Communications to the Editor

Oxidation of an α-Meso-Methyl-Substituted Heme to an α-Biliverdin by Heme Oxygenase. A Novel Heme Cleavage Reaction

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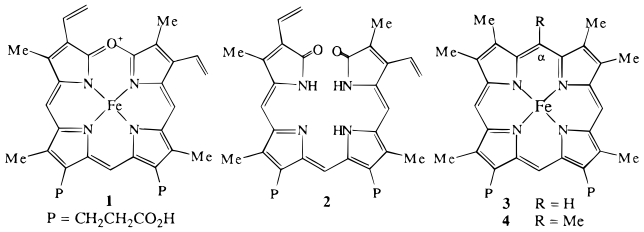
The physiological oxidation of heme to biliverdin is catalyzed by heme oxygenase in a reaction that consumes O_2 and reducing equivalents provided by NADPH-cytochrome P450 reductase. The catalytic sequence involves (a) NADPH- and O_2-dependent α-*meso*-hydroxylation of the heme, (b) O_2-dependent elimination of the α-*meso*-carbon as CO with concomitant formation of verdoheme (**1**), and (c) NADPH- and O_2-dependent conversion of verdoheme to biliverdin (**2**). ^{18}O-Labeling studies have



demonstrated that the oxygens incorporated into both the CO and the verdoheme oxygen bridge derive from O_2.[2] Reaction of heme oxygenase with preformed α-*meso*-hydroxyheme yields the normal reaction products.[3] Although the details of extrusion of CO and the formation of verdoheme remain obscure, the role of α-*meso*-hydroxyheme as a reaction intermediate appears to be well-established. In order to further define the oxidation mechanism, we have investigated the oxidation of a heme with an α-*meso*-methyl substituent that should block α-*meso*-hydroxylation and therefore abrogate the catalytic process. We report here that the normal α-biliverdin is formed despite the α-*meso*-methyl substituent and provide evidence that precludes the involvement of an α-*meso*-hydroxyheme intermediate in the oxidation of the modified heme.

To avoid complications due to the asymmetry of heme (iron protoporphyrin IX), the present studies were carried out with the symmetric heme analogue **3** and its α-*meso*-methyl derivative **4**.[4] The enzyme used for the studies was a recombinant, truncated version of human heme oxygenase-1 (hHO-1) without the 23 amino acid membrane anchor.[5] The absorption maxima of the ferric, ferrous−CO, and ferrous−O_2 hHO-1 complexes of **4** are red-shifted by 4−10 nm relative to those of **3**.[6] Reaction of the complex of hHO-1 with **3** (300 μg, 9.9 nmol) with P450 reductase (1.6 μg, 20 pmol) and NADPH (83 μg, 0.1 μmol) under a partial CO atmosphere arrests the reaction at a stage identified by its characteristic 614 nm absorption maximum as the Fe^{2+}−CO verdoheme complex.[3a,7,8] This complex produces a biliverdin upon further exposure to O_2.[9] Reaction of the complex of hHO-1 with the α-*meso*-methyl substituted derivative **4** (220 μg, 7.2 nmol) under identical conditions produces an identical absorption spectrum (Figure 1),[6] demonstrating the formation of an Fe^{2+}−CO verdoheme complex.

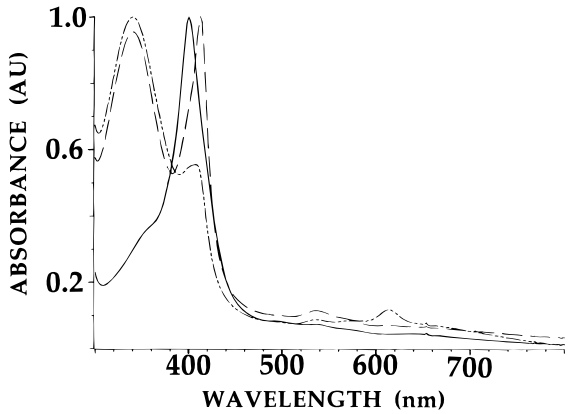


Figure 1. Absorption spectra observed in the oxidation of **4** by hHO-1: ferric complex (—); Fe^{2+}−CO complex (- - -); Fe^{2+}−CO verdoheme complex (-···-). The spectroscopic changes closely resemble those observed in the oxidation of **3** by hHO-1.

To obtain enough of the biliverdin for HPLC, UV, and mass spectrometric analyses, the reactions were scaled up and run in the absence of CO. Reaction of the hHO-1 complex with **3** (2.6 mg, 86.8 nmol) with P450 reductase (60 μg, 0.75 nmol) and NADPH (4.1 mg, 5.0 μmol) for 1 h at 25 °C produced a blue-green solution. After acidification to pH 3 with HCl and AcOH, the biliverdin was extracted with diethyl ether and was shown by HPLC analysis to be a single isomer with a retention time of 10.9 min and absorption maxima (MeOH) at 364 and 638 nm.[10] The mass spectrometric molecular ion of the biliverdin was at *m*/*z* (MH^+) 559 (calcd for $C_{31}H_{34}O_6N_4$ 558),

