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Comparison of the mechanisms of heme hydroxylation by heme oxygenases-1 and -2: kinetic and cryoreduction studies

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Abstract

The two isoforms of human heme oxygenase (HO1 and HO2) catalyze oxidative degradation of heme to biliverdin, Fe, and CO. Unlike HO1, HO2 contains two C-terminal heme regulatory motifs (HRMs) centered at Cys265 and Cys282 that act as redox switches and, in their reduced dithiolate state, bind heme (Fleischhacker, et al., 2015, *Biochemistry* **54**, 2693-708). Here, we describe cryoreduction/annealing and EPR spectroscopic experiments to study the structural features of the oxyheme moiety in HO2 and to elucidate the initial steps in heme degradation. We conclude that the same mechanism of heme hydroxylation to α -meso-hydroxyheme is employed by both isoforms and that the HRMs do not affect the physico-chemical properties of the oxy-Fe(II) and HOO-Fe(III) states of HO2. However, the absorption spectrum of oxy-Fe(II)-HO2 is slightly blue shifted relative to that of HO1. Furthermore, heme hydroxylation proceeds three times slower and the oxy-Fe(II) state is 100-fold less stable in HO2 than in HO1. These distinctions are attributed to slight structural variances in the two proteins, including differences in equilibrium between open versus closed conformations. Kinetic studies revealed that heme oxygenation by HO2 occurs solely at the catalytic core in that a variant of HO2 lacking the C-terminal HRM domain exhibits the same specific activity as one containing both the catalytic core and HRM domain; furthermore, a truncated variant containing only the HRM region binds but cannot oxidize heme. In summary, HO1 and HO2 share similar catalytic mechanisms and the HRMs do not play a direct role in the HO2 catalytic cycle.

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Supporting Information. Includes UV-VIS spectra of Fe(II)-heme HO1 and its complex with O₂ and comparison of the EPR spectra of 77 K cryoreduced oxy HO1 and oxy HO2. (<http://pubs.acs.org>)

Author Contributions

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Keywords

Electron Paramagnetic Resonance; Cryoreduction; Oxygenase

Heme oxygenase (HO) catalyzes the O₂- and NADPH-cytochrome P450 reductase-dependent conversion of heme to biliverdin, free iron, and CO through three successive oxygenation steps. Heme participates both as a prosthetic group and as substrate in the process.^{1–4} Biliverdin is subsequently converted to the antioxidant bilirubin by biliverdin reductase (BVR). This process is highly important process in metal ion homeostasis, given that both heme and iron are required, yet toxic at elevated levels. The HO/BVR system appears to be the sole mechanism for heme degradation and the sole source of CO in mammals. In all amniotes (from reptiles and birds to humans), there are two isoforms, HO1 and HO2, yet most work on the catalytic mechanism has been performed with HO1. The HO1 catalytic cycle begins with Fe(III)-heme coordination to the neutral imidazole of histidine to form a heme-protein complex (Fe(III)-HO1).^{1, 4–6} In the first monooxygenation step, heme is converted to the α -meso-hydroxyheme through the following process. NADPH cytochrome P450 reductase (CPR) donates one electron to reduce the ferric heme on HO1 to the ferrous state (Fe(II)-HO1), which promotes the binding of dioxygen to form an oxy-Fe(II)-HO1 complex. Then, oxy-Fe(II)-HO1 undergoes a second one-electron reduction by CPR to generate hydroperoxyferri-HO. Experiments with meta-chloroperoxybenzoic acid (*m*-CPBA) and H₂O₂ performed by Wilks and Ortiz deMontellano⁷ and Ishikawa² as well as cryoreduction/annealing experiments with oxy-Fe(II)-HO1⁸ demonstrated that the reactive hydroxylating species for HO1 is indeed the hydroperoxy ferric HO species, rather than a high-valent ferryl intermediate, which is the reactive species in most oxidases like peroxidases and cytochrome P450.⁹ In the second oxygenation step, the reactive α -meso-hydroxyheme is rapidly and spontaneously autoxidized. Lastly, in the third oxygenation step, O₂ is activated by verdoheme, which cleaves the bound porphyrin macrocycle to form biliverdin and free ferrous iron, which dissociate from the protein, preparing the enzyme for the next catalytic cycle. The last step is considered to be the major rate-determining step of HO catalysis.

