Published in final edited form as:

Biochem Biophys Res Commun. 2007 April 6; 355(2): 581–586.

***Arthromyces ramosus* peroxidase produces two chlorinating species**

Liusheng Huang and Paul R. Ortiz de Montellano*

Department of Pharmaceutical Chemistry, University of California, 600 16th Street, San Francisco, California 94158-2517

Abstract

We previously reported that the hemes of horseradish peroxidase (HRP) and *Arthromyces ramosus* peroxidase (ARP) undergo vinyl and *meso*-carbon modifications when the enzymes oxidize chloride ion. Here we demonstrate for ARP that, although both modifications exhibit the same pH profile with an optimum at ~pH 4.0, monochlorodimedone suppresses the vinyl but not *meso*-carbon modifications. Furthermore, *meso*-chlorination occurs when ARP reacts with exogenous HOCl, implicating an Fe(III)-O-Cl intermediate in the reaction. These results establish that (a) the chloro species involved in *meso*-modification differs from that which reacts with the vinyl groups, (b) equilibration of the vinyl modifying species (HOCl) into the medium occurs more rapidly than vinyl group modification, and (c) the oxidation of chloride by ARP produces two reactive species: HOCl, which adds to the heme vinyl but not *meso*-positions, and a distinct second species that adds to the *meso*-carbon.

Keywords

Arthromyces ramosus peroxidase; Horseradish peroxidase; Heme modification; Chloride oxidation; Hypochloric acid; Chloride radical

INTRODUCTION

Hemoprotein peroxidases catalyze two types of reactions. The most general reaction is one electron abstraction from electron rich substrates to produce substrate-derived radicals. A typical example is the conversion of phenols to phenoxy radicals [1,2]. The second type of reaction is the net two-electron oxidation of heteroatoms to give the corresponding oxides. An example of this is the conversion of Cl⁻ to HOCl by myeloperoxidase [3,4], a reaction that until recently was thought to be too energetically unfavorable to be catalyzed by plant peroxidases. This is consistent with the fact that the oxidation of halides by the mammalian peroxidases is physiologically important for the destruction of pathogenic microorganisms [5], but the oxidation of halides by plant peroxidases has no general physiological role.

The catalytic mechanism of all peroxidases involves reaction of the enzyme with a peroxide to generate an Fe(IV)=O species coupled to either a porphyrin or protein radical that is known as Compound I [5,6]. In the peroxidatic cycle, Compound I is reduced by two one-electron

To whom editorial correspondence should be addressed: Dr. Paul Ortiz de Montellano, University of California, Genentech Hall N572D, 600 16th Street, San Francisco, CA 94158-2517, TEL: (415) 476-2903, FAX: (415) 502-4728, e-mail: ortiz@cgl.ucsf.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

steps, first to Compound II in which the protein or porphyrin radical is quenched, and subsequently to the resting ferric state. In contrast, the oxidation of halide ions is thought to involve addition of the halide (X^-) to the ferryl of Compound I to generate a transient Fe(III)-OX species from which HOX dissociates as a diffusible hypohalide [7]. HOX is an antimicrobial agent involved in host defense [7,8]. It has been postulated that, in addition to the dissociated HOX, the Fe(III)-OX intermediate may also function as an oxidative agent, but the role of this second oxidizing species, if any, remains uncertain [7,9–12].

Recently we reported that under acidic conditions horseradish peroxidase (HRP), *Arthromyces ramosus* peroxidase (ARP), and soybean peroxidase oxidize chloride ion, albeit only at acidic pH and to a lower extent than a mammalian peroxidase like myeloperoxidase [13,14]. Based on the initial rate of chloride oxidation, lactoperoxidase is nearly 20-fold more active than HRP and 2-fold more active than ARP, and myeloperoxidase in turn is much more active than lactoperoxidase [15]. Interestingly, the prosthetic heme group in the plant and fungal, but not mammalian, peroxidases was modified at the vinyl- and *meso*-positions during chloride oxidation [14]. Comparison of HRP and ARP revealed that ARP is much more susceptible to *meso*-chlorination than HRP [13].