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(1) For recent reviews, see: (a) Maines, M. D. *Heme Oxygenase—Clinical Applications and Functions*; CRC Press: Boca Raton, FL, 1992. (b) Beale, S. I. *Chem. Rev.* **1993**, *93*, 785.
(2) (a) Tenhunen, R.; Marver, H.; Pimstone, N. R.; Trager, W. F.; Cooper, D. Y.; Schmid, R. *Biochemistry* **1972**, *11*, 1716−1720. (b) Docherty, J. C.; Schacter, B. A.; Firneisz, G. D.; Brown, S. B. *J. Biol. Chem.* **1984**, *259*, 13066−13069.
(3) (a) Yoshida, T.; Noguchi, M.; Kikuchi, G.; Sano, S. *J. Biochem. (Tokyo)* **1981**, *90*, 125−131. (b) Yoshinaga, T.; Sudo, Y.; Sano, S. *Biochem. J.* **1990**, *270*, 659−664. (c) Jackson, A. H.; Kenner, G. W.; Smith, K. M. *J. Chem. Soc. C* **1968**, 302−310. (d) Sano, S.; Sano, T.; Morishima, I.; Shiro, Y.; Maeda, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 531−535. (e) Morishima, I.; Fujii, H.; Shiro, Y. *J. Am. Chem. Soc.* **1986**, *108*, 3858−3860. (f) Bonnett, R.; Chaney, B. D. *J. Chem. Soc., Perkin Trans. 1* **1987**, 1063−1067.
(4) (a) Lee, D. A.; Xie, H.; Senge, M. O.; Smith, K. M. *J. Chem. Soc., Chem. Commun.* **1994**, 791−792. (b) Xie, H.; Lee, D. A.; Wallace, D. M.; Senge, M. O.; Smith, K. M. Submitted for publication.
(5) Wilks, A.; Black, S. M.; Miller, W. L.; Ortiz de Montellano, P. R. *Biochemistry* **1995**, *34*, 4421−4427.
(6) Absorption maxima of hHO-1 complexes of **3**: Fe^{3+} (394 nm), Fe^{2+}−CO (410, 526, 558 nm), Fe^{2+}−O_2 (402, 532, 566 nm), and Fe^{2+}−CO verdoheme (614 nm). Absorption maxima of hHO-1 complexes of **4**: Fe^{3+} (400 nm), Fe^{2+}−CO (414, 534 nm), Fe^{2+}−O_2 (408, 536 nm), and Fe^{2+}−CO verdoheme (614 nm).
(7) Lagarias, J. C. *Biochem. Biophys. Acta* **1982**, *717*, 12−19.
(8) Iron(III) porphyrins **3** and **4** were combined with hHO-1 in a 2:1 heme to enzyme ratio, and the mixture was purified over a BioRad HTP column to give a 1:1 complex in 100 mM potassium phosphate buffer (pH 7.4).
(9) Wilks, A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 22357−22362.
(10) Semipreparative reverse phase (C18) HPLC was done with 50:50 v/v acetone/0.1% aqueous formic acid at a flow rate of 3.0 mL/min. Mass spectra were obtained by (+)LSIMS using a 1:1 (1% trifluoroacetic acid) glycerol/thioglycerol matrix.

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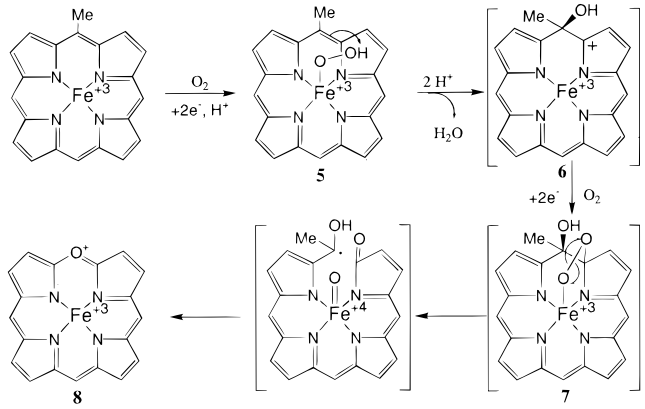
in accord with normal cleavage of **3** at the α-*meso*-carbon. Identical reaction, workup, and analysis of the hHO-1 complex with **4** (2.9 mg, 96.8 nmol) again yielded a single biliverdin isomer with the same retention time and absorption and mass spectra as those of the biliverdin product obtained with **3**. These results, particularly the mass spectrum, clearly establish that hHO-1 oxidizes **4** to the same α-biliverdin product as formed from **3**.

To determine if the α-*meso*-methyl group is catalytically removed from **4** by hHO-1, permitting normal α-hydroxylation and heme cleavage, hHO-1 was incubated with **3** and **4** and the production of CO was monitored by adding ferrous deoxymyoglobin (λ_{max} 434 nm) at the end of the incubation. Formation of the Mb−CO complex (λ_{max} 422 nm) was readily observed with **3**, but no such complex was detected with **4**. Cleavage of **4** to the α-biliverdin derivative does not involve the α-*meso*-hydroxylated intermediate.

A possible mechanism (Scheme 1: peripheral substituents not shown) for the oxidation of **4** that circumvents the α-*meso*-hydroxy intermediate incorporates elements similar to those proposed to explain the chemical cleavage of a cobalt porphyrin bearing a carboxy-substituted naphthalene moiety at the cleaved meso position.[11] Thus, reductive activation of O_2 to the level of H_2O_2 yields a ferric peroxide complex **5** that adds electrophilically to the heme ring. The resulting cation **6** may be stabilized as the epoxide or by combination with a protein nucleophile. Two-electron reduction and O_2 binding yield the peroxide **7** that undergoes iron-catalyzed homolytic cleavage.

(11) Chang, C. K.; Avilés, G.; Bag, N. *J. Am. Chem. Soc.* **1994**, *116*, 12127−12128.

Scheme 1



Elimination of the methyl-substituted α-*meso*-carbon, possibly as acetic acid, followed by intramolecular electron transfer produces the verdoheme derivative **8**. The steps beyond verdoheme are the same for the oxidation of **3** and **4**.

The role of this alternative pathway in the normal oxidation of heme by heme oxygenase remains to be explored, but the chemistry that it represents is of general significance. It supports the conclusion that the first step is electrophilic addition to the heme and makes the search for additional reaction intermediates attractive.

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