The two HO isoforms catalyze the same reaction, share ~45% sequence identity and have similar structures of their catalytic cores, which consist of residues 20–248 for HO1 and 1–228 for HO2.^{5, 10} However, HO1 and HO2 differ in several notable ways. HO1 exhibits inducible expression, whereas HO2 is expressed constitutively.¹¹ HO1 is localized mainly in the liver and spleen, where it serves to degrade excess heme, whereas HO2 is present largely in the brain where it is implicated in oxygen sensing by the carotid body,^{12, 13} mediating oxidative stress in neurons¹⁴ and regulating cerebral blood flow.^{5, 10, 14, 15}

A particularly intriguing difference between HO1 and HO2 is the complete lack of Cys residues in the former and the presence of three Cys residues as part of heme regulatory motifs (HRMs) in HO2.^{16–18} Spectroscopic and thiol-trapping studies of HO2 and variants that possess site-directed substitutions in the HRMs provided evidence that the cysteine residues of the two C-terminal HRMs act as a redox switch that interconverts between the disulfide and dithiolate states, and, upon reduction, Cys265 provides a free thiol group

capable of coordinating to heme.^{17, 19–21} The physiological relevance of this switch was questioned;¹⁸ however, various studies demonstrated that the redox state of these two HRMs change in accordance with the cellular redox state.^{17, 20} Although it was clearly shown that the thiolates can coordinate heme, the role of the HRMs remained unclear. We had conjectured the possibility of a so-called fishhook mechanism, in which one of the Cys thiolates from the HRMs coordinates to the heme bound to the catalytic core.¹⁷ Recently, the oxidized form of HO2 (HO2^O), with its HRM thiolate ligands tied up in a disulfide bond, was shown to only bind heme at the catalytic core, while HO2^R, the reduced form of HO2, containing the HRM Cys residues in the dithiolate state, was shown to bind up to two additional Fe(III)-heme moieties independently of the catalytic core, with an affinity ca. 10-fold (HRM1 containing Cys265) and 100-fold (HRM2 containing Cys282) lower, respectively, than that of the catalytic core.²² However, it was not clear if the HRM has catalytic activity to degrade its bound Fe(III)-heme.

This report tests for differences in function of HO1 and HO2 that might arise because HO1 has only a single heme binding site, while HO2 binds additional Fe(III)-heme at the HRMs at its C-terminal end.^{21, 22} The role for the HRM in HO2 has not yet been determined. Because the relatively less studied HO2 exhibits several modes of binding Fe(III)-heme, including the thiolate/Fe(III)-heme coordination in the HRMs, we had hypothesized that the mechanism of HO2 catalysis might differ from that of well-characterized HO1. For example, in the proposed fishhook mechanism,¹⁷ one of the Cys thiolates from the HRMs coordinates to the heme bound to the catalytic core. Recent NMR studies indicate that the C-terminal HRM region of HO2 does associate in a redox dependent manner with the catalytic core.²¹ Furthermore, although mutagenesis studies demonstrated that the HRMs are not necessary for catalysis at saturating heme concentrations,¹⁷ the rate limiting step in heme degradation is formation or release of biliverdin;²³ therefore, it was important to directly examine the spectra of catalytic intermediates to test the possibility that the HRM may be involved in one of the early steps in the mechanism. Finally, given that the role of the HRM is unknown, it was important to determine if there is any heme degradation activity associated with the HRMs or if all of the HO catalytic activity arises from the core.

We now report the combination of radiolytic cryoreduction/annealing with UV-VIS and EPR spectroscopy to study structural features of the oxy-Fe(II)-heme moiety in HO2 and to elucidate the mechanism of hydroxylation of bound heme, the first step of HO-catalyzed heme degradation.⁸ In general, our combined results suggest that the mechanisms of HO1 and HO2 are similar; however there are slight differences between the absorption spectra as well as significant differences in the stability of the oxy-Fe(II) states of HO1 and HO2. Furthermore, there is no evidence for a thiol-ligated heme-based intermediate during catalysis and of the three heme-binding sites in HO2 (the catalytic core and the two HRMs), only the catalytic core can catalyze heme oxidation.

