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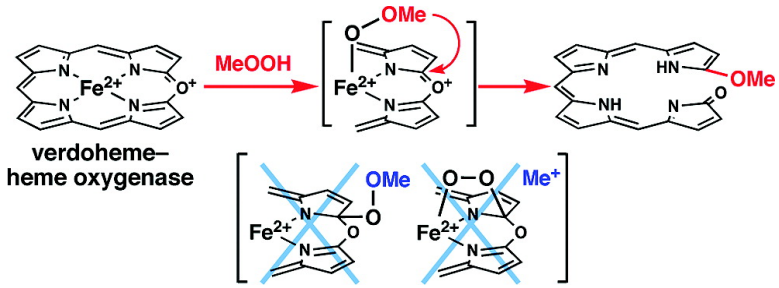


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# Alkyl Peroxides Reveal the Ring Opening Mechanism of Verdoheme Catalyzed by Heme Oxygenase

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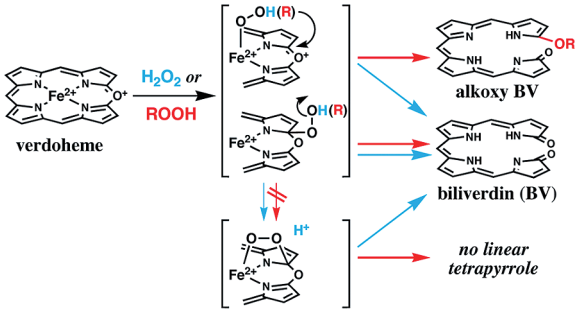
Heme oxygenase (HO) catalyzes the catabolism of heme (iron-protoporphyrin IX) to biliverdin, CO, and free iron.[1−3] The substrate heme itself reductively activates $O_2$ during three successive oxygenations of the HO catalysis: the first, *meso*-hydroxylation of heme; the second, conversion to verdoheme; and the third, ring-opening of verdoheme to yield biliverdin. The first heme hydroxylation has been extensively studied to identify an Fe−OOH heme as an active species.[4,5] The verdoheme ring-opening has been the least understood step in the HO catalysis, even though this third oxygenation is considered as the rate-determining step to regulate HO enzyme activity *in vivo*.[6]

While the HO enzyme was originally reported to exclusively utilize $O_2$ for the conversion of verdoheme to biliverdin,[2,7] we have recently demonstrated that $H_2O_2$ also supports this ring-opening reaction *in vitro*, and possibly *in vivo* under severe oxidative stress.[8] The $H_2O_2$ reaction proceeds in a manner similar to that of the $O_2$ reaction, and the two reactions are initiated by binding of either $O_2$ or $H_2O_2$ to verdoheme.[8] The $H_2O_2$ reaction indicates that the dioxygen species bound to verdoheme can be protonated prior to its ring cleavage. On the basis of the inhibitory and mutational studies, we have proposed a mechanism involving an Fe−OOH verdoheme complex which adds its terminal OH to an α-pyrrole carbon (Scheme 1, *top*).[8] We cannot rule out, however, a nucleophilic attack of $H_2O_2$ to the α-pyrrole carbon[2] (Scheme 1, *middle*) and formation of a bridged intermediate by putative deprotonation of the Fe−OOH or ring−OOH intermediate[2] (Scheme 1, *bottom*). In this study, we have examined reactions of a verdoheme−HO-1 complex with alkyl hydroperoxides, namely MeOOH, to discriminate the three possible pathways for the ring-opening mechanism (Scheme 1, *red*). The Fe−OOR pathway could add the alkoxy moiety to one end of biliverdin, while the ring−OOR pathway would give normal biliverdin. When the ring-opening requires the bridged intermediate, no linear tetrapyrrole should be observed because the alkyl group is hardly liberated.

Reactions of the verdoheme IXα−rat HO-1 complex[8] were performed under anaerobic conditions in 0.1 M HEPES, pH 7.5 at 20 °C. An aqueous solution of MeOOH[9,10] was treated by catalase and then bubbled by $N_2$ gas to remove $H_2O_2$ and $O_2$. The ferrous verdoheme−HO-1 complex (Figure 1A, *black*) reacted with MeOOH to exhibit an absorption spectrum similar to that of the *ferric* verdoheme complex[7] (Figure 1A, *red*). While following addition of sodium ascorbate partially regenerate the starting *ferrous* verdoheme complex (Figure 1A, *cyan*), its incomplete recovery implicates the presence of product(s) other than ferric verdoheme.

