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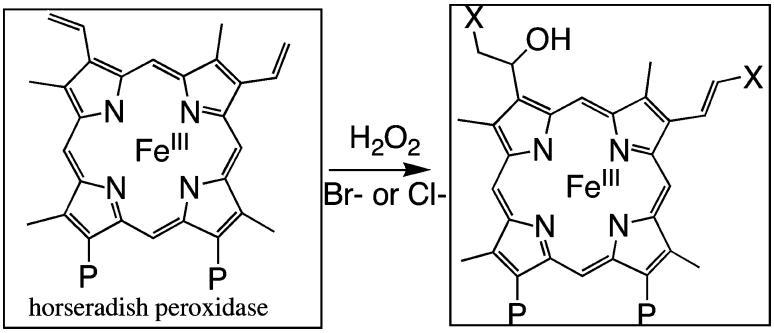

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Prosthetic Heme Modification during Halide Ion Oxidation. Demonstration of Chloride Oxidation by Horseradish Peroxidase

Liusheng Huang, Grzegorz Wojciechowski,† and Paul R. Ortiz de Montellano*

Contribution from the Department of Pharmaceutical Chemistry, University of California, 600 16th Street, San Francisco, California 94143-2280

Received January 15, 2005; E-mail: ortiz@cgl.ucsf.edu

Abstract: Myeloperoxidase (MPO), eosinophil peroxidase (EPO), and chloroperoxidase can oxidize iodide, bromide, and chloride, but most peroxidases, including the prototypical horseradish peroxidase (HRP), reportedly only oxidize iodide and, in some cases, bromide. We report here that incubation of HRP with Br^- and H_2O_2 at acidic pH results in both bromination of monochlorodimedone and modification of the heme group. Mass spectrometry indicates that the heme 2- and 4-vinyl groups are modified by either replacement of a vinyl hydrogen by a bromide or addition of HOBr to give a bromohydrin. These reactions do not occur if protein-free heme and Br^- are co-incubated with H_2O_2 or if the HRP reaction is carried out at pH 7. Surprisingly, similar prosthetic heme modifications occur in incubations of HRP with H_2O_2 and Cl^-. A mechanism is proposed involving oxidation of Br^- or Cl^- to give HOBr or HOCl, respectively, followed by addition to a vinyl group. In the reaction with Cl^-, a *meso*-chloro heme adduct is also formed. This first demonstration of Cl^- oxidation by HRP, and the finding that prosthetic heme modification occurs when Br^- or Cl^- is oxidized in the absence of a cosubstrate, show that only modest tuning is required to achieve the unique chloride oxidation activity of MPO and EPO. The results raise the question of how the prosthetic hemes of MPO and EPO, whose function is to produce oxidized halide species, escape modification.

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO), both of which have a histidine nitrogen as the proximal iron ligand, can oxidize chloride, bromide, and iodide ions.[1−4] The other enzyme that readily oxidizes chloride ion is chloroperoxidase,[5] which differs from conventional peroxidases in that the proximal heme iron ligand is a P450-like cysteine thiolate.[6] In contrast, most other peroxidases, including the prototypical horseradish peroxidase (HRP), are known to oxidize iodide and in some cases bromide, but not chloride.[7,8] Morrison and Schonbaum, in their early review, categorically stated that HRP, like thyroid peroxidase,[9] only oxidizes iodide.[10]

Among the halides other than fluoride, which is apparently not oxidized, chloride is always the least active and iodide the most active substrate. Furtmüller and colleagues compared the reactivity of the halides with MPO Compound I:[3] at pH 5 and 15 °C, the second-order rate constants for the reactions of MPO Compound I with Cl^-, Br^-, and I^- were $(3.9 \pm 0.4) \times 10^6$, $(3.0 \pm 0.2) \times 10^7$, and $(6.3 \pm 0.7) \times 10^7$ M^{-1} s^{-1}, respectively. EPO is a less powerful oxidizing enzyme than MPO and has an even higher selectivity, preferentially oxidizing Br^- in the presence of at least a 1000-fold excess of Cl^-.[4] In the case of lactoperoxidase, at pH 7.0 and 15 °C, the rate of reduction of Compound I by iodide was reported as $(1.2 \pm 0.04) \times 10^8$ M^{-1} s^{-1} and that of reduction by bromide as $(4.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, but no reaction could be detected with chloride.[11] In the case of HRP, it is well established that it can oxidize I^-, and limited evidence exists for oxidation of bromide.[7,8] To the best of our knowledge, no evidence whatever exists for the oxidation of chloride by HRP.[10]

The oxidation of halides by peroxidases is initiated by reaction of the enzyme with H_2O_2 to form Compound I. In HRP Compound I, the ferric iron is oxidized to a ferryl species (Fe^{IV}=O) and the porphyrin to a porphyrin radical cation.[12,13] Compound I subsequently undergoes a two-electron reduction in which the halide adds to the oxygen atom, forming a transient ferric hypohalous complex (Fe^{III}−OX, where X is the halide).

† On temporary leave from the Faculty of Chemistry, A. Mickiewicz University, Grunwaldzka 6, 60-780 Pozna, Poland.

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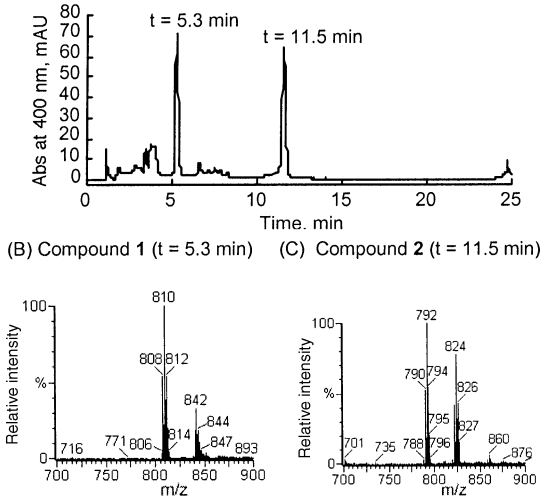


Figure 1. HPLC and mass spectrometric analysis of the reaction mixture of KBr and HRP in the presence of H_2O_2: (A) HPLC trace of the reaction mixture; (B) and (C) mass spectra of the two major products from (A). The molecular ion of the porphyrin is followed by a second molecular ion at m/z +32 due to the complex of the iron porphyrin with methanol.

This intermediate is itself an oxidizing species, but most of the evidence suggests that the complex dissociates into the ferric enzyme and free hypohalous acid (HOX), which in turn is responsible for substrate oxidation.

We report unambiguous evidence that at acidic pH HRP catalyzes the oxidation not only of bromide but, to a lower extent, also of chloride. Interestingly, in these reactions, the prosthetic heme group of the enzyme undergoes autocatalytic modifications that involve reaction of the hypohalous acid with the 2- and 4-vinyl groups of the prosthetic heme group. This vinyl reactions represent a new type of prosthetic heme modification.