We have investigated the *meso*-modification of ARP to clarify the nature of the reaction and to explore the features of ARP that favor it. Our results indicate that (a) HOCl equilibrates into the medium prior to vinyl group modification, (b) a different oxidizing species is responsible for *meso*-chlorination than vinyl modification, and (c) the second oxidizing species, like HOCl, probably derives from a common Fe(III)-O-Cl intermediate.

Material and Methods

Materials

ARP, 30% (w/w) H_2O_2 , monochlorodimedone (MCD), trifluoroacetic acid, NaOCl, and hemin were from Sigma-Aldrich (St. Louis, MO). d_4 -Mesoheme was provided by Grzegorz Wojciechowski [16]. Citrate buffer was prepared by titrating citric acid to pH 4.4 with 1 M KOH. Phosphate buffer (25 mM) was prepared by titrating dibasic potassium phosphate to pH 7.0 with 0.1 M KOH. Water was double glass distilled prior to use.

Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentrations of HRP and ARP were determined using the molar extinction coefficients $\epsilon_{403} = 102,000 \text{ M}^{-1} \text{ cm}^{-1}$ [17] and $\epsilon_{403} = 108,000 \text{ M}^{-1} \text{ cm}^{-1}$ [18], respectively. The H_2O_2 concentration was standardized spectrophotometrically at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$) [19]. Liquid chromatography-mass spectrometry (LC/MS) was performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system (2695 separations module, Waters 2487 Dual λ Absorbance detector) employing an Xterra® MS C₁₈ column (2.1 x 50 mm, 3.5 μ m).

HPLC was performed on a Hewlett-Packard 1090 Series II instrument equipped with a photodiode array detector. Unless otherwise specified, analyses were performed on a Vydac protein C₄ column (4.6 x 150 mm, 5 μ m) fitted with a precolumn filter (MAC-MOD, Chadds Ford, PA). Solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% trifluoroacetic acid. The gradient program consisted of linear segments with 30% B (0–3 min), from 30 to 40% B (3–23 min), 95% B (23.1–26 min), and 30% B (26.1–30 min) at a flow rate of 1 mL/min. The eluent was monitored at 278 and 400 nm.

Reaction of d₄-mesoheme-reconstituted ARP with chloride

ARP (10 mg) dissolved in 1 mL of water was acidified with 10 μ L of 6 N HCl. The solution was extracted with 2-butanone (2 mL $\times$ 2) and the aqueous phase, after passing through a PD-10 column and concentration on an Amicon® ultra centrifugal filter device (MWCO 10K), afforded apoARP (0.23 mM, 1.1% residual heme). An aliquot of the apoARP solution (50 μ L) was added to 300 μ L of phosphate buffer (25 mM, pH 7.0), followed by addition of 1.2 equivalents of d₄-mesoheme (0.4 mM, 3.4 μ L). After incubation for 2 hr at ~25 °C, the solution was changed to citrate buffer (50 mM, pH 4.4) on an Amicon® ultra centrifugal filter device (MWCO 10K). The final volume was 80 μ L.

A 20 μ L aliquot of the reconstituted ARP (final concentration 0.4 μ M) was mixed with KCl (final concentration 0.6 M) in 30 mM citrate buffer and H₂O₂ (final concentration 0.2 mM) was then added to initiate the reaction. After 20 min, the reaction mixture was analyzed by LC/MS.

Effect of MCD on heme modification

Native ARP (60 μ M, 10 μ L) and KCl (4 M, 20 μ L) were added to 20 μ L of citrate buffer (0.5 M, pH 4.0). To this mixture was added MCD (1 mM) 0, 40 μ L, and 60 μ L, respectively, followed by addition of citrate buffer to bring the volume to 110 μ L. Each solution was then mixed with H₂O₂ (2.4 mM, 10 μ L) to initiate the reaction. The final concentrations were: ARP, 5 μ M; Cl⁻, 0.67 M; MCD, 0, 0.33, or 0.50 mM; H₂O₂, 0.20 mM. After 20 min at ~25 °C, the solutions (84 μ L) were injected into the HPLC.

