemes (Fig. 6, traces B and C) reveals several differences. (a) The major bands at 1612 and 1258 cm\(^{-1}\) (Fig. 6, *trace B*) shift to 1608 and 1252 cm\(^{-1}\), respectively, in the ferrous verdoheme spectrum (*trace C*); (b) the band at 1463 cm\(^{-1}\) (trace B) splits into two bands at 1441 and 1484 cm\(^{-1}\) in ferrous verdoheme (*trace C*); and (c) the band at 1353 cm\(^{-1}\) in

3 In contrast to what is observed when the reaction is carried out anaerobically, in the aerobic reaction of the ferric heme-HO-1 complex with \( \text{H}_2\text{O}_2 \), complete conversion to verdoheme can be achieved by increasing the \( \text{H}_2\text{O}_2 \) concentration (1.5 eq of \( \text{H}_2\text{O}_2 \) was used in this particular experiment).
essentially identical to that reported by Matera only significant difference is that the bands at 1338 and 1366 (413 nm excitation).

trace C characteristic of ferrous heme (18) comes from the reduction of the unreacted ferric heme (1375 cm\(^{-1}\) in trace B). This change in the residual heme peak serves to confirm that the iron atoms in the solution are in the ferrous state. Thus, it is seen that the ferrous verdoheme spectrum (Fig. 6, trace C) is essentially identical to that reported by Matera et al. (42). The only significant difference is that the bands at 1338 and 1366 cm\(^{-1}\) in their spectrum are masked in trace C by the unreacted ferrous heme band at 1353 cm\(^{-1}\), but shoulders at higher and lower frequencies are noted. Further support for identification of trace C as that of ferrous verdoheme is provided by the similarities between it and the resonance Raman spectrum of a model Fe(II) verdoheme in pyridine. \(^4\) These data prove that the iron in the verdoheme obtained from reaction of \(\alpha\)-meso-hydroxyheme with oxygen in the absence of reducing equivalents is in the ferric state, as expected.

Another piece of evidence for the formation of a ferric product is provided by the finding that the UV-visible spectrum of the verdoheme complex generated by aerobic reaction of heme-hHO-1 complex with 1 eq of \(\text{H}_2\text{O}_2\) (Fig. 7, ––) with a distinct UV-visible spectrum (\(\lambda_{\text{max}} = 400, 534,\) and 690 nm). The addition of CO to the reduced sample yields a UV-visible spectrum with maxima at 412 and 638 nm that clearly identifies the product as the Fe(II) verdoheme-CO complex (Fig. 7, ––) (16, 37, 38). Thus, reaction of the heme-hHO-1 complex with oxygen in the absence of an external electron source readily yields Fe(III) verdoheme.

EPR spectroscopy provides additional evidence for the aerobic conversion of \(\alpha\)-meso-hydroxyheme to Fe(III) verdoheme. When anaerobically generated \(\alpha\)-meso-hydroxyheme (Fig. 8, trace A; obtained from Fig. 4, trace B) is exposed to \(\text{O}_2\), the \(g = 2.008\) radical species disappears, and the \(g = 6\) signal increases (trace B). The remaining \(g = 6\) axial signal comes from the unreacted ferric heme species also observed in the resonance Raman experiments. A fairly weak rhombic signal in the \(g = 2\) region (\(g_x = 2.57, g_y = 2.14,\) and \(g_z = 1.86; g_{av} = 2.19\)) is also obtained that corresponds to a low spin (\(S = 1/2\)) ferric signal. Comparison of this spectrum with that obtained when 1 eq of \(\text{H}_2\text{O}_2\) is added aerobically to the heme-hHO-1 complex (Fig. 8, trace C) shows that, in the aerobic reaction, the \(g = 6\) signal is similarly suppressed, and the new low spin ferric iron signal is also generated. However, the residual \(g = 6\) signal due to unreacted heme is smaller (Fig. 8, traces B and C), which suggests that the conversion of heme to verdoheme is more complete when the reaction with \(\text{H}_2\text{O}_2\) is carried out aerobically.