Results

Formation of Bromo Heme Adducts. Incubation of HRP with bromide in the presence of H_2O_2 (40 equiv) in citrate buffer (0.5 M, pH 4.4) at ~25 °C for 30 min produced two major HPLC-separable heme products with retention times of 5.3 and 11.5 min (Figure 1). The formation of these adducts was prevented by inclusion of 1 mM monochlorodimedone (MCD) ($\geq[H_2O_2]$) in the incubation (data not shown). The mass spectra of the new heme-derived products exhibit molecular ion patterns at *m*/ *z* (intensity ratio) 808:810:812 (1:2:1) and 790:792:794 (1:2:1), respectively. The isotopic peak ratios indicate that they are both di-bromo heme adducts. Comparison with the mass spectrum of heme, with a molecular ion at 616, indicates that the new compound at $t = 5.3$ min with a mass of 808 contains two new hydroxyl groups in addition to the two bromide atoms [i.e., MW = 616 + 158 (2Br) + 34 (2OH)], and the compound at $t = 11.5$ min with a mass of 790 contains one additional oxygen atom [i.e., MW = 616 + 158 (2Br) + 16 (O)]. The mass spectra also show a second set of molecular ion peaks due to the iron porphyrin complexed with methanol ($M^+ + 32$). The UV−visible spectra of the new compounds in MeOH, with Soret bands at 391 and 395 nm, respectively, are slightly blue shifted relative to the value of 398 nm for the Soret peak of native heme. These are the approximate shifts expected for loss of two or one double bond, respectively. These compounds were identified as **1** and **2**, respectively, based on experiments described below.

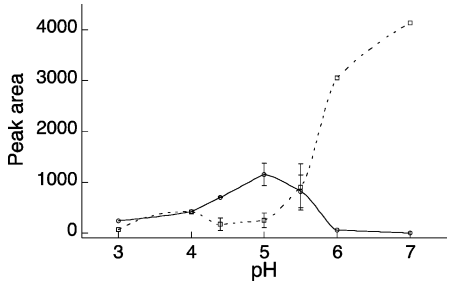


Figure 2. The pH dependence of bromoheme adduct formation. The data with the error bars represent the average of three experiments, with the error bars indicating the standard deviation. The solid line represents the amount of bromoheme adduct (retention time $t = 11.5$ min), and the dashed line represents unreacted heme. The second adduct at $t = 5.3$ min was present in lower amounts in the final HPLC traces, and a good correlation could not be established with either pH or bromide concentration.

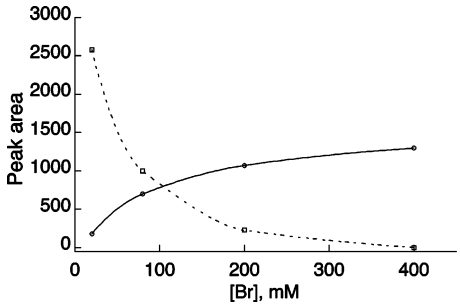


Figure 3. Effect of the bromide concentration on the heme modification reaction. The data represent the average of two experiments. The solid line represents the amount of the bromo-heme adduct (retention time $t = 11.5$ min), and the dashed line represents the remaining unmodified heme. The *Y*-axis represents the integrated peak area from HPLC.

pH Dependence of Heme Adduct Formation. The heme adducts were first identified in an incubation of HRP at pH 4.4. Subsequent experiments over a range of pH values demonstrated that pH 5.0 is optimal for formation of the bromo heme adducts (Figure 2). At pH 4.4−5.5, the adducts were generated in good yield, but at pH 7.0 no adducts were obtained. At very low pH (pH < 4.0), the amount of heme adducts decreased dramatically, possibly because they were sensitive to the highly acidic conditions and were degraded to undetected products.

Dependence of Heme Adduct Formation on the Bromide Ion Concentration. An excess of H_2O_2 is required for formation of the heme adduct. When HRP was incubated with 5 equiv of H_2O_2 in the presence of 0.4 M KBr, the reaction only went to partial completion. When HRP was incubated with more than 10 equiv of H_2O_2 in 0.4 M KBr, essentially no unmodified heme was detected (data not shown). Increasing concentrations of Br^- decreased the amount of recovered unreacted heme and concomitantly increased, albeit to a lesser extent, the amount of the heme adduct (Figure 3). When HRP was incubated with 0.4 M Br^- in the presence of 20 equiv of H_2O_2, the reaction went to completion within a few minutes and no unreacted heme remained. These are therefore the optimal conditions employed hereafter.

Mesoheme-Reconstituted HRP. To determine if the bromo atoms are attached to the vinyl groups, we reconstituted *apo*HRP

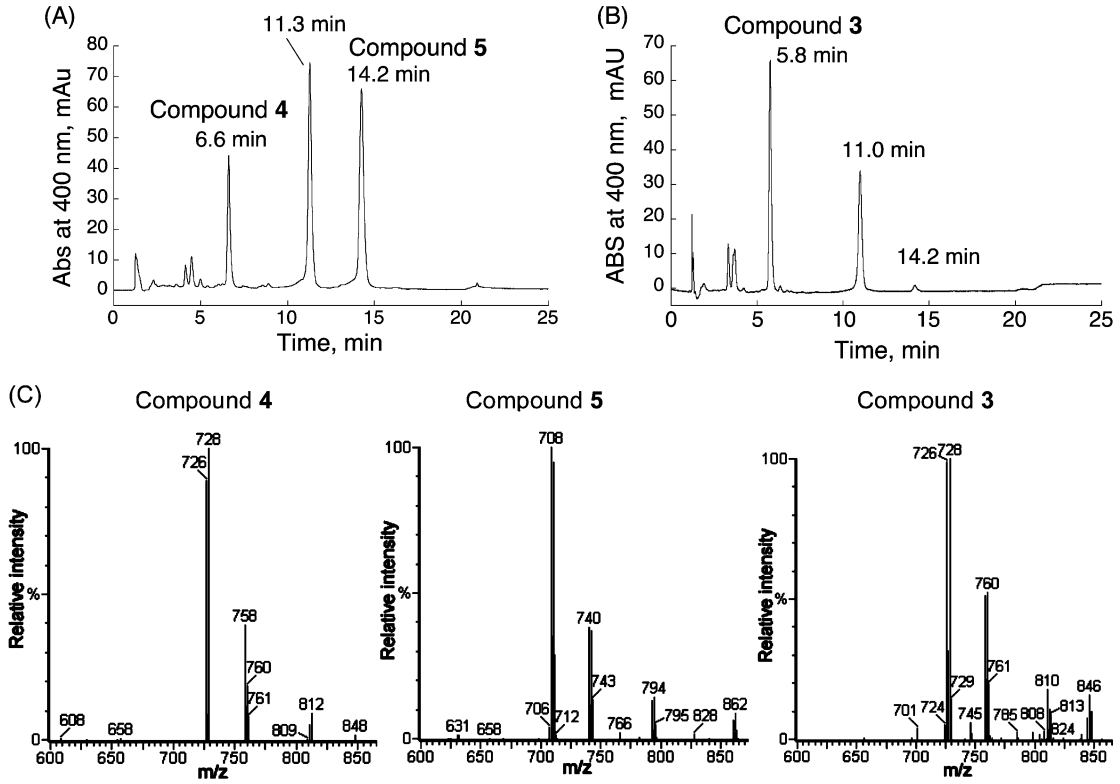
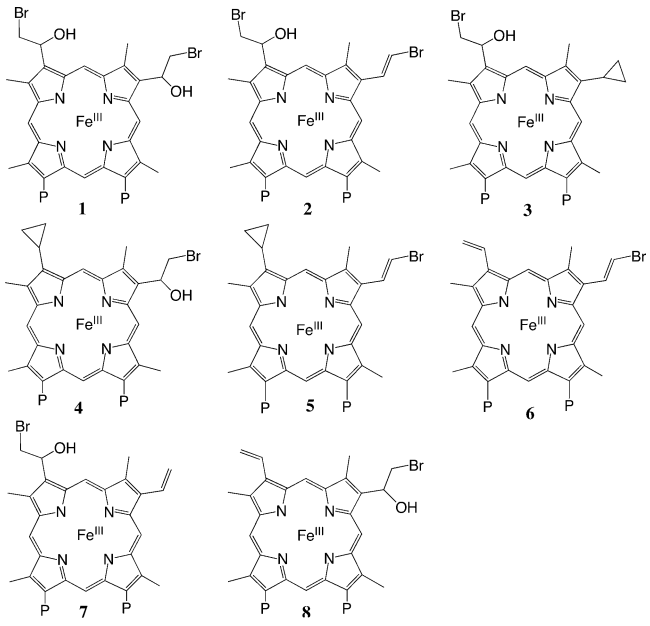


