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Radical Energies and the Regiochemistry of Addition to Heme Groups. Methylperoxy and Nitrite Radical Additions to the Heme of Horseradish Peroxidase

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Abstract: The heme of hemoproteins, as exemplified by horseradish peroxidase (HRP), can undergo additions at the *meso* carbons and/or vinyl groups of the electrophilic or radical species generated in the catalytic oxidation of halides, pseudohalides, carboxylic acids, aryl and alkyl hydrazines, and other substrates. The determinants of the regiospecificity of these reactions, however, are unclear. We report here modification of the heme of HRP by autocatalytically generated, low-energy $NO_2^{\bullet}$ and $CH_3OO^{\bullet}$ radicals. The $NO_2^{\bullet}$ radical adds regioselectively to the 4- over the 2-vinyl group but does not add to the *meso* positions. Reaction of HRP with *tert*-BuOOH does not lead to heme modification; however, reaction with the F152M mutant, in which the heme vinyls are more sterically accessible, results in conversion of the heme 2-vinyl into a 1-hydroxy-2-(methylperoxy)ethyl group [$-CH(OH)CH_2OOCH_3$]. [^{18}O]-labeling studies indicate that the hydroxyl group in this adduct derives from water and the methylperoxide oxygens from O_2. Under anaerobic conditions, methyl radicals formed by fragmentation of the autocatalytically generated *tert*-$BuO^{\bullet}$ radical add to both the δ-*meso* carbon and the 2-vinyl group. The regiochemistry of these and the other known additions to the heme indicate that only high-energy radicals (e.g., $CH_3^{\bullet}$) add to the *meso* carbon. Less energetic radicals, including $NO_2^{\bullet}$ and $CH_3OO^{\bullet}$, add to heme vinyl groups if they are small enough but do not add to the *meso* carbons. Electrophilic species such as HOBr, HOCl, and HOSCN add to vinyl groups but do not react with the *meso* carbons. This *meso*- versus vinyl-reactivity paradigm, which appears to be general for autocatalytic additions to heme prosthetic groups, suggests that *meso* hydroxylation of the heme by heme oxygenase occurs by a controlled radical reaction rather than by electrophilic addition.

Introduction

Hemoproteins, including the cytochromes P450, peroxidases, and globins, oxidize diverse substrates to chemically reactive intermediates. The reactive species include radicals, for example alkyl, aryl, halide, and azidyl radicals, and electrophilic agents such as HOCl and HOBr. The prosthetic heme group of hemoproteins can undergo addition reactions with these agents at the 2- and 4-vinyl groups,[1,2] the four *meso* carbons,[3−7] the four porphyrin nitrogen atoms,[8] or the iron atom.[9,10] Additions to the iron and the porphyrin nitrogens are related in that addition of an aryl radical to the iron yields an aryl−iron complex that, under suitable oxidative conditions, shifts to give an *N*-aryl heme adduct.[8,10] However, the parameters that control addition to the heme vinyls versus the *meso* carbons remain uncertain. The two hemoprotein systems that have been primarily employed to investigate these reactions are horseradish peroxidase (HRP) and the globins because these proteins are readily available, reasonably stable, and easily investigated. HRP undergoes additions of alkyl,[4] aryl,[3] azidyl,[5] acetyl,[7] cyanyl,[11] and chloride[1] radicals at the δ-*meso* carbon of the heme. The regiospecificity for the δ-*meso* rather than the α-, β-, or γ-*meso* carbons is the result of steric control by the protein active-site structure. Additions to the vinyl groups of HRP have been observed for HOBr, HOCl, and HOSCN.[1,2] In the case of myoglobin, addition of alkyl[5] radicals to the γ-*meso* carbon, aryl radicals to the iron and porphyrin nitrogens,[9] and haloalkyl and NO_2[12] radicals to the vinyls[13] has been reported.