pH dependence of the heme vinyl- and meso-modifications

ARP (60 μ M, 10 μ L) and KCl (4 M, 20 μ L) were added to 60 μ L of citrate/phosphate buffer (0.5 M) at pH 3.0, 3.5, 4.0, 4.4, 5.0, 6.0, and 7.0, respectively. To each of these solutions was added H₂O₂ (2.4 mM, 10 μ L). The total reaction volume was 100 μ L, and the final concentrations were: ARP, 6 μ M; Cl⁻, 0.80 M; H₂O₂, 0.24 mM. After incubation at ~25 °C for 15 min, a 70 μ L aliquot of the solution was directly injected into the HPLC. The modified and unmodified heme peaks were integrated and the peak areas were plotted against the pH values with KaleidaGraph 3.5.

Reaction of ARP with exogenous hypochlorite

To ARP (100 μ M, 10 μ L) in 60 μ L of citrate buffer (0.5 M, pH 4.4) was added NaOCl (3.2 mM, 10 μ L $\times$ 3) at 10 min intervals. An experiment with free hemin instead of ARP was performed as a control: hemin (0.6 mM in tetrahydrofuran, 10 μ L) and NaOCl (16 mM, 10 μ L) were added to 80 μ L citrate buffer (0.5 M, pH 4.4). After 30 min, the solutions were injected into the HPLC for heme product analysis.

Results

Reaction of d₄-mesoheme-reconstituted ARP with chloride

We previously demonstrated that a single chloride was autocatalytically added to a *meso*-carbon of the prosthetic group when mesoheme-reconstituted ARP oxidized chloride ion [13]. This suggested that the chlorination occurred at a *meso*-carbon because in mesoheme the vinyl groups are replaced by ethyls, but it did not unambiguously establish the site of the reaction. We therefore reconstituted ARP with d₄-mesoheme in which a deuterium replaces each of the four *meso*-hydrogen atoms. Reaction of the reconstituted ARP with chloride and H₂O₂ at pH 4.4 gave a modified heme with a molecular ion at *m/z* 657 [624 (d₄-mesoheme) – 2 (deuterium) + 35 (Cl)] (Fig. 1), which corresponds to replacement of one *meso*-deuterium by a chlorine atom. The mass spectrum also exhibits the commonly observed (M + 32) peak

that results from coordination of the solvent methanol to the iron atom. Clearly, the chloride is introduced at one of the four *meso*-carbons.

Chloride oxidation by native ARP in the presence of MCD

MCD, an enolized β -diketo cyclic compound, reacts with HOCl or HOBr to generate a non-enolizable tetrasubstituted carbon. The loss of absorbance associated with conversion of the enolized keto structure to an unconjugated diketone can be monitored spectroscopically. In sufficiently large amounts, MCD can effectively compete for HOCl or HOBr and thereby inhibit or suppress alternative reactions of these agents.

We previously reported that MCD protected the prosthetic heme vinyl groups from modification during the oxidation of bromide by HRP [14]. To determine whether MCD similarly protects the heme of ARP during chloride oxidation, we incubated native ARP with H₂O₂ and chloride ion in the presence of excess MCD. Strikingly, only the vinyl modification was suppressed, leaving the *meso*-chlorination unchanged (Fig. 2). With 2.5 eq of MCD (relative to H₂O₂), the vinyl addition was negligible, whereas *meso*-chlorination proceeded unimpeded. This is clearly shown by the HPLC trace, in which the peak at 10 min due to heme with both vinyl- and *meso*-modifications is suppressed in the presence of MCD, whereas the peak at ~19 min of the singly modified *meso*-chloroheme is unaffected by MCD (Fig. 2, lower panel). As the MCD to H₂O₂ ratio is increased (Fig. 2, upper panel), the peak with the vinyl- and *meso*-modifications progressively decreases to essentially zero, that of *meso*-chloroheme remains unchanged, and that of unreacted heme increases.