Figure 4. HPLC and mass spectrometric analyses of the reaction mixtures of KBr and 2- or 4-cyclopropylheme-reconstituted HRP in the presence of H_2O_2. (A) HPLC trace of the reaction mixture from 2-cyclopropylheme-reconstituted HRP. (B) HPLC of the reaction mixture from 4-cyclopropylheme-reconstituted HRP. (C) Mass spectra of the two major products in (A) and the single major product in (B). Note again the presence of both the molecular ion and the molecular ion of the methanol complex 32 *m*/*z* units higher.

with mesoheme, the heme analogue in which the vinyl groups are replaced by ethyls. Mesoheme-reconstituted HRP had a catalytic activity similar to that of native HRP when tested with the substrate ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt). However, when it was incubated with Br^- under the conditions that normally lead to adduct formation, no modified heme products were detected by HPLC. This result establishes that the 2-and 4-vinyl groups are the sites modified in the oxidation of bromide by HRP.

Reaction with 2- or 4-Cyclopropylheme-Reconstituted HRP. To determine which of the two vinyl groups was modified and how they were modified, *apo*HRP was reconstituted with either 2-cyclopropylheme or 4-cyclopropylheme. 2-Cyclopropylheme and 4-cyclopropylheme retain only the vinyl group, the other being replaced by a cyclopropyl group. HRP reconstituted with each of these two heme analogues exhibited no active site perturbation, as judged by the spectra of the reconstituted enzymes, and retention of full catalytic activity, as determined by the ABTS assay. The Soret maximum of HRP reconstituted with either of the two mono-cyclopropylhemes was at 400 nm, only slightly blue-shifted relative to that of the native protein at 402 nm (50 mM phosphate buffer, pH 7.0). Incubation of Br^- with 2-cyclopropylheme-reconstituted HRP gave two major modified heme products, whereas incubation with 4-cyclopropylheme-reconstituted HRP gave only one such product (Figure 4). The single product from 4-cyclopropylheme-reconstituted HRP had an HPLC retention time of 5.8 min, and its mass spectrum exhibited a molecular ion at *m*/*z* (intensity ratio) 726:728 (1:1) (MW = 630 + Br + OH), in agreement with addition of a Br and an OH to the 2-vinyl group to give **3**. The first product from 2-cyclopropylheme-reconstituted HRP had an HPLC retention time of 6.6 min and, like the product from 4-cyclopropylheme-reconstituted HRP, a molecular ion at *m*/*z* 726:728 (1:1). The second product had a retention time of 14.2 min and exhibited a molecular ion at *m*/*z* 708:710 (1:1) (MW = 630 + Br − H). These two products thus correspond to the addition of HOBr to the 4-double bond to give bromohydrin **4**, and replacement of a hydrogen of the 4-vinyl group by a bromide to give bromovinyl adduct **5**.



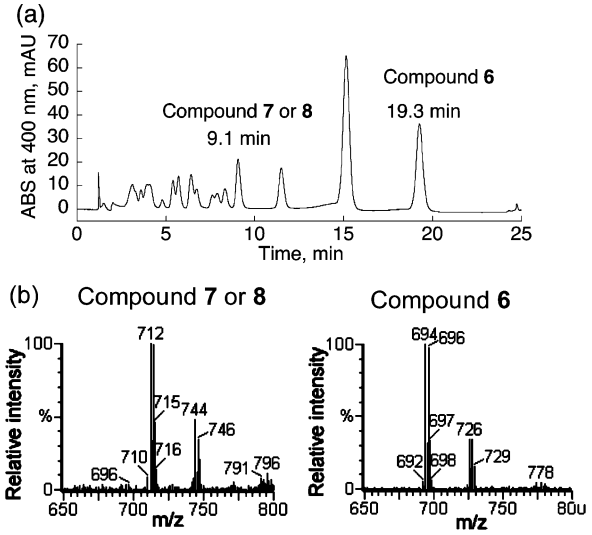


Figure 5. Identification of alternative products from incubation of HRP and Br^- with low concentrations of H_2O_2. Reaction conditions: HRP, 10 μM; KBr, 400 mM; H_2O_2, 50 μM (5 equiv); citrate buffer, 300 mM, pH 4.4; incubated 30 min. (a) HPLC trace of the reaction solution. In addition to the unmodified heme peak at $t = 15.2$ min, two major products are observed at $t = 19.3$ min and $t = 9.1$ min. (b) Mass spectra of the products at $t = 9.1$ min (left) and $t = 19.3$ min (right), clearly showing molecular ions and ions at 32 m/z units higher due to the methanol complexes.