Steric effects limit and impose regioselectivity on these hemoprotein reactions. However, this steric control does not alone explain the observed reaction regiochemistries. To determine if an intrinsic differential reactivity of the vinyl groups

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and *meso* carbons is superimposed on the steric control of specificity, we have sought additional information on low-energy free radicals, as most of the available data involves high-energy radicals. Here we examine the reactivity of the nitrite ($NO_2^{\bullet}$) and alkylperoxy ($ROO^{\bullet}$) radicals, both of which are of relatively low energy. Because the reactivity of the heme vinyl groups in HRP is limited by the steric protection afforded by the active-site structure, in some experiments we have utilized the F152M HRP mutant in which the bulky phenylalanine side chain is replaced by the smaller methionine side chain. This mutation makes the vinyls more sterically accessible. Our findings, combined with an analysis of the previously reported reactions of the heme with free radicals and electrophilic agents, provide general insights into the intrinsic and protein-dependent factors that control the regiospecificity of additions to the heme group in hemoproteins. The results have implications, for example, for the catalytic mechanism of heme oxygenase.

Results

Nitrite NO_2^- is a powerful oxidant formed by variety of cell types from the NO that is produced by nitric oxide synthases.[14,15] The synthesis of NO and NO_2^- is induced during inflammatory processes[15−17] because nitric oxide is believed to be a part of the self-defense system against invading microorganisms.[18,19] NO_2^- does not accumulate in organisms because it is rapidly oxidized to NO_3^- by oxyhemoglobin[20] and oxymyoglobin.[21] Myeloperoxidase also oxidizes NO_2^- to $NO_2^{\bullet}$ radicals and thereby nitrates tyrosine residues.[22−26] In lactoperoxidase (LPO) an iron-bound peroxynitrite is proposed to be the nitrating agent instead of $NO_2^{\bullet}$ [27−29] because nitrite is oxidized to nitrate NO_3^- in an extremely fast reaction ($\sim 2 \times 10^7\ M^{-1}\ s^{-1}$) by compound I of LPO.[30] Eosinophil recruitment and enhanced production of NO are characteristic features of asthma,[31] and eosinophil peroxidase is at least 4-fold more effective than myeloperoxidase at nitration of tyrosine-containing peptides, a reaction that involves the $NO_2^{\bullet}$ radical.[32,33] Horseradish peroxidase (HRP) also produces $NO_2^{\bullet}$ and nitrates tyrosines,[24] but at high nitrite concentrations peroxynitrite may also be a nitrating agent.[27−29,34] The 2-vinyl of the heme of equine metmyoglobin has been shown to add an NO_2 moiety upon incubation with nitrite,[11] but no information is available on possible nitrite-dependent modifications of the heme in plant or fungal peroxidases.

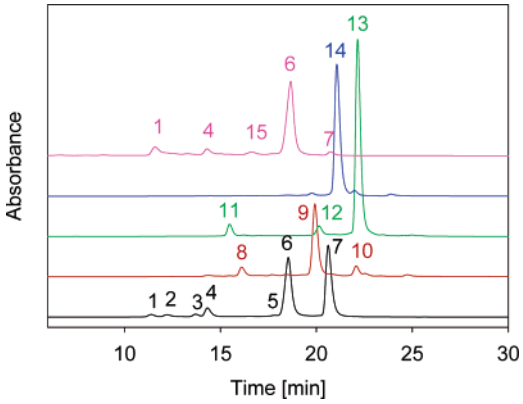

Figure 1. LC/MS chromatograms monitored at 400 nm. HRP with 10 mM NO_2^- and 2 mM H_2O_2: black; 2-cyclopropylheme HRP with 10 mM NO_2^- and 2 mM H_2O_2: red; 4-cyclopropylheme HRP with 10 mM NO_2^- and 2 mM H_2O_2: green; mesoheme-d_4 HRP with 10 mM NO_2^- and 2 mM H_2O_2: blue; HRP with 0.8 M NO_2^- without H_2O_2: pink.

LC/MS analysis indicates that six modified hemes are formed on incubation of HRP with H_2O_2 and nitrite at pH = 7 (Figure 1). This heme modification reaction is strongly pH- (Figure 2A) and time-dependent (Figure 2B). Furthermore, heme modification by $NO_2^{\bullet}$ appears to occur by different mechanisms at low and high nitrite concentrations.