Reaction of ARP with exogenous sodium hypochlorite

To determine if exogenously added HOCl modifies the heme of ARP, the native enzyme was incubated with NaOCl at pH 4.4. The *meso*-chlorinated heme was formed in the incubation. The ratio of *meso*- to vinyl-modification of the heme was 10–15:1, in contrast to a ratio of ~1–3:1 in incubations of ARP with H₂O₂ and Cl[−]. Decreased vinyl modification was thus observed with exogenous HOCl. Incubation of hemin with NaOCl in aqueous medium at pH 4.4 yielded traces of modified hemes but no detectable trace of the *meso*-chloro addition.

pH Dependence of the vinyl- and meso-modifications

Incubations of ARP with H₂O₂ and chloride performed at pH = 3.0, 3.5, 4.0, 4.4, 5.0, 6.0, and 7.0 indicate that the pH profiles of the two heme modification reactions are similar (Fig. 3). At pH > 5, both modifications decrease dramatically, such that at neutral pH no modification is detected. At pH < 3.5, the signals from not only the two products but also unmodified heme decrease greatly, presumably due to degradation or precipitation of the hemes. The optimal pH for both modification reactions is approximately pH = 4.0.

Discussion

As previously reported [13,14], the prosthetic hemes of HRP and ARP undergo vinyl and *meso*-modifications when the enzymes oxidize chloride ion. Although the heme modification patterns differ, they are qualitatively similar and involve addition of HOCl to one (or both) of the vinyl groups to give a chlorohydrin and/or replacement of a *meso*-hydrogen by a chloride atom. The differences in the modification patterns presumably reflect differences in the partitioning of Compound I into different reactive species, and/or topological or physical differences in the enzyme active sites.

The key finding here is that, although MCD completely protects the heme of ARP from vinyl-modification during chloride oxidation, it has a negligible protective effect on *meso*-chlorination. As MCD is unlikely to penetrate far into the active site because the distal heme

site in plant and fungal peroxidases is sterically constricted [20–22], HOCl must equilibrate into the medium, where it is trapped by MCD, at a faster rate than it reacts with the heme vinyl groups. Given the demonstration with d₄-mesoheme-reconstituted ARP that *meso*-chlorination occurs, and the fact that *meso*-substitution reactions invariably occur at the δ -*meso*-carbon in plant and fungal peroxidases [23], the δ -*meso*-carbon must be the site of chloride addition in ARP. The preferential reactivity of the δ -*meso*-carbon is consistent with the crystal structures of HRP and ARP, which show that it is the most accessible *meso*-carbon [24–26]. Furthermore, the δ -*meso*-carbon is less sterically protected and is more accessible to exogenous agents than either of the two heme vinyl groups. The species responsible for *meso*-halogenation thus must differ from that responsible for vinyl-modification, as steric effects cannot explain the differential reactivities of the two sites. The product profiles, our earlier studies with ¹⁸O-labeled H₂O₂ and H₂O, and the protection afforded by MCD unambiguously implicate HOCl (or more generally HOX) [14] as the agent that reacts with the vinyl groups. Therefore, by exclusion, HOCl itself cannot be directly responsible for *meso*-chlorination.

What is the second oxidizing agent responsible for *meso*-chlorination? One possibility is that homolytic scission of the O-Cl bond in the Fe(III)-O-Cl intermediate produces the Fe(IV)=O compound II intermediate and a chloride radical (Fig. 4). Addition of the Cl• to the *meso*-carbon of Compound II would produce a transient isoporphyrin intermediate with a tetravalent *meso*-carbon. Electron transfer to the Fe(IV)=O would then reduce the iron to the ferric state and generate a cation that rearomatizes by loss of the *meso*-proton. An alternative possibility is that the Fe(III)-O-Cl intermediate directly delivers the chloro atom to the *meso*-carbon, although models suggest that the *meso*-carbon is too far for this direct transfer unless there is significant ruffling of the porphyrin skeleton and/or tilting of the Fe(III)-O-Cl bond towards the *meso*-carbon.