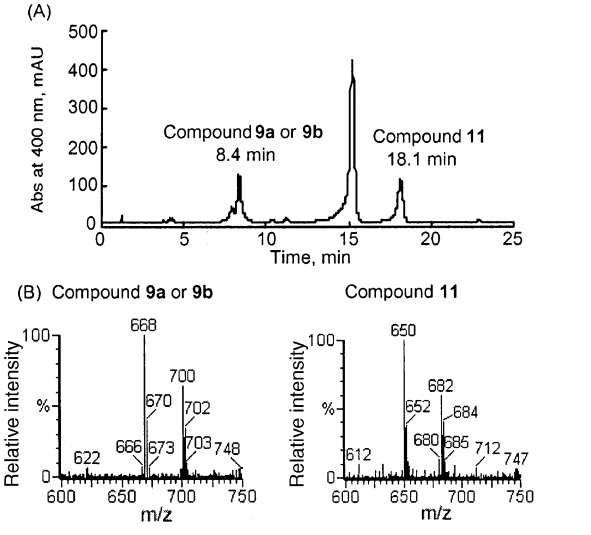
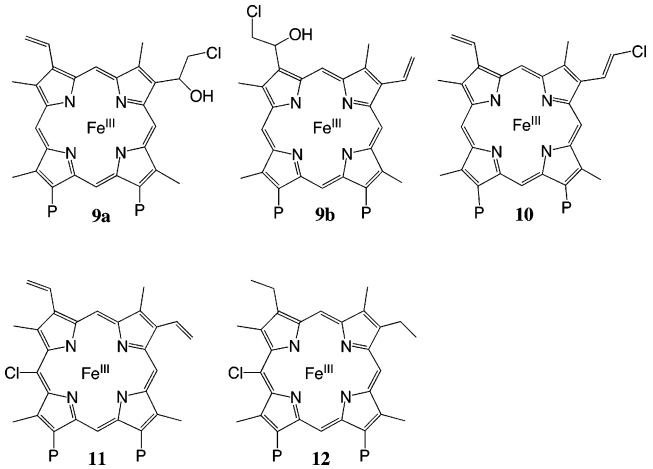


Figure 6. HPLC and mass spectrometric analyses of the reaction mixture from HRP incubated with Cl^- in the presence of H_2O_2. (A) HPLC trace of the reaction mixture. (B) Mass spectra of the products eluted at 8.4 min (left) and 18.1 min (right), indicating addition by [Cl + OH] and substitution by Cl, respectively. Note the presence of a molecular ion as well as an ion 32 m/z units higher due to the methanol complex.

Minor Heme Products Formed in the Oxidation of Bromide by HRP. In addition to the products already discussed, minor heme products were formed when Br^- was oxidized by native HRP. The amounts of these minor products increased as the concentration of H_2O_2 was decreased. This was particularly clear for the product eluting at $t = 19.3$ min (Figure 5a). It was the major product with 5 equiv of H_2O_2, but with greater than 10 equiv of H_2O_2 it disappeared and two other heme-derived compounds became the major products (Figure 1). The mass spectrum of the product obtained at low H_2O_2 concentration ($t = 19.3$ min) had a molecular ion peak at m/z (intensity ratio) 694:696 (1:1) (Figure 5b, right). This molecular mass, the presence of a mono-bromo isotope pattern, and the fact that the compound is a precursor of one of the major products (**1** and **2**) at higher H_2O_2 concentrations identified it as 4-(2′-bromo)vinyl heme **6**. A second minor product ($t = 9.1$ min) was identified as either 2-(1′-hydroxyl, 2′-bromo)ethyl heme **7** or the 4-(1′-hydroxyl, 2′-bromo)ethyl heme **8** by its mass spectrometric molecular ion at m/z 712:714 (1:1) (Figure 5b, left). These two products are intermediary adducts in the reaction of Br^- with HRP in the presence of H_2O_2.

Identification of the Chloro Heme Adduct. Incubation of HRP with Cl^- and H_2O_2 (25 equiv) in citrate buffer (0.5 M, pH 4.4) at ~25 °C for 30 min produced two major products with retention times of 8.4 and 18.1 min (Figure 6). The mass spectra of the two new products exhibited molecular ions at m/z (intensity ratio) 668:670 (3:1) and 650:652 (3:1), respectively. The isotopic peaks indicated they were both monochloroheme adducts. Comparison with the mass spectrum of heme, which has a molecular ion at 616, established that the product at $t = 8.3$ min also contains a hydroxyl group [i.e., MW = 616 + 35 (Cl) + 17 (OH)], whereas the compound at $t = 18.1$ min contains only a Cl atom [MW = 616 + 35 (Cl) − 1 (H)]. The compound at 8.4 min is therefore assigned to a structure in which either the 2- or the 4-vinyl has been converted to the chlorohydrin (**9a or 9b**). Efforts to identify the position modified in 2- or 4-cyclopropylheme-reconstituted HRP during the chloride reaction were unsuccessful. The mono-chlorinated product could involve formation of the 2-chlorovinyl analogue equivalent to that formed with bromide (i.e., **10**) or could bear a chlorine atom at a *meso*-position (i.e., **11**). To resolve this question, we carried out experiments with mesoheme-reconstituted HRP.



Reaction of Chloride with Mesoheme-d_4-Reconstituted HRP. Unlike our findings in the oxidation of Br^-, mesoheme-reconstituted HRP was converted during the oxidation of Cl^- into a monochlorinated mesoheme adduct (m/z 654, data not shown). To determine if the chloride was bound to a *meso*-carbon of the heme, as there were no vinyls in the parent structure, mesoheme-d_4 was synthesized and used to reconstitute *apo*HRP. Mesoheme-d_4 stands for mesoheme in which a deuterium replaces the hydrogen at each of the four *meso*-carbons. HRP reconstituted with the deuterated mesoheme was incubated under the conditions that yield a monochloro adduct, and the heme product was subjected to LC−MS analysis (Figure

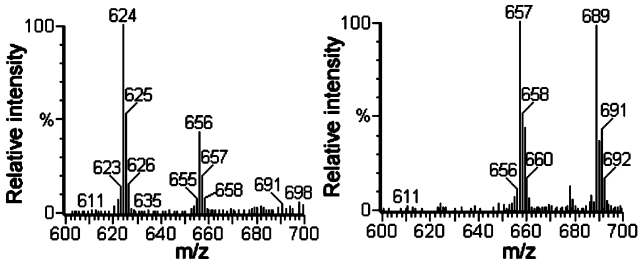


Figure 7. Comparison of mass spectra of mesoheme-d_4 and its monochloro adduct. Left, mesoheme-d_4; right, chloro adduct. Molecular ions are seen for the iron porphyrin and for its methanol complex 32 *m*/*z* units higher.

Table 1. Activity of HRP and Chloroperoxidase in the Oxidation of Br^- and Cl^- As Measured by the MCD Assay

	V_0,[a] Br^- oxidation	V_0, Cl^- oxidation
HRP	72 ± 3	11 ± 1
chloroperoxidase	$(1.3 \pm 0.2) \times 10^4$	$(8.7 \pm 1.4) \times 10^3$

[a] V_0 is the initial rate, expressed in moles of MCD oxidized per minute per mole of enzyme. Data represent the average of three independent experiments ± standard deviation.

7, right panel). Comparison of the molecular ion peaks of the starting mesoheme with those of the product establishes that the chloride is indeed bound to a *meso*-carbon. The starting mesoheme-d_4 exhibits a molecular ion peak at *m*/*z* 624. The chloro-mesoheme adduct exhibits a new molecular ion at *m*/*z* 657 [M − 2 (D) + 35 (Cl)]. It is therefore clear that the chloro substituent has been introduced at one of the four *meso*-positions, as illustrated for *δ*-*meso*-substitution in structure **12**. Notably, the mass spectra of starting mesoheme-d_4 and its chloro adduct both exhibit an (M + 32) peak, which results from coordination of the solvent methanol to the iron atom.