The loss of the \(g = 6\) axial signal associated with verdoheme formation indicates that Fe(III) verdoheme has a different iron spin state relative to that of ferric heme or \(\alpha\)-meso-hydroxyheme, possibly a low spin \((S = 1/2)\) that gives rise to the rhombic EPR signal at \(g_{av} = 2.19\). The same results, disappearance of the \(g = 2.008\) radical species and appearance of a weak rhombic EPR signal at \(g_{av} = 2.19\), are observed (data not shown) when the CO-complexed ferrous \(\pi\) neutral radical (sample in Fig. 4, trace D) is exposed to \(\text{O}_2\). This finding indicates that the ferrous \(\pi\) neutral radical resonance structure of the \(\alpha\)-meso-hydroxyheme most clearly represents the species that reacts with molecular oxygen. The new \(g_{av} = 2.19\) rhombic EPR signal generated in these reactions suggests that it is a signature of Fe(III) verdoheme, although the reason why it is a weak signal is unclear.

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\(^4\) J. Sun, T. M. Loehr, and C. K. Chang, unpublished results.
Heme Oxygenase Intermediates

The first step in heme degradation is thought to be the cytochrome P450 reductase-, NADPH-, and O₂-dependent α-meso-hydroxylation of heme by heme oxygenase. This transformation requires reduction of the ferric to the ferrous heme-protein complex by an electron from NADPH-cytochrome P450 reductase, oxygen binding to the iron to give the ferrous dioxygen (Fe(II)–OO) complex, and a second reduction to give an undetected species formally equivalent (after protonation) to a ferric peroxide (Fe(III)–OOH) complex. The formation of α-meso-hydroxyheme (data not shown), but the reaction product was not detected in earlier EPR studies of α-meso-hydroxyheme.

α-meso-Hydroxymes have been synthesized, reconstituted into myoglobin and heme oxygenase, and shown to be converted to the corresponding verdohemes and biliverdins (29, 31, 42). α-meso-Hydroxyme, however, has never actually been identified by spectroscopic or other means as an intermediate in the enzymatic conversion of heme to verdoheme. This is a significant shortcoming because the synthetic species could be converted to the observed product without actually being an intermediate in the normal process. Evidence that this is possible is provided by the recent finding that α-meso-methyl-substituted hemes are oxidized to give normal α-biliverdin, but not CO (34, 35). The kinetic, optical, resonance Raman, and EPR studies of the intermediate formed when 1 eq of H₂O₂ is added anaerobically to the heme-hHO-1 complex provide the missing evidence that α-meso-hydroxyme is a true intermediate in the catalytic reaction.

The electronic structure of α-meso-hydroxyme can be presented by three resonance structures: a ferric phenolate ion, a ferric keto enol neutral radical, and a ferrous keto enol neutral radical (Scheme 2, 4). The rhombic EPR signal of α-meso-hydroxyme, which represents the contribution of the ferric phenolate and keto resonance structures, suggests that the iron is in the high spin state (S = 5/2). This finding agrees with the results obtained when α-hydroxyme is reconstituted into either HO-1 or apomyoglobin (31, 42). Matera and co-workers (48) and Bogumil et al. (49) also reported that the complex is a five-coordinate species based on the similarity of its rhombic high spin EPR signal to that of pentacoordinate myoglobin mutants. Rhombic high spin signals are often observed for five-coordinate high spin ferric hemoproteins (48, 50). The observation that CO binds to and increases the contribution of (Fig. 4, trace D) the ferrous π neutral radical at the expense of the ferric keto-enol resonance structures, suggests that the iron is in the high spin state (S = 5/2). This finding agrees with the results obtained when α-hydroxyme is reconstituted into either HO-1 or apomyoglobin (31, 42).