Methods

Chemicals

Sodium dithionite, glycerol, glycerol- d_3 were bought from Sigma-Aldrich. D_2O was obtained from Cambridge Isotope Laboratories Inc. O_2 was purchased from Cryogenic Gas Co.

Protein Expression and Purification

The soluble forms of HO2 were expressed and purified as described previously.^{21, 22} Briefly, HO2 spanning residues 1–288 (HO2_{sol}) or residues 1–248 (HO2_{core}) were expressed with an N-terminal six-His tag from pET28a, purified on a Ni-nitrilotriacetic acid affinity column (Qiagen), and treated with thrombin to remove the affinity tag. HO2 spanning residues 213–288 (HO2_{tail}) was expressed with N-terminal six-His tag and a protein G B1 (GB1) domain in a modified version of pMCSG7, purified on a Ni-nitrilotriacetic acid affinity column (Qiagen), and treated with tobacco etch virus protease to remove both the six-His tag and the GB1 domain. After purification and separation from the cleaved affinity tags, all proteins were in the apo form and, additionally, HO2_{sol} and HO2_{tail} were in the oxidized form with a disulfide bond between Cys265 and Cys282 (here termed HO2_{sol}^O and HO2_{tail}^O).

Oxy-Fe(II)-HO1^{3, 8} and the truncated variant 1–265 HO1^{3, 7, 8} were expressed and purified as described previously.

The soluble form of human cytochrome P450 reductase (CPR) lacking the first 66 amino acids that span the membrane anchor and human biliverdin reductase (BVR) were expressed and purified as described previously.²⁴

Preparation of Fe(III)-Heme Bound HO2

Fe(III)-heme was freshly prepared in 50 mM Tris buffer (pH 8.0) containing 50 mM KCl with 15% dimethyl sulfoxide and 0.1M NaOH. The concentration was determined by using $\epsilon_{385}=58.4 \text{ mM}^{-1} \text{ cm}^{-1}$ in 0.1 M NaOH.²⁵ As described previously,²² excess Fe(III)-heme was added to HO2_{sol}^O or HO2_{core} and passed through PD-10 desalting columns (GE Healthcare) to remove unbound heme to yield the Fe(III)-heme-bound forms (Fe(III)-HO2_{sol}^O and Fe(III)-HO2_{core}, respectively). The Fe(III)-heme-bound forms of the dithiolate forms of HO2_{sol} and HO2_{tail} (Fe(III)-HO2_{sol}^R and Fe(III)-HO2_{tail}^R, respectively) were prepared similarly except under anaerobic conditions with proteins that had been reduced with tris(2-carboxyethyl)phosphine prior to addition of Fe(III)-heme, as described.²²

Samples of Fe(III)-HO2_{sol}^O for cryoreduction were prepared as described previously²¹ but with the addition of approximately 1:1 protein: Fe(III)-heme rather than an excess as described above. Thus, the protein used in all cryoreduction conditions contains heme bound only to the catalytic core.

Oxy-ferrous HO₂_{sol}^O Sample Preparation

All experiments on cryoreduction were conducted using 0.5 mM oxy-Fe(II)-HO₂_{sol}^O in 50% glycerol (v/v) – 50 mM Tris buffer pH 8.0. When needed, the protein was exchanged into buffers made using D₂O and d₃-glycerol. In D₂O samples, the pH was adjusted to pH 7.6.²⁶

The preparation of oxy-ferrous HO₂_{sol}^O complex (oxy-Fe(II)-HO₂_{sol}^O) was conducted as follows. The ferrous HO₂_{sol}^O samples (Fe(II)-HO₂_{sol}^O) were made first by incubating the Fe(III)-HO₂_{sol}^O in aqueous buffer in an anaerobic glovebox overnight at 3 °C to remove oxygen from the protein solution. An aliquot of a standardized solution of sodium dithionite was added to reduce Fe(III)-HO₂_{sol}^O to Fe(II)-HO₂_{sol}^O, using 10% excess of dithionite. An extinction coefficient of 8000 M⁻¹ cm⁻¹ at 315 nm was used to determine the concentration of dithionite. Then, the reduced protein solution was mixed with oxygen-free glycerol. Complete reduction of the protein was confirmed spectrophotometrically. The sample of Fe(II)-HO₂_{sol}^O was transferred into EPR tubes. Oxy-Fe(II)-HO₂_{sol}^O complexes were made by bubbling the Fe(II)-HO₂_{sol}^O sample at –25 °C with 20 ml cold oxygen gas for 40–60s. The samples were then stored in quartz EPR tubes at 77 K until cryoreduction.