The second product was successfully accumulated by the MeOOH reaction in the presence of excess sodium ascorbate (Figure 1B). Repetitive addition of MeOOH up to 30 equiv caused a spectral change with distinct isosbestic points. The final spectrum (Figure 1B, *red*) is similar to but slightly different from that of biliverdin ($\lambda_{max}$(biliverdin): 381 and 690 nm). A reversed phase HPLC

***Scheme 1.*** Three Possible Pathways and Their Expected Products for the Verdoheme Ring-Opening by HO When Using $H_2O_2$ (*Cyan*) and ROOH (*Red*) as Oxygen Sources[a]




[a] Peripheral substituents and a left-half of the macrocycle are omitted for clarity.

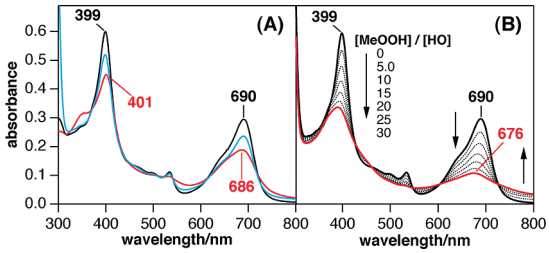



***Figure 1.*** Absorption spectral changes during the anaerobic reactions of the ferrous verdoheme−HO-1 complex (12 μM) with MeOOH. (A) Spectra before and after addition of 84 μM MeOOH (*black* and *red*, respectively) and following addition of sodium ascorbate (*cyan*). (B) Spectra after addition of the indicated amounts of MeOOH in the presence of 2 mM sodium ascorbate. Arrows indicate directions of the spectral change.

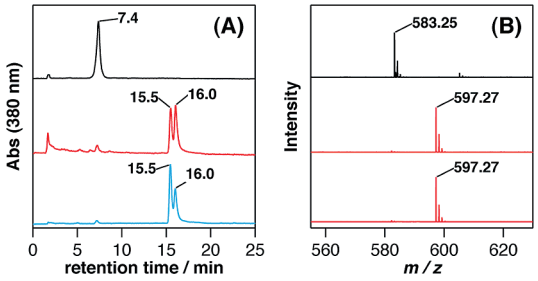
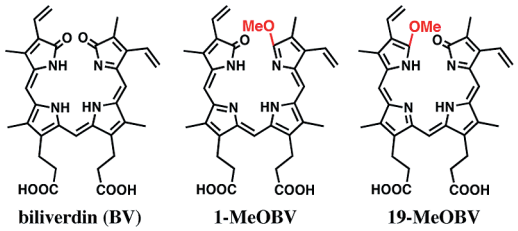



***Figure 2.*** Product analysis of the MeOOH-dependent reaction of the verdoheme−HO-1 complex. Panel A shows HPLC chromatograms of authentic biliverdin (*top*), the HO/MeOOH products (*middle*), and the verdoheme/NaOMe products (*bottom*). Panel B exhibits ESI-MS spectra of biliverdin (*top*) and "15.5 min" and "16.0 min" species of the HO/MeOOH products (*middle* and *bottom*, respectively).

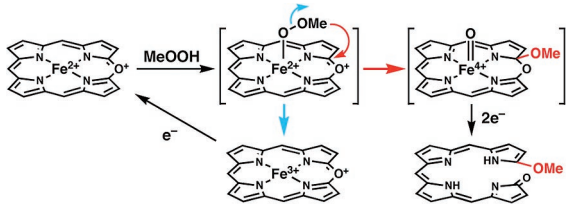
analysis of the reaction mixture (Figure 2A, *middle*) showed two peaks at 15.5 and 16.0 min with a negligible amount of biliverdin (7.4 min). The two HO/MeOOH products seem to be an isomeric mixture of 1- and 19-methoxy-deoxy-biliverdin IXα (1- and 19-


10.1021/ja710495z CCC: $40.75 

***Chart 1***




***Scheme 2.*** Reactions of the Verdoheme−HO-1 Complex with MeOOH[a]




[a] Peripheral substituents of the macrocycle are omitted for clarity.