Efforts to detect the α-meso-hydroxyme intermediate using NADPH-cytochrome P450 reductase and stoichiometric amounts of O₂ have failed, presumably because oxygen reacts faster with α-meso-hydroxyme than it does with heme. Attempts to add 1 reducing eq “anaerobically” to the preformed oxy-ferrous heme complex yielded a product with UV-visible and EPR spectra similar to those of α-meso-hydroxyme (data not shown), but the reaction product was not homogeneous.

**DISCUSSION**

The first step in heme degradation is thought to be the cytochrome P450 reductase-, NADPH-, and O₂-dependent α-meso-hydroxylation of heme by heme oxygenase. This transformation requires reduction of the ferric to the ferrous heme-protein complex by an electron from NADPH-cytochrome P450 reductase, oxygen binding to the iron to give the ferrous dioxygen (Fe(II)–OO) complex, and a second reduction to give an undetected species formally equivalent (after protonation) to a ferric peroxide (Fe(III)–OOH) complex. The formation of α-meso-hydroxyheme (data not shown), but the reaction product was not detected in earlier EPR studies of α-meso-hydroxyheme-recon-
stituted apomyoglobin or HO-1 (31, 42). We have found that the $g = 2.008$ radical signal is increasingly saturated when the instrument power is increased from 1 to 500 μW at 6 K (data not shown). When the power is raised above 400 μW at 6 K, the radical signal is completely saturated and disappears. It is therefore very likely that, under the conditions (10 K, 1000 μW) used in the earlier studies, the radical signal was saturated and thus was not observed.

The reaction of α-meso-hydroxymethylene with O$_2$ to give verdoheme, as shown here by kinetic, optical, EPR, and resonance Raman studies, is rapid and proceeds without a requirement for additional reducing equivalents. This finding is consistent with the reaction mechanism in Scheme 2. The ferrous π neutral radical of α-meso-hydroxymethylene (1) binds O$_2$ on the ring carbon adjacent to the keto group to form a ferrous hydroperoxy radical (O–O•••O; 2). Intramolecular electron transfer from the iron to the peroxo radical produces a ferric peroxide intermediate (3), the terminal oxygen of which coordinates to the iron to form a peroxo-bridged intermediate (4). Heterolytic dioxygen bond cleavage then yields a ferryl (Fe(IV)=O) species and an alkoy radical (5) that fragments with the elimination of CO. Internal electron transfer from the resulting pyrrole ring A carbon radical to the ferryl oxygen followed by reaction with the pyrrole ring B carbonyl group produces ferric verdoheme (6). Formation of ferric rather than ferrous verdoheme is established by several lines of evidence. (a) The addition of CO to the verdoheme generated from the heme-hHO-1 complex and 1 eq of H$_2$O$_2$ does not alter the UV-visible spectrum, which suggests that verdoheme is in the ferric state (Fig. 7, ---). (b) The addition of 1 reducing eq and CO to the verdoheme-hHO-1 complex yields the characteristic Fe(II) verdoheme-COUV-visible spectrum (Fig. 7, ———). (c) The addition of 1 reducing eq to the verdoheme produces a resonance Raman spectrum (Fig. 6, trace C) that closely resembles that reported for Fe(II) verdoheme (42). (d) The generation of a weak low spin signal associated with loss of the $g = 6$ signal in the aerobic reaction of the heme-hHO-1 complex with H$_2$O$_2$ suggests that the spin state of the verdoheme intermediate is $S = 1/2$ Fe(III) (Fig. 8, traces B and C).

Past studies with HO-1 (37, 38, 42) and apomyoglobin (31) suggest that the iron oxidation state of the verdoheme product is Fe(II). For example, Yoshida et al. (37, 38) reported for the NADPH-cytochrome P450 reductase-dependent reaction that a 688 nm intermediate located after hydroxyheme but before the reaction of the ferric heme complex preformed by anaerobic reaction with 1 eq of H$_2$O$_2$ suggests that the spin state of the verdoheme intermediate is $S = 1/2$ Fe(III) (Fig. 8, traces B and C).