Bromide and Chloride Oxidation Assay. MCD is a broadly used substrate for the determination of the formation of HOCl, HOBr, and other halogenating species. MCD was therefore employed to quantitate the formation of halohydrin species by HRP. The assay was performed in 50 mM citrate buffer at pH 4.4. Mass spectrometric analysis of the product formed in the reaction established that it was the expected 2-bromo-2-chloro-5,5-dimethyl-1,3-cyclohexanedione [*m*/*z* (intensity %) = 253:255:257 (81:100:30) ($M^+ + 1$)] for the Br^- reaction and 2,2-dichloro-5,5-dimethyl-1,3-cyclohexanedione [*m*/*z* (intensity %) = 209:211:213 (100:67:10) ($M^+ + 1$)] for the Cl^- reaction. The initial rate (V_0) was calculated and expressed as moles of MCD oxidized per min per mole of HRP (Table 1). HRP, with initial rates of 72 ± 3 for Br^- and 11 ± 1 for Cl^-, is a much slower enzyme for the oxidation of these halides than chloroperoxidase, for which the initial rates under similar conditions are $(1.3 \pm 0.2) \times 10^4$ for Br^- and $(8.7 \pm 1.4) \times 10^3$ for Cl^-.

Stopped-Flow Analysis of the Reaction of HRP Compound I with Bromide. Under the conditions that normally lead to adduct formation, HRP is converted to Compound I in 80 ms, and Compound I then reacts directly with bromide ion within seconds to regenerate the resting ferric state without the detectable intervention of a Compound II intermediate (data not shown). This result is consistent with a two-electron reaction of the bromide with the ferryl species, as expected if the oxygen is transferred to the halide without an initial electron transfer.

Inactivation of HRP by Bromide. Incubation of HRP with 0.4 M Br^- at pH 5.0 in the presence of H_2O_2 resulted in rapid

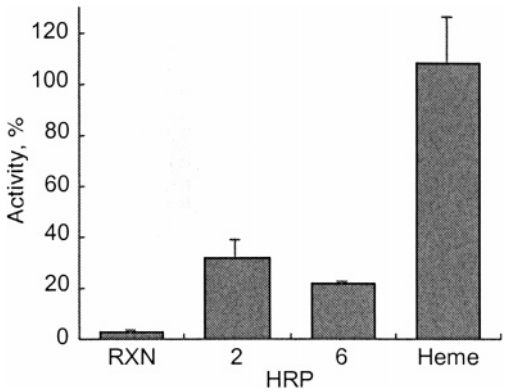


Figure 8. Inactivation of HRP by bromide. Data represent the average of three independent experiments for the activity assay of the mixture after preincubation with Br^- and H_2O_2 for 1 min (RXN), and two independent experiments for the activity assay of HRP reconstituted with compound **2**, **6**, or heme, with triplicate measurements in each case. The percentage activity is relative to native wild-type HRP.

inactivation of HRP (Figure 8). The residual activity after 1 min was only $2.5 \pm 0.5\%$ of the original wild-type activity. Coincidentally, all of the prosthetic heme group was converted to bromoheme adducts, as determined by HPLC after the same 1 min incubation period (data not shown). To check whether the inactivation is due to heme modification and/or protein modification, HRP was reconstituted with 2-(1′-hydroxyl, 2′-bromo)ethyl, 4-(2′-bromo)vinyl heme (compound **2**), and 4-(2′-bromo)vinyl heme (compound **6**). The reconstituted HRP had significantly reduced activity ($32 \pm 6\%$ and $21 \pm 1\%$) when compared to heme-reconstituted HRP, which exhibited nearly the same activity as native HRP. This result indicates that the loss of HRP activity is due in part to modification of the prosthetic heme group, but probably also in part to modification of the protein.

Discussion

Unlike MPO and EPO, which oxidize Cl^-, Br^-, and I^-, only the oxidation of iodide has been extensively studied with HRP.[1,10] It has been reported that the oxidation of ethylenediaminetetraacetic acid (EDTA) by HRP is facilitated by the presence of bromide ions,[7] and the formation of Br_3^- in incubations of Br^- with HRP and H_2O_2 has been detected spectroscopically.[8] Nevertheless, the evidence for bromide oxidation by HRP is relatively thin. The present study demonstrates that bromide is readily oxidized by HRP to a species that brominates monochlorodimedone, a standard test for the presence of HOBr. A direct, two-electron reaction of Compound I of HRP with bromide is supported by the finding that it is reduced on reaction with bromide to the ferric state without the intervention of a detectable Compound II intermediate. Furthermore, in the absence of a reducing substrate, HRP undergoes a pH-dependent reaction with the activated bromide that results in modification of its prosthetic heme group. This heme modification is most efficient at pH 5.0 (Figure 3) and is greatly attenuated below pH 4.0 and above pH 7.0. Kinetic studies have shown that Compound I of HRP is readily formed with H_2O_2 in the pH range from 4.0 to 8.0,[12] so that formation of Compound I is not the pH-dependent step that limits modification of the heme by activated bromide. Observation that the activation of Br^- by HRP is favored at acidic pH mirrors

the finding with MPO and EPO that the rate of oxidation of Br^- and Cl^- is dramatically increased in going from pH 8 to pH 4.[2,3] In the case of HRP, one interpretation of the pH dependence is that a protonated amino acid residue facilitates bromide activation and heme modification. The residue in question is unlikely to be the distal histidine, His42, as its pK_a is close to 2.5.[12] The decline in the amount of adduct obtained at very acidic pH values probably reflects chemical instability of the adducts at very low pH.

The sites of heme modification are unambiguously established for Br^- by the fact that (a) the adducts are not formed when HRP is reconstituted with mesoheme, in which the vinyl groups are replaced by ethyl groups, (b) a single adduct is formed when HRP is reconstituted with 4-cyclopropylheme in which the 2-vinyl group is converted to the 2-(1′-hydroxyl, 2′-bromo) ethyl derivative **3**, and (c) two products are formed when HRP is reconstituted with 2-cyclopropylheme, in which the 4-vinyl group is converted into either the 4-(1′- hydroxyl, 2′- bromo) ethyl derivative **4** or the 4-(2′-bromo) vinyl adduct **5**. The combined results from 2-cyclopropylheme- and 4-cyclopropylheme-reconstituted HRP argue that the two bromo-heme adducts from native HRP are the 2,4-di(1′- hydroxyl, 2′-bromoethyl) derivative **1** and the 2-(1′-hydroxyl, 2′-bromo)ethyl, 4-(2′-bromo)vinyl heme **2**.