MeOBV in Chart 1) because of their similar absorption spectra (*data not shown*) and their monocationic mass signals both at 597.27 (Figure 2B, *m*/*z*: calculated for $[C_{34}H_{36}N_4O_6 + H^+]$, 597.27).

Authentic MeOBVs were prepared by chemical ring opening of verdoheme IXα according to a method reported for a corresponding reaction of octaethylverdoheme.[11] Treatment of the ferrous verdoheme IXα pyridine complex with sodium methoxide (NaOMe) followed by acidification with acetic acid under anaerobic conditions gave two products. These products, designated as verdoheme/NaOMe products, are essentially the same as the HO/MeOOH products in terms of HPLC retention times (Figure 2A, *bottom*), absorption spectra (*data not shown*), and *m*/*z* values of the molecular ions (597.28). $^1$H NMR spectra of the isolated verdoheme/NaOMe products (Supplemental Figure S1) show that these compounds, and hence the HO/MeOOH products, are the mixture of 1- and 19-MeOBV (Chart 1).

The addition of a methoxy group at one end of the linear tetrapyrrole leads to a clear conclusion that HO catalyzes the MeOOH-dependent ring-opening of verdoheme through the Fe−OOMe intermediate (Schemes 1 and 2). The Fe−OOMe formation is also consistent with the ferric verdoheme production in the MeOOH reaction (Figure 1A). The iron oxidation would be a result of the methoxy liberation from the Fe−OOMe complex (Scheme 2, *cyan*). Thus, MeOOH and probably $H_2O_2$ as well bind to the ferrous iron of verdoheme rather than its macrocycle. Once the Fe−OOH verdoheme is generated, the OH moiety can rebind to the α-pyrrole carbon as the methoxy group does. The OH transfer of the Fe−OOH verdoheme can proceed more efficiently than the methoxy rearrangement as discussed below.

The Fe−OOH mechanism of the verdoheme reaction is analogous to the well-defined mechanism of the first *meso*-hydroxylation of heme catalyzed by HO.[4,5] In the first step, the OH transfer of the Fe−OOH heme to the *meso*-carbon is enhanced by a nearby water molecule fixed at a proper position by a distal Asp residue (Asp140 in mammalian HO-1).[12−15] The distal Asp mutation significantly retards the $O_2$- and $H_2O_2$-supported reactions of both the heme-hydroxylation[12−15] and the verdoheme opening.[8] Therefore, the OH transfer in verdoheme also appears to be assisted by the critical water molecule which may activate the Fe−OOH species and/or can block the escape of the OH group from the active site.[8,14] This critical interaction with the nearby water should be weaker in the Fe−OOMe species due to its terminal methoxy group. In addition, the small OH group can be more easily attached to the α-pyrrole-carbon than the larger methoxy group. As the size of the alkyl group increases, the rebinding efficiency of the alkoxy group is further lowered: ethoxy biliverdin formation with EtOOH requires more peroxide (40 equiv) than in the MeOOH reaction, and *t*-BuOOH and $Ph(CH_3)_2COOH$ did not afford any linear tetrapyrroles (*data not shown*). A very similar peroxide-dependence was reported for the first *meso*-hydroxylation in the HO catalysis.[7,16]

Here we present the first evidence for the alkoxy rearrangement of $Fe^{2+}$−OOR verdoheme to the α-pyrrole carbon in HO. The corresponding OH transfer of the $Fe^{2+}$−OOH species is highly probable in the verdoheme degradation using $H_2O_2$. The $O_2$-dependent reaction is also proposed to involve the OH transfer step; however, there remains some ambiguity in the oxidation state of the reactive intermediate. The $Fe^{2+}$−OOH formation in the $O_2$ reaction requires reduction of a putative $Fe^{3+}$−OOH complex, and the O−O bond of the ferric complex may be cleaved without generating the ferrous species. Although the different redox state might affect the mode of the ring cleavage, our mutational study has pointed out a mechanistic similarity between the $O_2$ and $H_2O_2$ reactions.[8] Characterization of the "peroxy-verdoheme" would clarify the complex $O_2$-dependent reaction.

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**Supporting Information Available:** Detailed procedures of product analysis and $^1$H NMR spectra of MeOBVs. This material is available free of charge via the Internet at http://pubs.acs.org.

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