Interestingly, the maximum modification of the heme of HRP caused by low (10 mM) nitrite concentrations and H_2O_2 occurs at pH = 7 rather than at the pH of 4.5−5 that is optimal for many heme modification reactions. Thus, no heme modification is detected below pH 4.5 with 10 mM $NaNO_2$ and 2 mM H_2O_2. However, at high (∼0.8 M) nitrite concentrations at which H_2O_2 addition is not required for formation of modified heme **6**, heme modification occurs best at pH = 5 (Figures 1, 3, and 4).

The time course (Figure 2B) shows that 1 h of incubation at pH = 5 is required for nearly full heme modification with 0.8 M nitrite and no added H_2O_2, whereas a longer time is required at pH = 7 with 10 mM nitrite and 2 mM H_2O_2. After 30 min a plateau is reached and no further increase of modified heme products is observed, probably because the enzyme is inactivated by reaction with $NO_2^{\bullet}$. At high nitrite concentrations and pH = ∼7, heme modification is more effective, with ∼95% heme modification compared to the system with 10 mM $NaNO_2$ and H_2O_2, which only gives ∼50−60% heme modification. At high nitrite concentrations no heme modification occurs above pH = 7. This difference shows that at pH = 5, in the absence of added H_2O_2, the heme is probably modified by NO_x products of HNO_2 decomposition, whereas when H_2O_2 is present, $NO_2^{\bullet}$ is generated. This radical can modify the heme but also deactivates the enzyme due to both heme modification and tyrosine/tryptophan nitration, explaining the lower yield of this reaction. At pH = 5 heme modification only occurs at high nitrite concentrations, whereas at pH = 7 the addition of H_2O_2

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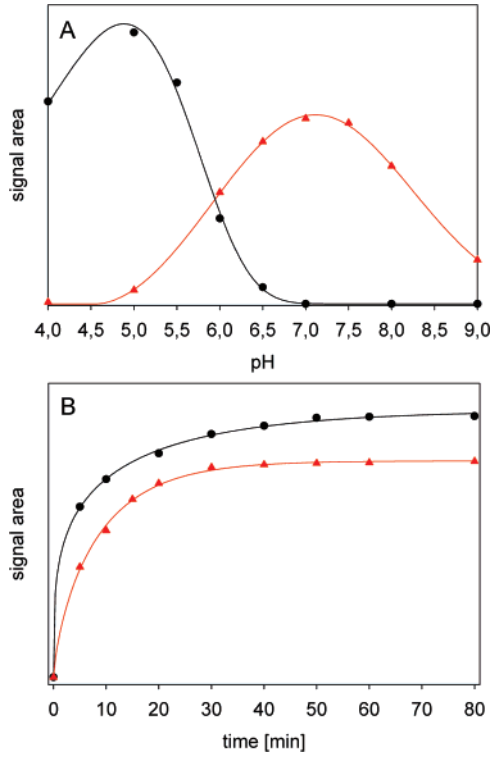

Figure 2. (A) pH profile of the yield of modified heme after a 1 h incubation: (●) HRP with 0.8 M $NaNO_2$ without H_2O_2; (▲) HRP with 10 mM $NaNO_2$ and 2 mM H_2O_2; (B) time course of the heme modification reaction (●) HRP with 0.8 M $NaNO_2$ without H_2O_2 at pH 5; (▲) HRP with 10 mM $NaNO_2$ and 2 mM H_2O_2 at pH = 7.