The differences in modification of the 2- and 4-vinyl groups are notable. Both vinyl groups undergo addition of HOBr to give the corresponding bromohydrin (i.e., a 1-hydroxy-2-bromoethyl substituent), but only the 4-vinyl appears to also give rise to the 2-bromovinyl group. Addition of HOBr to double bonds is a well-established reaction that involves transfer of a bromonium ion (Br^+) to the double bond followed by addition of water from the medium to the internal carbon, as expected from Markovnikov's rule. This clearly explains formation of the bromohydrins at both vinyl groups. Formation of the 4-(2′-bromo)vinyl group requires that the internal cation formed upon addition of Br^+ be quenched by deprotonation of the terminal carbon, regenerating the double bond, rather than by addition of water. As the 2- and 4-vinyl groups do not differ in their intrinsic reactivity, their different reactivities must reflect differences in their environments within the protein active site. The crystal structure of HRP (PDB 1h5a)[13] shows that two water molecules are present near the heme 2-vinyl group, but no fixed waters are adjacent to the 4-vinyl. Water-333 is 3.158 Å from the terminal carbon and 4.014 Å from the internal carbon of the 2-vinyl group, and water-334 is 4.018 Å from the terminal carbon and 4.075 Å from the internal carbon. The presence of these waters would favor bromohydrin formation at the 2-vinyl, whereas the absence of similar water molecules near the 4-vinyl would permit deprotonation to the vinyl bromide to compete with bromohydrin formation.

As found for MPO and EPO,[2,3] the oxidation of chloride by HRP is less efficient than that of bromide. Indeed, the major surprise is that chloride is oxidized at all, as it is widely accepted that this reaction does not occur (e.g., ref 7). The reaction is sufficiently sluggish that it is not readily detected by the MCD assay, but it is detected, and unambiguously established, by the finding that the heme of HRP undergoes modifications comparable to those observed on oxidation of bromide. As with Br^-, Cl^- oxidation is facilitated by acidic pH. EPO likewise only oxidizes Cl^- to HOCl below pH 6.5.[2] In contrast to the reaction with Br^-, only one Cl^- is incorporated into the prosthetic heme in the reaction mediated by HRP, possibly because HOCl is less reactive than HOBr as an electrophilic reagent.[4] Furthermore, a monochlorinated product was unexpectedly formed on incubation of both native HRP and mesoheme-reconstituted HRP with H_2O_2 and Cl^-, a finding that requires reaction of an activated chloride with a position other than the vinyl groups. Based on the results obtained with *meso*-d_4-mesoheme-reconstituted HRP, the chloro atom is covalently bound to one of the *meso*-carbons. As was found in all previous reactions of HRP involving a *meso*-carbon of the heme, it is probably the *δ-meso*-position (i.e., **12**). Again, by analogy with the known reactions that modify the *δ-meso*-position, it is likely that *meso*-chlorination involves a chloride radical rather than HOCl as the reactive species. Interestingly, no such product was formed in the oxidation of bromide by HRP, either because the bromide radical is not formed or is subject to alternative reactions that outcompete *meso*-bromination of the heme. Thus, the oxidation of chloride by HRP results in modification of the heme by both electrophilic addition and probably radical substitution, whereas only electrophilic addition is observed in the oxidation of bromide.

The striking similarities among the reactions of halides with HRP, EPO, and MPO suggest that they proceed via a common mechanism (Scheme 1). Oxidation of HRP to Compound I is followed by addition of Br^- (or Cl^-) to the ferryl oxygen. Dissociation of the HOBr (or HOCl) regenerates the ferric peroxidase, with the HOBr (or HOCl) either directly reacting with the heme vinyl groups or diffusing from the active site to react with substrates in the medium. As MCD protected the heme group of HRP from modification in the oxidation of Br^-, the HOBr apparently dissociates from the protein and, as its concentration increases, eventually reassociates and reacts with the heme 2- and 4-vinyl groups. The addition proceeds in two steps with initial attack by the electrophilic halogen of HOBr to give the more stable internal carbocation. The carbocation is then trapped by water to give the bromohydrin or is deprotonated to generate the 4-(2′-bromo)vinyl moiety.

This study provides two key findings. The first is Cl^-, in addition to Br^-, can be oxidized by HRP. Although only a fraction of the prosthetic heme was modified with Cl^- under our reaction conditions (~10% for each of the two adducts), this is the first demonstration that Cl^- is oxidized by HRP. The second is that the HOBr or HOCl formed in these oxidation reactions covalently modifies the prosthetic heme group of HRP and inactivates the enzyme. Does this reaction occur with the mammalian peroxidases, whose physiological function is specifically to oxidize halide or pseudohalide ions? According to Thomas and co-workers, in the absence of a substrate (an amine), HOBr generated from Br^- by MPO or EPO was reduced by H_2O_2 and the enzymes were not inactivated.[14] If the heme vinyls in enzymes such as MPO, EPO, and lactoperoxidase are not modified, how is this reaction suppressed? One intriguing possibility in the case of MPO is provided by the finding that the 2-vinyl is already modified to a vinyl sulfonium structure by covalent attachment to a methionine sulfur atom. This strongly electron-drawing substitution would severely attenuate addition of Br^+ or Cl^+ to the double bond. An analysis of the

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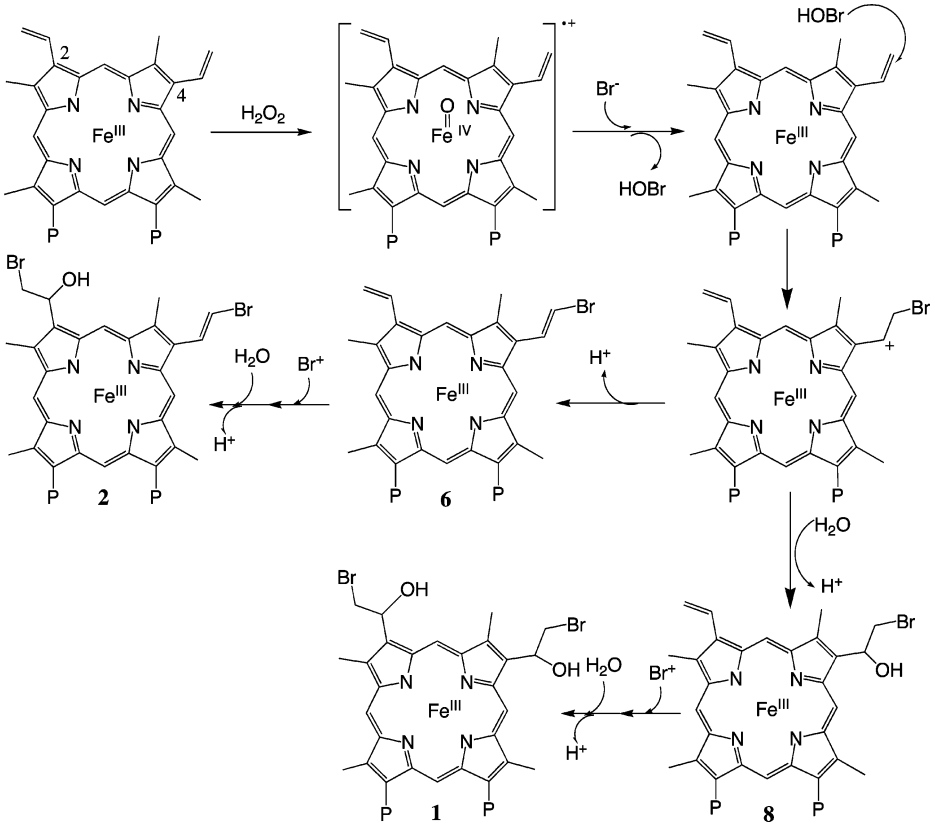
Scheme 1. Proposed Mechanism for the Formation of the Bromoheme and Chloroheme in the Reactions of HRP with Bromide and Chloride in the Presence of H_2O_2[a]