The Fe(III)-O-Cl intermediate appears, nevertheless, to be the precursor of both the HOCl responsible for vinyl modifications and the agent involved in *meso*-chlorination, as both reactions were observed when NaOCl was added to ARP. The ratio of *meso*- to vinyl-modified hemes differed in the reaction with H₂O₂ and Cl[−] from that with added NaOCl, the ratio being ~1–3:1 in the former and ~10–15:1 in the latter. This difference may stem from the slower generation of HOCl in the catalytic process. Interestingly, *meso*-chlorination was not observed when free heme was incubated with NaOCl, which implies that the reaction requires the structural organization or catalytic groups of the enzyme active site. These results support the conclusion that Fe(III)-O-Cl is the common precursor of both chlorinating species, a proposal consistent with the finding that both heme modification reactions exhibit similar pH profiles (Fig. 3).

The reason for the higher susceptibility of ARP to *meso*-chlorination - i.e., to formation of the second chlorinating species - is unclear. HOCl may dissociate less readily from the Fe(III)-OCl intermediate in ARP than in HRP due to active site differences, thus permitting a relatively slow homolytic scission of the O-Cl bond to compete more effectively with HOCl release. Visual comparison of the HRP [24] and ARP [25,26] crystal structure active sites shows that ARP has a larger and less polar site than HRP, and its two distal cavity waters are farther from the iron [27]. These differences could decrease the rate of dissociation of HOCl from the iron atom. ARP also has a higher reduction potential than HRP: the E₀' values of ARP Compounds I and II are 0.915 and 0.982 V, respectively, while the corresponding values for HRP are 0.880 V, and 0.900 V [28]. One reason for this is the fact that the proximal histidine is deprotonated to a greater extent in HRP than in ARP, and the increased imidazolate character stabilizes the hypervalent Compound I more than a neutral imidazole. The higher potential of the ARP Compound I and II intermediates could also favor homolytic scission of the Fe(III)-O-Cl. Thus, both slower dissociation of HOCl from the heme iron and an enhanced rate of O-Cl homolysis may contribute to higher *meso*-chlorination in ARP.

The arguments above are based on the assumption that a chloride radical is the *meso*-chlorinating agent. Analysis of the reactions of electrophiles versus radicals with the *meso*-carbon of HRP have led to the conclusion that the *meso*-position reacts with radicals but not with electrophilic agents [29], and among radicals only with those having a bond dissociation energy greater than $DH_{298} = 90$ kcal/mol. This includes the chloride radical (103.15 ± 0.03 kcal/mol) but not the bromide radical (87.54 ± 0.05) [30]. Interestingly, no *meso*-bromination is observed in the reactions of HRP and ARP with bromide ion even though the heme of both enzymes is extensively modified on the vinyl groups by HOBr [14]. Despite the strong support of these results for a chloride radical mechanism, we cannot exclude a concerted or quasi-concerted/homolytic mechanism in which the chloride is delivered to the *meso*-carbon directly from the Fe(III)-O-Cl intermediate. A homolytic scission followed by directed transfer to the *meso*-carbon has been postulated in a computational analysis for the *meso*-hydroxylation of the heme catalyzed by heme oxygenases [31].

Acknowledgements

This work was supported by National Institutes of Health Grant GM32488.