γ-Irradiation of the frozen hemoprotein solutions at 77 K typically was performed for 20h (dose rate of 0.1 Mrad/h, total dose 2 Mrad) using a Gammacell 220 ⁶⁰Co. Annealing at temperatures over the range 77–270 K was performed by placing the EPR sample in the appropriate bath (e.g., *n*-pentane or methanol cooled with liquid nitrogen) and then refreezing in liquid nitrogen.

Spectroscopic Techniques

X-band CW EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 910 continuous He flow cryostat. As shown previously, γ-irradiation at 77 K yields an intense EPR signal at g=2.0 from radiolytically generated organic radicals; such signals are truncated in the reported spectra for clarity.

UV-Visible spectra at 77 K were acquired directly on the EPR samples, which were in 4 mm (outer diameter) quartz tubes that were immersed in a liquid nitrogen-filled “finger dewar” placed in the cuvette-holder of a USB 2000 spectrophotometer (Ocean Optics, Inc.).

Steady-state activity assays with Fe(III)-HO₂

With minor modifications of the assay described previously,²⁴ HO₂ was assayed in a 200 μL reaction containing 0.1 μM Fe(III)-HO₂, 15 μM Fe(III)-heme, 0.35 μM BVR, 0.25 μg/μL BSA, 20 U/μL catalase, and 5 μM CPR. After a 2 min incubation at 37 °C, the reaction was initiated with the addition of 2 μL of 100 mM NADPH and monitored spectrophotometrically at 468 nm. The activity was calculated using a difference extinction coefficient of 43 mM⁻¹ cm⁻¹ between Fe(III)-heme and the product, bilirubin.

Results and Discussion

Absorption spectra of Fe(II)-HO2_{sol}^O and its complex with O₂

The absorption spectra of Fe(II)-HO2_{sol}^O and its O₂ complex are presented in Figure 1. The spectrum of Fe(II)-HO2_{sol}^O at room temperature shows poorly resolved α - and β -bands at ~530 and 558 nm and a broad Soret band at 430 nm (not shown) that is similar to that reported previously.^{17, 19} The observed spectrum resembles that of the high-spin, five-coordinate Fe(II)-heme HO1 complex.²⁷ However, as shown previously,¹⁹ in the low temperature spectrum of Fe(II)-HO2_{sol}^O (Figure 1), two Soret bands can be resolved and the α - and β -bands are more distinct.¹⁹ To our knowledge, the observed temperature effect does not occur for Fe(II)-HO1 (Figure S1) nor for any other studied high-spin pentacoordinate ferrous hemoprotein. Rather, the temperature effect observed for Fe(II)-HO2_{sol}^O has previously been suggested to be due to the formation of a low-spin hexacoordinate Fe(II)-HO2_{sol}^O species at low temperatures.¹⁹ However, the identity of the sixth ligand (besides His and the tetrapyrrole nitrogens) in low-spin Fe(II)-HO2_{sol}^O remains uncertain. While one of the Cys residues in the two HRMs was considered to be a candidate based on the speculation (described above) that Cys may act as a ligand to Fe(III)-heme bound to the core,¹⁷ it now appears that this is not so, as the same hexacoordinate Fe(II)-heme species was observed with a variant containing substitutions at the HRM Cys residues.²² An obvious second possibility is a water molecule.²⁸

Addition of O₂ to Fe(II)-HO2_{sol}^O at -25 °C results in the appearance of a new absorption spectrum (Figure 1) with peaks at 536 nm and 572 nm that can be assigned to the dioxygen adduct of the Fe(II)-heme of HO2, termed oxy-Fe(II)-HO2_{sol}^O. The absorption spectrum of the oxy adduct is slightly (2–3 nm) blue shifted relative to that for oxy-Fe(II)-HO1 (Figure S2). The observed species is unstable at room temperature and rapidly ($t_{1/2} \sim 1.5$ min) converts into ferriheme HO2 with a rate constant that is approximately 100-fold faster than that for oxy-Fe(II)-HO1 ($t_{1/2} \sim$ several hours).⁸