[a] The bromination reaction is used to illustrate the mechanism. The propionic acid substituents ($-CH_2CH_2CO_2H$) on the bottom two pyrrole rings of the heme are indicated by the letter P. The 2- and 4-positions are labeled in the first structure. The reaction could also involve reaction at the 2-vinyl to give **7**, but this is not shown to simplify the scheme.

prosthetic heme groups in mammalian peroxidases is underway to explore this question.

Experimental Section

Materials. HRP (EIA grade) was obtained from Roche. The 30% (w/w) hydrogen peroxide, ABTS, MCD, methanol-*d*, 2-butanone, KBr, KCl, dibasic potassium phosphate, and trifluoroacetic acid were purchased from Sigma-Aldrich. Hydrochloric acid, potassium hydroxide, citric acid, methanol, and acetonitrile were obtained from Fisher. Citrate buffer was prepared from citric acid titrated to pH 4.4 with 1 M KOH. Phosphate buffer (25 mM) was from dibasic potassium phosphate titrated to pH 7.0 with 0.1 M KOH. Water was double distilled prior to use. 2-Cyclopropylheme and 4-cyclopropylheme were synthesized from protoporphyrin IX dimethyl ester by modification of a published procedure.[15]

Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using $\epsilon_{402} = 102\,000\ M^{-1}\ cm^{-1}$ and MW = 44 000.[16] The H_2O_2 concentration was standardized spectrophotometrically at 240 nm by using the molar extinction coefficient $\epsilon = 43.6\ M^{-1}\ cm^{-1}$.[17] HPLC was performed on a Hewlett-Packard 1090 Series II instrument equipped with a photodiode array detector. 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 C_{18} column (2.1 × 50 mm, 3.5 μm).

HPLC Analysis. Unless otherwise mentioned, HPLC analysis was performed with an Alltech Alltima C_{18} column (4.6 × 150 mm, 5 μm) fitted with a guard column. 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 40% B (0−2 min), from 40% to 50% B (2−22 min), from 50% to 95% B (22−23 min), 95% B (23−26 min), from 95% to 40% B (26−27 min), and 40% B (27−30 min) at a flow rate of 1 mL/min. The eluent was monitored at 278 and 400 nm.

Formation of the Heme Adduct: HPLC and Mass Spectrometry. HRP (30 μM) was incubated with KBr (0.6 M) and H_2O_2 (1.2 mM, 40 equiv) in 100 μL of citrate buffer (0.5 M, pH 4.4) at ∼25 °C for 30 min. A 70 μL aliquot of the solution was directly injected onto HPLC. Fractions containing the peaks at $t = 5.3$ and 11.5 min were individually collected, concentrated, and analyzed by mass spectrometry. The settings of the mass spectrometer were as follows: capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 300 °C; source temperature, 120 °C.

pH Dependence of Heme Adduct Formation. HRP (0.1 mM in water, 10 μL), KBr (2 M in 2 mM H_2O_2 aqueous solution, 10 μL), and citrate/phosphate buffer (0.5 M, 80 μL) at different pH values (pH 3.0, 4.0, 4.4, 5.0, 5.5, 6.0, and 7.0) were mixed. The resulting mixtures were then incubated at ∼25 °C for 30 min before a 70 μL aliquot of each was analyzed by HPLC. The peaks of the heme adduct (retention time: 11.5 min) and unmodified heme (retention time: 15 min) were individually integrated, and the data acquired were analyzed with kaleidaGraph 3.5.

Dependence of Heme Adduct Formation on Bromide Concentration. To 10 μL aliquots of 100 μM aqueous HRP was added 80 μL of citrate buffer (0.5 M, pH 5.0), and then added 10 μL of 2 mM H_2O_2

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(20 equiv) in 0.2, 0.8, 2, or 4 M KBr, respectively. The solutions were mixed well. After 30 min, the reaction mixtures were analyzed by HPLC as described above. The peaks of the heme adduct (retention time: 11.5 min) and unmodified heme (retention time: 15 min) were integrated. The data were analyzed with kaleidaGraph 3.5.

Removal of Heme from HRP. *apo*HRP was prepared according to the method described previously.[18] HRP (10 mg) was dissolved in 1 mL of 25 mM phosphate buffer (pH 7.0), and into this was added 100 μL of 3 M HCl to give a solution with pH = 1.3. The HRP solution was extracted with 2-butanone (2 mL × 2) to remove the heme, and then immediately neutralized with 1 M NaOH. The apoprotein in the aqueous phase was passed through a PD-10 column eluted with 25 mM phosphate buffer (pH = 7.0). Fractions containing *apo*HRP were combined and condensed with an ultracentrifugal filter device (10 000 MWCO, Millipore Amicon), affording ~0.4 mL of *apo*HRP with AU_{278} = 0.55 after 8-fold dilution, Rz = 0.03 (Rz = ReinheitZahl). The residual heme in the apoHRP was less than 1%.

Reaction of Bromide with Mesoheme-Reconstituted HRP. The dimethyl ester of commercial mesoporphyrin IX was hydrolyzed with 2 M aqueous KOH−tetrahydrofuran (1:1 v/v) overnight, and then Fe^{3+} was inserted as described previously.[18] The resulting mesoheme was dissolved in 0.2 mL of MeOH, AU_{390} = 1.05 after 500-fold dilution. A 7 μL (~2 equiv) aliquot of mesoheme in MeOH was added to 800 μL of *apo*HRP (AU_{278} = 0.55) in phosphate buffer (pH 7.0), and the mixture was incubated at ~25 °C for 2 h. The solution was passed through a DEAE (DE52) column. Fractions containing HRP were combined and concentrated, affording mesoheme-reconstituted HRP with Rz = 2.6 and AU_{394} = 0.77 (after 10-fold dilution). The activity of the reconstituted HRP was tested with the ABTS assay as follows.[19] Into a disposable cuvette were added 50 μL of 2.5 mM aqueous ABTS and 2 μL of HRP (20 μg/mL) in 25 mM phosphate buffer (pH 7.0) before mixing with 950 μL of 50 mM citrate buffer at pH 4.4. The absorbance at 414 nm was immediately recorded as a function of time. The initial rate was calculated from the change in absorbance between 2 and 22 s.[17] To a 10 μL aliquot of the reconstituted HRP solution was added 30 μL of KBr (2 M) and 60 μL of H_2O_2 (2 mM) in citrate buffer (0.5 M, pH 4.4), incubated at ~25 °C for 20 min and then analyzed by HPLC.