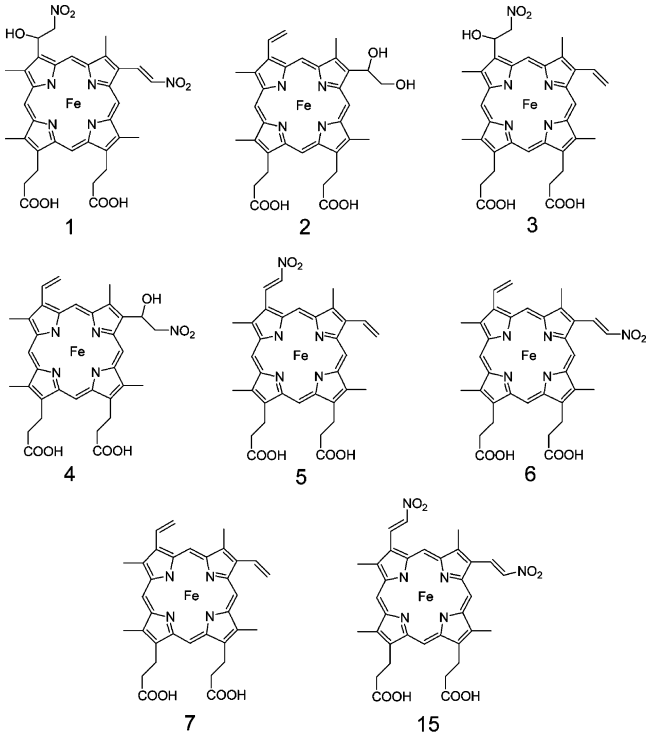

Figure 3. Proposed structures of the modified hemes isolated from HRP treated with nitrite.

is required. Using concentrations of NO_2^- higher or lower than 10 mM at pH = 7 in the presence of 2 mM H_2O_2 decreases the yield of heme products. HNO_2, which is formed at pH 5 (the pK_a of HNO_2 is 3.15[35] or 3.36[36]), or its decomposition products (NO_2 or other unidentified NO_x) oxidize 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), known as ABTS, (without H_2O_2 and peroxidase addition) also form compound II of HRP that can be further reduced by NO_2^-, forming $NO_2^{\bullet}$. NO is not involved in heme modification in either system because incubation of HRP with NO independently generated in situ does not cause heme modification (not shown). The possibility that peroxynitrite was formed in the system was ruled out by the absence of heme modification after incubation of HRP with $ONOO^-$ at both pH 5 and 7. Furthermore, a role for H_2O_2 formed in the reaction mixture itself was ruled out by the addition of catalase, which had no effect on the formation of modified heme products. Thus, two different and strongly pH-dependent reactions yielding the same heme products are observed, although they are formed in higher yield at high nitrite concentrations, conditions that also give an additional product (**15**, see later discussion, Figure 3). Having optimized the conditions for heme modification for both high and low nitrite concentrations, we focused on the physiologically relevant lower nitrite concentration conditions.

To determine the site of heme modification, we reconstituted HRP with mesoheme-d_4 (**14**). No heme modification occurred on incubation of this reconstituted enzyme with nitrite and H_2O_2 at pH = 7 (Figure 1). It is clear from this result that only the heme vinyl groups react with the oxidized nitrite species. The number of products in the chromatogram of HRP incubated with nitrite and H_2O_2 indicates that both vinyl groups can be modified, but the presence of a single major product (**6**) shows that one of the two vinyl groups is more reactive. To identify the more reactive vinyl group, we reconstituted HRP with either 2-cyclopropyl- (**10**) or 4-cyclopropylheme (**13**) and incubated the reconstituted proteins with nitrite and H_2O_2. Incubation of 2-cyclopropylheme/HRP gives **8** with m/z = 693 [2-cyclopropylheme (630) + NO_2 (46) + OH (17)] and **9** with m/z = 675 [2-cyclopropylheme (630) + NO_2 (46) − H (1)]. The corresponding structures are presented in Figure 5 and the molecular compositions in Table 1. In the analogous 4-cyclopropylheme/HRP reaction only small amounts of **11** (m/z = 693) and **12** (m/z 675) (Table 1, Figures 1 and 5) were formed.

The mass spectra of products **8** and **11** as well as **9** and **12** (Supporting Information) show that they correspond to regioisomeric structures. This confirms that both the 2- and 4-vinyl groups can be modified by $NO_2^{\bullet}$ but the 4-vinyl group is more reactive than the 2-vinyl group, presumably as the result of differential steric obstruction to reaction with the nitrite radical. Two kinds of reaction products are observed with both vinyl groups, one in which the vinyl is replaced by a 1-hydroxy-2-nitroethyl moiety and another in which a hydrogen on the β-carbon of the vinyl group is replaced by an NO_2 moiety.