References

1. Raven EL. Understanding functional diversity and substrate specificity in haem peroxidases: what can we learn from ascorbate peroxidase? *Nat Prod Rep* 2003;20:367–381. [PubMed: 12964833]
2. Veitch NC. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* 2004;65:249–259. [PubMed: 14751298]
3. Furtmüller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C, Obinger C. Active site structure and catalytic mechanisms of human peroxidases. *Arch Biochem Biophys* 2006;445:199–213. [PubMed: 16288970]
4. Vlasova II, Arnhold J, Osipov AN, Panasencko OM. pH-Dependent regulation of myeloperoxidase activity. *Biochemistry (Moscow)* 2006;71:667–677. [PubMed: 16827659]
5. Hurst JK, Barrette WC Jr. Leukocyte oxygen activation and microbicidal oxidative toxins. *CRC Crit Rev Biochem Mol Biol* 1989;24:271–328. [PubMed: 2548810]
6. Dunford, HB. Heme Peroxidases. Wiley-VCH; NY: 1999. Horseradish peroxidase I: Ligand binding, redox potentials, formation of its compounds, and some of their reactions; p. 58-91.
7. Dunford, HB. Chloroperoxidase and *Pseudomonas* cytochrome c peroxidase, Heme Peroxidases. Wiley-VCH; NY: 1999. p. 323-385.
8. Gaut JP, Yeh GC, Tran HD, Byun J, Henderson JP, Richter GM, Brennan ML, Lusi AJ, Belaouaj A, Hotchkiss RS, Heinecke JW. Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis. *Proc Natl Acad Sci USA* 2001;98:11961–11966. [PubMed: 11593004]
9. Marquez LA, Dunford HB. Chlorination of taurine by myeloperoxidase. Kinetic evidence for an enzyme-bound intermediate. *J Biol Chem* 1994;269:7950–7956. [PubMed: 8132515]
10. Woggon WD, Wagenknecht HA, Claude C. Synthetic active site analogues of hemethiolate proteins. Characterization and identification of intermediates of the catalytic cycles of cytochrome P450cam and chloroperoxidase. *J Inorg Biochem* 2001;83:289–300. [PubMed: 11293549]
11. Manoj KM. Chlorinations catalyzed by chloroperoxidase occur via diffusible intermediate(s) and the reaction components play multiple roles in the overall process. *Biochim Biophys Acta* 2006;1764:1325–1339. [PubMed: 16870515]
12. Lambeir AM, Dunford HB. A steady state kinetic analysis of the reaction of chloroperoxidase with peracetic acid, chloride, and 2-chlorodimedone. *J Biol Chem* 1983;258:13558–13563. [PubMed: 6685730]
13. Huang L, Ortiz de Montellano PR. Heme-protein covalent bonds in peroxidases and resistance to heme modification during halide oxidation. *Arch Biochem Biophys* 2006;446:77–83. [PubMed: 16375846]

14. Huang L, Wojciechowski G, Ortiz de Montellano PR. Prosthetic heme modification during halide ion oxidation. Demonstration of chloride oxidation by horseradish peroxidase. *J Am Chem Soc* 2005;127:5345–5353. [PubMed: 15826172]
15. Furtmüller PG, Jantschko W, Regelsberger G, Jakopitsch C, Arnhold J, Obinger C. Reaction of lactoperoxidase compound I with halides and thiocyanate. *Biochemistry* 2002;41:11895–11900. [PubMed: 12269834]
16. Wojciechowski G, Huang L, Ortiz de Montellano PR. Autocatalytic modification of the prosthetic heme of horseradish but not lactoperoxidase by thiocyanate oxidation products. A role for heme-protein covalent crosslinking. *J Am Chem Soc* 2005;127:15871–15879. [PubMed: 16277530]
17. Ohlsson PI, Paul K. Horseradish peroxidase with 2,4-modified hematin, including vinyl homologues. *Biochim Biophys Acta* 1973;315:293–305.
18. Farhangrazi ZS, Copeland BR, Nakayama T, Amachi T, Yamazaki I, Powers LS. Oxidation-reduction properties of compounds I and II of *Arthromyces ramosus* peroxidase. *Biochemistry* 1994;33:5647–5652. [PubMed: 8180190]
19. Hildebrandt AG, Roots I, Tjoe M, Heinemeyer G. Hydrogen peroxide in hepatic microsomes. *Methods Enzymol* 1978;52:342–350. [PubMed: 672638]
20. Ortiz de Montellano PR. Control of the catalytic activity of prosthetic heme by the structure of hemoproteins. *Accts Chem Res* 1987;20:289–294.
21. Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL. Crystal structure of horseradish peroxidase C at 2.15 Å resolution. *Nature Struct Biol* 4:1032–1038. [PubMed: 9406554]
22. Sharp KH, Mewies M, Moody PCE, Raven EL. Crystal structure of the ascorbate peroxidase-salicylhydroxamic acid complex. *Nature Struct Biol* 2003;1997;10:303–307. [PubMed: 12640445]
23. Ortiz de Montellano PR. Free radical modification of prosthetic heme groups. *Pharmac Ther* 1990;48:95–120.
24. Berglund G, Carlsson G, Smith A, Szoke H, Henrikson A, Hajdu J. The catalytic pathway of horseradish peroxidase at high resolution. *Nature* 2002;417:463–468. [PubMed: 12024218]
25. Fukuyama K, Sato K, Itakura H, Takahashi S, Hosoya T. Binding of iodide to *Arthromyces ramosus* peroxidase investigated with x-ray crystallographic analysis, ^1H and ^{127}I NMR spectroscopy, and steady-state kinetics. *J Biol Chem* 1997;272:5752–5756. [PubMed: 9038188]
26. Fukuyama K, Kunishima N, Amada F, Kubota T, Matsubara H. Crystal structures of cyanide- and triiodide-bound forms of *Arthromyces ramosus* peroxidase at different pH values. Perturbations of active site residues and their implication in enzyme catalysis. *J Biol Chem* 1995;270:21884–21892. [PubMed: 7665612]
27. Battistuzzi G, Bellei M, De Rienzo F, Sola M. Redox properties of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple in *Arthromyces ramosus* class II peroxidase and its cyanide adduct. *J Biol Inorg Chem* 2006;11:586–592. [PubMed: 16791642]
28. Nakayama T, Amachi T. Fungal peroxidase: its structure, function, and application. *J Mol Catalysis B: Enzymatic* 1999;6:185–198.
29. Wojciechowski G, Ortiz de Montellano PR. Radical energies and the regiochemistry of addition to heme groups. Methylperoxy and nitrite radical additions to heme of horseradish peroxidase. *J Am Chem Soc* 2007;129 In press
30. Berkowitz J, Ellison GB, Gutman D. Three methods to measure RH bond energies. *J Phys Chem* 1994;98:2744–2765.
31. Kumar D, de Visser SP, Shaik S. Theory favors a stepwise mechanism of porphyrin degradation by a ferric hydroperoxide model of the active species of heme oxygenase. *J Am Chem Soc* 2005;127:8204–8213. [PubMed: 15926850]

