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Compound I of Heme Oxygenase Cannot Hydroxylate Its Heme *meso*-Carbon

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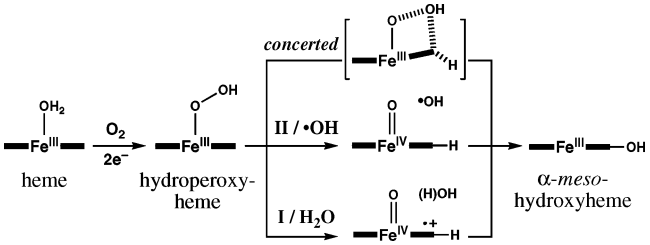
Heme oxygenase (HO) catalyzes the degradation of heme (iron-protoporphyrin IX) to biliverdin, CO, and free iron through three successive oxygenations in which the substrate heme itself reductively activates O_2.[1,2] The first oxygenation of heme occurs regiospecifically at the α-meso position to afford α-*meso*-hydroxyheme (Scheme 1). The meso-hydroxylating species has been thought to be a hydroperoxy-ferric heme intermediate ($Fe^{III}-OOH$), while most heme enzymes employ an oxo ferryl porphyrin coupled with a porphyrin or protein radical, so-called compound I, as a reactive species. The noninvolvement of compound I in HO catalysis was first proposed from the fact that H_2O_2 but not alkyl peroxides and peracids can be a surrogate of O_2 and electrons.[3] Mutational studies also suggested the ferryl heme to be a dead-end product for the heme catabolism.[4,5] The Fe−OOH species of HO has been observed at low temperatures and converts to α-*meso*-hydroxyheme in a single kinetic step, without detectable intermediates.[6] This Fe−OOH reaction exhibits a significant kinetic solvent isotope effect, indicating activation by proton transfer, but also shows a small secondary isotope effect on deuteration of the α-meso position.[7] This finding suggested that hydroxylation by Fe−OOH may be concerted and may involve a tetrahedral transient state (Scheme 1, top).

Recent theoretical studies,[8−11] however, predicted high activation energy for the concerted hydroxylation due to significant folding of the porphyrin ring. Shaik and co-workers proposed a related, but stepwise, pathway in which initial O−O bond *homolysis* of Fe−OOH is followed by addition of the resulting •OH to the meso position of the ferryl porphyrin (compound II) that was formed (Scheme 1, middle).[8,9] Although compound II alone is inactive for self-hydroxylation,[3] the reactivity of a transient •OH radical generated above the *meso*-carbon has not yet been evaluated. This compound II/•OH mechanism can also account for the solvent isotope effect observed.[7,9] Kamachi and Yoshizawa proposed a different stepwise mechanism where compound I is formed by O−O bond *heterolysis*, and the H_2O thus liberated attacks the compound I (Scheme 1, bottom).[11] This proposal appears to contradict the experimental evidence suggesting noninvolvement of compound I in the HO catalysis; however, the compound I/H_2O mechanism cannot be dismissed because there has been no investigation of compound I reactivity. In this study, we report the first direct observation of compound I of rat HO-1, formed in the reaction with *m*-chloroperbenzoic acid (*m*CPBA). Subsequent reactions of HO compound I have been examined to evaluate its involvement in the first step of HO catalysis.

Reactions of the ferric heme-HO-1 complex[12] with *m*CPBA were examined by means of a rapid-scan stopped-flow apparatus in 0.1 M potassium phosphate, pH 7.0 at 20 °C. Upon mixing with *m*CPBA, a biphasic spectral change was observed as shown in Figure 1. The first reaction, with isosbestic points at 376, 428, and

Scheme 1. Three Pathways Proposed for the Heme meso-Hydroxylation by Heme Oxygenase



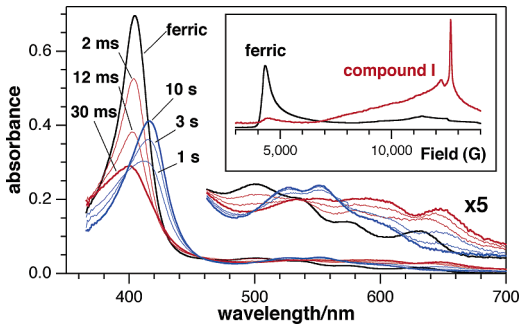


Figure 1. Absorption spectral changes during the reaction of the ferric heme-HO-1 complex (4.5 μM) and *m*CPBA (10 μM). Spectra were recorded before mixing with *m*CPBA (black line) and at an indicated time after mixing (red and blue lines). Inset: Absorption EPR spectra of HO-1 ferric heme and compound I, taken under rapid-passage conditions: $\nu_{MW} \approx 35.4$ GHz, $T = 2$ K, 100 kHz field modulation (amplitude 4 G).

524 nm, is completed within 30 ms after mixing. The Soret absorption decreased to ~40% that of the starting ferric form, with a slight shift of the peak from 404 to 400 nm. A broad absorption having a peak at 648 nm appears in the visible region. The observed rate constant for this phase increased proportionally with *m*CPBA concentration ($k_{mCPBA} = 1.9 \pm 0.2 \times 10^7$ M^{-1} s^{-1}).