With HO-1 catalytically oxidized heme peptide-heme, it has been demonstrated that the spin state of the verdoheme intermediate is $S = 1/2$ Fe(III) (Fig. 8, traces B and C).

The reaction of α-meso-hydroxymethylene with O$_2$ yields Fe(III)-biliverdin, which in turn reacts with O$_2$ to give biliverdin unless the high affinity of ferrous verdoheme for CO is used to arrest the reaction. The universal finding that ferrous verdoheme is the product of the reaction of α-meso-hydroxymethylene with O$_2$ simply reflects the fact that exogenous reducing agents were present in the experimental systems that were used. In the present studies carried out without exogenous reducing agents, the reaction product is clearly identified as ferric verdoheme. A case can be made that the formation of Fe(III) verdoheme is desirable to prevent the CO that is released in the reaction from binding to the intermediate and stopping the reaction process.

Sano et al. (31) reported that reaction of the ferric α-meso-hydroxymethylene-myoglobin complex with O$_2$ yields Fe(III)-biliverdin via Fe(II) verdoheme without the addition of reducing equivalents. Matera et al. (42) reported that both O$_2$ and 1 reducing eq are required for formation of Fe(II) verdoheme from the α-meso-hydroxymethylene-HO-1 complex. We find that oxygen alone is required for the conversion of α-meso-hydroxyheme to Fe(III) verdoheme, although an electron is required to reduce the Fe(III) verdoheme complex to the ferrous state. It is not clear why Matera et al. (42) failed to observe the formation of verdoheme in the reaction of the α-meso-hydroxymethylene-HO-1 complex with O$_2$. They apparently monitored verdoheme formation under CO by observing the Fe(II) verdoheme-CO complex at $\lambda_{max} = 402$ and 638 nm (37, 38). One possible explanation is that CO binds to the ferrous π neutral radical species (see Scheme 2, 7), causing the reaction to stop at the ferrous- CO peroxo radical adduct (8) due to inhibition of electron transfer from the iron to the peroxo radical by the CO ligand. An external electron might then be required to reduce the peroxo radical to the hydroperoxide (9), leading to formation of Fe(II) verdoheme (10) and the conclusion that an exogenous electron is required for the reaction to occur. This scheme would also explain their observation of an organic radical (8) when they exposed the α-meso-hydroxymethylene-hHO-1 complex to O$_2$ in the presence of CO. However, we have been unable to reproduce their observations by reacting the α-meso-hydroxymethylene-hHO-1 complex with O$_2$ for 5 min under a 50% CO atmosphere. Although these conditions may not closely reproduce theirs, our observation that the reaction does not stop at the ferrous CO-peroxo radical adduct (8), but continues to Fe(III) verdoheme (6) under these conditions, suggests that the affinity of the peroxo radical species for CO is not high.

Spectroscopically identical verdoheme species are formed when 1 eq of H$_2$O$_2$ is added aerobically to the heme-hHO-1 complex (one step) or by exposure to O$_2$ of the α-meso-hydroxymethylene-hHO-1 complex preformed by anaerobic reaction with 1 eq of H$_2$O$_2$ (two steps). The verdoheme yields obtained by the two approaches differ, however, with a ratio of ~1:0.6 for the one- and two-step processes, respectively. The higher intensity of the $g = 6$ signal in the EPR spectrum of the two-step reaction suggests that the lower yield of verdoheme is matched by a higher fraction of unreacted heme complex. This finding suggests that the α-meso-hydroxymethylene intermediate may react with H$_2$O$_2$ to give alternative reaction products, a possibility that is currently under investigation.

In summary, the formation of α-meso-hydroxymethylene in the HO-1-catalyzed oxidation of heme has been directly demonstrated, and its subsequent conversion to ferric verdoheme has been shown to require O$_2$, but not an exogenous electron. The exogenous electron is only required to reduce ferric verdoheme to the ferrous state.

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