Thus, the differences in the UV-visible spectra of the Fe(II)- and oxy-Fe(II)- states of HO1 and HO2 and the marked instability of the oxy-Fe(II) state of HO2 relative to that of oxy-Fe(II)-HO1 indicate that there are functionally significant differences between these states that may reflect the small differences in the crystal structures of the catalytic cores of these proteins, which appear to be restricted to the proximal and distal helices that directly interact with the heme.¹⁰ In addition, HO2 appears to more favor the open conformation than HO1. These differences also may relate to the influence of the 20 additional N-terminal residues of HO2 or the C-terminal HRM domain, neither of which were observed in the HO2 crystal structure and both of which are conformationally mobile regions.²¹ Indeed, NMR studies indicated a redoxdependent interaction between the HO2 catalytic core and its C-terminal HRM region.²¹

EPR spectroscopy of cryoreduced oxy-Fe(II)-HO2

After radiolytic cryoreduction at 77 K, oxy-Fe(II)-HO2 shows a rhombic EPR spectrum with $g = [2.38, 2.19, 1.93]$ (Figure 2), which is very similar to that reported for cryoreduced oxy-Fe(II)-HO1, i.e., $g=[2.37, 2.19, 1.93]$ (Figure S2) and has been assigned to the hydroperoxy

ferric intermediate.^{8, 29} Such a primary product of cryoreduction as that of oxy-Fe(II)-HO2 trapped at 77K retains the conformation of the parent dioxygen complex, which often differs from that of the equilibrium state of the cryoreduced species. The EPR spectra presented in Fig.2 imply that oxy-Fe(II)-HO2 exhibits a single conformer of the parent Fe-O-O moiety. The spectroscopic equivalence of the cryoreduced oxy complexes of HO1 and HO2 suggests that there is significant structural similarity between the distal environments of the hydroperoxy-heme moiety in the two isoforms. As with HO1, the EPR signal of the cryo-generated hydroperoxy intermediate of HO2 is characterized by higher rhombicity than that of other studied hemoproteins, including globins,^{30, 31} cytochromes P450,³²⁻³⁵ peroxidases;^{36, 37} and nitric oxide synthase (NOS).³⁸ The results may be the consequence of unusual geometric/H-bonding constraints in the distal heme environment to promote the formation of a Fe-OOH bond, which is the catalytically active species that hydroxylates the heme in HO1 and HO2.^{4, 39} It was noted that HO1 exhibits an unusually acute Fe-O-OH angle, which provides van der Waals contact between the terminal oxygen of the ferric-hydroperoxide and the α -meso carbon.⁴ The similarity of the EPR spectra of cryoreduced oxy-Fe(II)-HO2 to that of oxy-Fe(II)-HO1 suggests that the ferric-hydroperoxide intermediate in HO2 enforces a similar geometry to promote the heme ring hydroxylation.

Because cryoreduction of the diamagnetic oxy-Fe(II)-HO2 complex at 77 K generates an EPR-active state that retains the conformation of the oxy precursor,^{32, 35, 40, 41} this procedure generates a sensitive EPR probe of the diamagnetic precursor. *In situ* cryoreduction of oxy-Fe(II)-heme first forms the peroxo-ferriheme species, which must then undergo proton transfer at 77 K to form the observed hydroperoxy-ferric state. Previous cryoreduction studies with a variety of oxy-hemoproteins, including cytochrome P450,³² NOS,³⁸ oxy globins,^{30, 31} peroxidases^{36, 37} and HO1⁸ showed that the observation of proton transfer to the basic peroxo heme ligand trapped at 77 K or below implies the existence of a hydrogen-bonded proton delivery network that includes an ordered active-site water molecule that is hydrogen bonded to the terminal oxygen atom. Observation of the hydroperoxy ferriheme as the product of 77 K cryoreduction of oxy-Fe(II)-HO2 implies the presence of such a network, which was shown to promote very efficient proton transfer to the cryogenerated peroxy ligand in HO1 even at helium temperatures.²⁹ The transferred proton can originate either from acid/base groups provided by amino acid residues within the active site,^{8, 32} from bound substrate^{38, 39} or from water clusters connected to the active site.⁴² The X-ray crystal structure of HmuO, which is structurally similar to that of HO1 and HO2, in its oxy complex showed the terminal oxygen atom of the peroxo ligand interacting with a water molecule as part of a hydrogen-bonding network containing a water cluster and a catalytically critical distal Asp residue.⁴³ Site-specific substitution of the analogous distal Asp140 in HO1 to Ala inhibits proton delivery and consequently the conversion of cryogenerated peroxo to the hydroperoxy species.⁸