The spectral features of this first product are reminiscent of horseradish peroxidase (HRP) compound I (i.e., an oxo ferryl porphyrin cation radical species).[13] To test this assignment, the HO intermediate was trapped by rapid freeze-quenching (rfq) at liquid nitrogen temperature[14] and its 2 K Q-band EPR spectrum was taken. The inset to Figure 1 shows that in the intermediate the high-spin ferriheme signal of ferric-HO-1 is almost completely replaced by a broad signal that peaks near *g*-2 and trails off to high and low fields. This is characteristic of a compound I in which the exchange between Fe(IV)═O ($S = 1$) and porphyrin radical ($S = {}^1/_2$) is weak and the exchange-coupling parameter, J, is distributed over a range of values around $J \approx 0$.[15] Thus, the EPR confirms that reaction of *m*CPBA with ferric heme-HO-1 indeed rapidly gives compound I.

In the second phase reaction of Figure 1, the Soret band slowly (0.41 s^{-1}) increases in intensity and red-shifts to 416 nm, with an appearance of two visible peaks at 526 and 551 nm.[16] The resulting spectrum identifies the second product as compound II of HO.[3] At longer times (several hours), compound II returns to the resting

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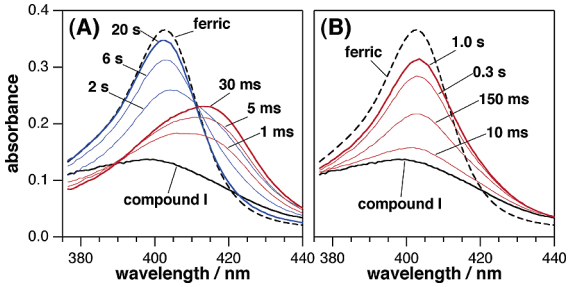

Figure 2. Reactions of HO compound I with (A) 7.5 μM guaiacol and (B) 50 μM thioanisole. Black broken and solid lines, respectively, represent the starting ferric form (2.5 μM) and compound I prepared by 1.5 mol equiv of *m*CPBA (aging time: 55 ms). Spectra were recorded at indicated time after mixing with the substrates (red and blue lines).

ferric state (Supporting Information) *without* conversion to the α-*meso*-hydroxyheme-HO.

To further characterize compound I, we examined its reaction with guaiacol and thioanisole in double-mixing stopped-flow experiments; these are typical substrates for one- and two-electron oxidation, respectively[17,18] (Figure 2). The ferric heme-HO-1 was initially mixed with 1.5 mol equiv of *m*CPBA to generate compound I in approximately 80% yield. A second mixing with guaiacol promptly (~200 s^{-1} with 7.5 μM guaiacol) gave compound II, which then slowly but almost completely returned to the starting ferric heme (Figure 2A). In contrast, upon the second mixing with thioanisole, compound I converted directly to the ferric heme at a rate of 7.5 s^{-1} (Figure 2B). The smaller recovery of the ferric heme (~75%) is due to ~20% accumulation of compound II, which is inactive with thioanisole. Product analysis showed formation of the corresponding sulfoxide with 59% enantiomeric excess.[19] These results further confirm that the first intermediate is compound I, oxidized 2 equiv above the ferric state, and located in the chiral heme pocket.

As shown in Figure 1, HO compound I is not able to produce a meso-hydroxylated product. One may think that this inability is a result of using the bulky aromatic peracid. In the HO heme distal pocket there is a well-conserved extended hydrogen bond network involving several water molecules.[20,21] One of the water molecules located in the close vicinity of the sixth ligand of heme plays a critical role in the meso-hydroxylation.[22,23] *m*CPBA binding to the heme iron would likely displace the critical water and perturb the hydrogen bond network, possibly inhibiting the compound I/H_2O mechanism for meso-hydroxylation. The product of oxidation, *m*-chlorobenzoic acid (*m*CBA), might also be transiently retained in the distal pocket, disrupting this mechanism. However, the lifetime of compound I is quite long enough ($t_{1/2}$ = 1.7 s) for displacement of the *m*CBA and re-formation of the entire hydrogen bond/water network. Moreover, *m*CBA, if retained, would block access of a substrate to the ferryl oxygen of compound I, but the large, hydrophobic thioanisole can access and accept this oxygen within the compound I lifetime (Figure 2B). In addition, during the physiologically relevant, HO reaction of heme degradation in the presence of O_2, NADPH, and cytochrome P450 reductase, co-incubation of guaiacol does not lead to its oxidation[3] despite its high reactivity with compound I (Figure 2A). We have further confirmed that guaiacol has negligible effect on the reaction rates and products of meso-hydroxylation (data not shown).

On the basis of these findings, we conclude that HO compound I is *inactive* for meso-hydroxylation and is not generated during the course of HO catalysis. This conclusion is consistent with the following facts: (i) the active site of HO is not designed to promote the O−O bond *heterolysis* of Fe−OOH[20,24] and (ii) no compound I formed in other heme enzymes has been reported to oxidize its *meso*-carbon, despite possible water access. The recently proposed compound I/H_2O pathway[11] thus is unambiguously ruled out for the heme meso-hydroxylation (Scheme 1, bottom). To discriminate between the remaining two mechanisms (Scheme 1, top and middle), further studies of the kinetics of hydroxylation of multiply isotope-labeled hemes by the Fe−OOH are in progress.

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Supporting Information Available: Absorption spectra during the reaction of ferric-heme-HO-1 with *m*CPBA at longer times. This material is available free of charge via the Internet at http://pubs.acs.org.

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