Fig. 1. LC/MS trace of the modified prosthetic group extracted from the reaction of d_4 -mesoheme-reconstituted ARP. The incubation contained ARP ($4 \mu\text{M}$), KCl (0.6 M), and H_2O_2 ($200 \mu\text{M}$). The peak at 17.85 min is δ -*meso*-chloro mesoheme and that at 16.22 min is unmodified mesoheme. The small peak at 15.04 min has not been identified. The Y-axis indicates relative absorbance with respect to the principal peak, which is set to 100.

Fig. 2.

Effect of the MCD-H₂O₂ ratio on heme modification. *Upper panel*: (circles, solid line) *meso*-modified heme; (diamonds, dotted line) *meso*- and vinyl-modified heme; and (squares dashed line) unmodified heme. *Lower panel*: HPLC of the reaction of ARP (5 μ M) with H₂O₂ (200 μ M) and KCl (0.6 M) in the absence (---) or presence (—) of MCD (500 μ M). The trace in the absence of MCD is offset upwards by 20 mAU for clarity. The peak at $t = 10$ min is the product with both vinyl and *meso*-chlorinations and is suppressed greatly by 2.5 eq of MCD. The peak at ~19 min is *meso*-chlorinated heme and that at ~14.5 min is unmodified heme. Only a trace of heme with only a vinyl group modification is observed at ~11 min.

Fig. 3. pH Dependence of the *meso*- and vinyl-modifications of the heme in ARP. The incubations contained ARP (6 μ M), KCl (0.8 M), and H₂O₂ (0.24 mM) in citrate/phosphate buffer at the indicated pH values: (diamonds, solid line) *meso*-modified heme; (circles, dotted line) vinyl-modified heme; (squares, dashed line) unmodified heme.

Fig. 4. Proposed mechanisms for modification of the prosthetic heme in the reaction of ARP with H_2O_2 and Cl^- . The substituent Pr is $-CH_2CH_2CO_2H$.