Reaction with 2- or 4-Cyclopropylheme-Reconstituted HRP. *apo*HRP (50 μL, AU_{278} = 0.4 after 10-fold dilution) was added to 450 μL of phosphate buffer (25 mM, pH 7.0). To this was added 2-cyclopropylheme in DMSO (15.8 μL, 2 equiv, AU_{396} = 0.811 after 100-fold dilution). After standing for 2 h, the solution was passed through a DEAE (DE52) column and was concentrated with a centrifugal filter device (Amicon, 10K MWCO): AU_{400} = 1.07 after 2-fold dilution, Rz = 1.6. *apo*HRP (100 μL, AU_{278} = 0.4 after 10-fold dilution) was added to 900 μL of phosphate buffer. To this was added 4-cyclopropylheme in DMSO (4 μL, 2 equiv, AU_{394} = 0.729 after 1000-fold dilution). The same procedure was then followed as above. The final solution had AU_{398} = 0.38 after 10-fold dilution, Rz = 2.5. The reconstituted HRP proteins were tested for activity as described above before being treated with Br^- under the same conditions as were used for native HRP except for the HPLC solvent gradient. With these proteins, the following gradient was employed: 46% B, 0−2 min; 46−54% B, 2−18 min; 54−95% B, 18−19 min; 95% B, 19−25 min. LC−MS was performed by the procedure described above.

Minor Heme Products Formed in the Oxidation of Bromide by HRP. To 60 μL of 0.5 M citrate buffer at pH 4.4 were added sequentially KBr (2 M, 20 μL), HRP (0.1 mM, 10 μL), and H_2O_2 (0.5 mM, 10 μL, 5 equiv). After 30 min, the reaction was analyzed by HPLC. Fractions containing the peaks at t = 9.1 min and t = 19.3 min were individually collected and analyzed by LC−MS. The HPLC and LC−MS were performed as described above.

HPLC and Mass Spectrometric Identification of the Chloro Heme Adduct. To 40 μL of HRP (100 μM) were subsequently added KCl (2 M, 20 μL) and H_2O_2 (2.5 mM, 40 μL, 25 equiv) in citrate buffer (0.5 M, pH 4.4). The mixture was incubated at ~25 °C for 30 min. The solution was analyzed by HPLC. Fractions containing the peaks at t = 8.3 and 18.1 min were individually collected, concentrated, and analyzed by mass spectrometry. The settings of the mass spectrometer were the same as described above.

Preparation of Mesoheme-d_4. Deuterium substitution at the four methine carbons was accomplished as reported.[20] A mixture of magnesium (160 mg), iodine (300 mg), and anhydrous diethyl ether (5 mL) was refluxed under N_2 until colorless before being filtered. The filtrate was evaporated to dryness under vacuum, and the residue was dissolved in anhydrous pyridine (5 mL) and methanol-*d* (1 mL). The resulting solution was added to a solution of mesoporphyrin IX dimethyl ester (15 mg) in 1 mL of dry pyridine, and the combined solution was heated overnight at reflux under an atmosphere of N_2. The cooled reaction mixture was diluted with $CHCl_3$ (20 mL), extracted sequentially with 3% citric acid (20 mL) and water (20 mL × 2), and dried over Na_2SO_4. The solvent was then removed under vacuum. The residue was submitted to a second round of deuteration by repeating the above process. The final residue was allowed to stir overnight in 5% (v/v) H_2SO_4/MeOH and was then purified by preparative TLC with toluene−THF (95:5). The residue was hydrolyzed with 2 M KOH−THF (1:1), and Fe^{3+} was inserted into the resulting porphyrin as previously reported.[18] LC−MS showed the presence of a single mesoheme peak with *m*/*z* (intensity %) 624 (100).

Bromide and Chloride Oxidation Assay. MCD was used as the substrate to test for Br^- and Cl^- oxidation by HRP.[21] MCD (1 mM, 50 μL), KBr or KCl (2 M, 50 μL), and HRP (5 μM for Br^- and 20 μM for Cl^-, 50 μL) were mixed well in a 1-mL quartz cuvette. To this was added 850 μL of H_2O_2 (0.54 mM) in 50 mM citrate buffer at pH 4.4. The resulting solution was mixed, and the absorbance at 292 nm was immediately recorded as a function of time: scan cycle, 0.5 s; scan time, 0−60 s. The initial velocity was calculated from the change in absorbance between 10 and 30 s and was converted to moles of MCD oxidized per min per mole of HRP, using ϵ_{292} = 20 000 M^{-1} cm^{-1}.

Stopped-Flow Analysis of the Reaction of Compound I with Bromide. Stopped-flow measurements were performed in single-mixing mode on an SF-61 DX2 Double Mixing Hi-Tech stopped-flow spectrophotometer. Channel C was filled with a solution containing 4 μM HRP and 1 M Br^-, and channel D was filled with 16 μM H_2O_2 in citrate buffer (50 mM, pH 4.4). The final concentrations after mixing were 0.5 M Br^-, 2 μM HRP, and 8 μM H_2O_2 (4 equiv).

Catalytic Activity of HRP with Modified Hemes. 2-(1′-Hydroxyl, 2′-bromo)ethyl, 4-(2′-bromo)vinyl heme, isolated from 2 mg of HRP after reaction with Br^- and H_2O_2, was purified by HPLC as already described: MS, *m*/*z* (intensity ratio) 790:792:794 (1:2:1) (M^+). The modified heme was dissolved in 25 μL of methanol, AU_{396} = 0.656 (after 50-fold dilution). This solution (16 μL) was added to *apo*HRP (20 μL, AU_{278} = 0.80 after 8-fold dilution) in 380 μL of phosphate buffer (25 mM, pH 7.0). After standing for 2 h, the solution was passed through a DEAE (DE52) column and was concentrated with a centrifugal filter device (Amicon, 10K MWCO). Two independent reconstitution experiments were performed. The resulting HRP solutions had Rz values of 2.1 and 2.7, respectively. Heme-reconstituted HRP solutions (Rz = 2.4 and 2.0) were also prepared as controls. The activity of the reconstituted HRP was determined with the ABTS assay as already described and converted to relative activity as compared to native HRP.

Inactivation of HRP by Bromide. To a mixture of HRP (1.9 mg/mL, 40 μL) and KBr (2 M, 20 μL) was added 40 μL of H_2O_2 (0.86

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mM) in 0.5 M citrate buffer at pH 5.0. After 1 min, a 2 μL aliquot of the reaction mixture was taken and diluted to 76 μL (20 μg/mL HRP), immediately followed by determination of activity with the ABTS assay as already described. In the meantime, an 80 μL aliquot of the remaining solution was analyzed by HPLC.

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