These cryoreduction and EPR spectroscopic studies of the cryoreduced oxy complex of HO2 show its significant similarity to HO1, and strongly suggest that the distal environments of the hydroperoxy-heme moiety in the two isoforms are highly similar, with a very effective H-bonding pathway for rapid proton delivery to the peroxo-ferriheme species. Furthermore,

the results indicate that, like HO1, the hydroperoxide state exhibits an unusual geometry that is poised for heme ring hydroxylation.

Annealing of cryoreduced oxy-Fe(II)-HO2

EPR spectroscopy shows that during annealing of cryoreduced oxy-Fe(II)-HO2 at progressively higher temperatures (Figure 2) the trapped hydroperoxy-heme state relaxes to other such states. Annealing at temperatures between 145–175K produced a negligible shift in the g_1 feature, from 2.38 to ~2.39. However, upon annealing at 185K for 2 min, the g_1 feature showed asymmetric broadening to lower field. Further, annealing at 200K converts some of the $g_1 = 2.39$ state to a relaxed hydroperoxoferri conformer with $g_1 = 2.36$, but no noticeable changes of g_2 or g_3 . A similar relaxation has been observed with cryoreduced oxy-Fe(II)-HO1.⁸

The relaxation to two spectroscopically similar equilibrium hydroperoxo-forms, $g_1 = 2.36$ and 2.39, which are likely in equilibrium, presumably involves rearrangement of the distal pocket, the putative water network, and possibly the protein side chains. After the presumed rearrangement, the equilibrium hydroperoxo-ferric intermediates of HO2 are prepared for the actual tetrapyrrole hydroxylation step. During annealing at 215K, both hydroperoxy intermediates decay (Figure 2) and their disappearance is accompanied by the formation of a new rhombic high spin signal with $g_x = 6.1$ and $g_y = 5.72$, characteristic of the α -*meso*-hydroxyheme product (Figure 3).^{8, 44}

Decay of the hydroperoxy intermediates of HO2 to form the α -*meso*-hydroxyheme exhibits a notable solvent kinetic isotope effect of ~ 3.1 (210K) (Figure 4), as reported for HO1,⁴⁵ which indicates that for both isoforms, protonation of the hydroperoxy ligand is the rate limiting hydroxylation step at this temperature. Figure 5 shows that at 210K the hydroxylation of heme by HO1 proceeds ~ 3 times faster than that of HO2.

Although Cys265 and Cys282 are present in their reduced states and potentially available to bind heme, we saw no evidence for any thiolate-ligated heme species at any step in the HO2 catalytic cycle. In fact, all of the spectral intermediates described so far exhibit EPR spectral features that are similar to those that have been previously observed for HO1. Even the kinetic isotope effect of 3.1 observed here for decay of the ferric hydroperoxy intermediates of HO2 is similar to that for HO1. These results indicate that the HRMs do not play a direct role in conversion of ferric hydroperoxy intermediate to α -*meso*-hydroxyheme. However, there are differences among the rates of the various conversion steps, which reflect structural differences in the active sites of HO1 and HO2 and/or the influence of the HRMs.

Steady-state activity of Fe(III)-HO2

Previous studies have indicated that the HRMs are not necessary for catalysis at saturating heme concentrations¹⁶ and studies described above suggest that the HRMs do not ligate to heme at any of the steps in the catalytic cycle. We performed steady state assays with Fe(III)-heme-bound proteins in which the HRMs were either not present (HO2_{core}) or reduced (HO2_{tail} and HO2_{sol}^R), since, in the oxidized state, the disulfides are not available to participate in turnover. Fe(III)-HO2_{tail}, which contains two bound hemes and spans only

the C-terminal HRM region but lacks the catalytic core, has no detectable activity. Further, there was no difference in activity amongst Fe(III)-HO2_{core} (443± 13 nmol/min/mg), which contains a single heme bound to the core and lacks the HRM region; Fe(III)-HO2_{sol}^O (388± 14 nmol/min/mg), which also contains a single heme because the HRMs are oxidized; and Fe(III)-HO2_{sol}^R (377± 28 nmol/min/mg), which contains heme bound both to the core and the HRMs.

Therefore, the combined kinetic and spectroscopic results clearly demonstrate that all heme oxidation activity comes from the HO2 catalytic core, consisting of residues 1–248. Given the structural homology between HO1 and HO2, we suggest that the catalytic activity of HO2 derives from highly conserved residues 21–248 of HO2. Furthermore, residues 213–288 from the C-terminal HRM region, containing a highly divergent sequence relative to that of HO1, and is not directly involved in the mechanism of heme turnover. The steady-state kinetic results are consistent with the cryoreduction studies, which showed that the mechanisms of HO1 and HO2 are the same.

Conclusion

This report tests for differences in function of HO1 and HO2 that might arise because HO1 binds a single heme within its catalytic core, while HO2 binds additional Fe(III)-heme at the HRMs at its C-terminal tail.^{21, 22} Cryoreduction and spectroscopic experiments, which allow trapping of early intermediates in the catalytic cycle, reveal that the distal environments of the oxy heme centers in HO1 and HO2 as well as the catalytically active hydroperoxo intermediates of the two proteins are very similar. Thus, it is clear that heme hydroxylation by HO2 operates through the same intermediates as HO1. On the other hand, the oxy-Fe(II)-states of HO2 and HO1 display notable differences in their absorption spectra as well as differences in the rates at which early intermediates interconvert. For example, oxy-Fe(II)-HO2 is significantly less stable than oxy-Fe(II)-HO1, converting into ferri-heme HO2 approximately 100-fold faster than oxy-Fe(II)-HO1. Furthermore, heme hydroxylation by HO1 proceeds ~ 3 times faster than that by HO2. These kinetic differences are suggested to relate to the small differences between HO1 and HO2 in the conformations of the proximal and distal helices that interact with the heme and/or the equilibrium between the open and closed conformations of the catalytic core, with HO2 more favoring the open conformation than HO1.¹⁰ Our results strongly indicate that the HRMs are not involved in thiol ligation to the catalytic heme in any of the observed catalytic intermediates and that the HRMs have no HO activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

EPR	Electron Paramagnetic Resonance
HO	heme oxygenase
m-CPBA	meta-chloroperoxybenzoic acid
HRM	heme regulatory motif
CPR	cytochrome P450 reductase
BVR	biliverdin reductase

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Figure 1.
Absorption spectra of ferrous HO₂ in 50% glycerol/buffer (pH 8.2) measured at room temperature (black) and at 77 K (green) and of its dioxygen adduct (red) measured at 77 K.

Figure 2.
X-band EPR spectra of cryoreduced oxy-Fe(II)-HO_{2sol} annealed at indicated temperatures. Instrument settings: temperature, 28 K; field modulation, 100 kHz; modulation amplitude, 10 G; microwave power, 10 mW; microwave frequency, 9.362 GHz.

Figure 3.

X-band EPR spectra of cryoreduced oxy-Fe(II)-HO_{2sol} annealed at indicated temperatures. Instrument settings: temperature, 9 K; modulation amplitude, 10 G; field modulation, 100 kHz; Microwave power, 2 mW; microwave frequency, 9.362 GHz.

Figure 4. Kinetics of the decay of the hydroperoxy intermediate of $\text{HO}_{2\text{sol}}$ in 50% glycerol/ H_2O buffer, pH 7.4, and in d_3 -glycerol/ D_2O buffer (pH 7.0) at 210 K.

Figure 5.
Kinetics of the decay of the hydroperoxy intermediates of HO1 and of HO2_{sol} in 50% glycerol/ buffer, pH 7.4, at 210 K.