$NO_2^{\bullet}$ thus only reacts with the vinyl groups. Compound **9,** the major product of the reaction, is formed by $NO_2^{\bullet}$ radical attack on the β-carbon of the 4-vinyl group (Figure 6), resulting in formation of the resonance-stabilized α-carbon radical. Transfer of the unpaired electron to the ferryl iron atom, reducing it to the ferric state, results in formation of a methylene cation from which either a proton can be eliminated to generate the NO_2-substituted vinyl group or the cation can be trapped by a water molecule.

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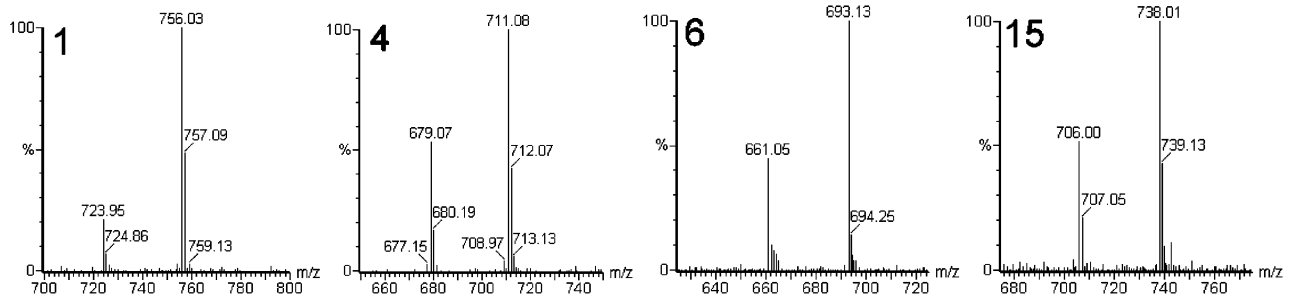

Figure 4. Mass spectra of the modified hemes formed in HRP. The full set of mass spectra is included in the Supporting Information.

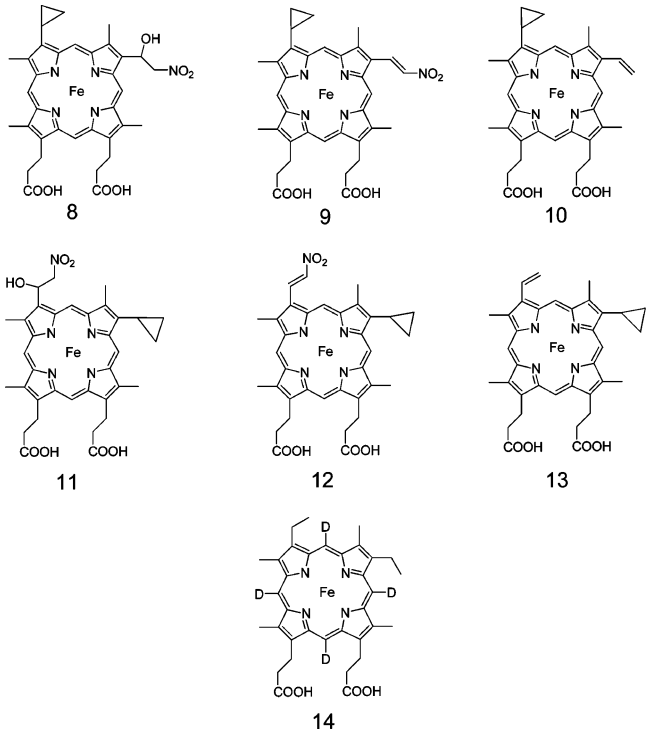

Figure 5. Proposed structures of the 2-cyclopropyl-, 4-cyclopropyl-, and mesoheme-d_4 products isolated from the reaction of appropriately reconstituted HRP with nitrite.

Table 1. Mass Spectrometric Data for the Modified Heme Products

cmpd	M^+ ion (m/z)[a]	heme	NO_2[b] (#)	OH (#)	H	molecular mass
1	724	616	92 (2)	17 (1)	−1	724
2	650	616	−	34 (2)	−	650
3	679	616	46 (1)	17 (1)	−	679
4	679	616	46 (1)	17 (1)	−	679
5	661	616	46 (1)	−	−1	661
6	661	616	46 (1)	−	−1	661
7	616	616	−	−	−	616
8	693	630[c]	46 (1)	17 (1)	−	693
9	675	630[c]	46 (1)	−	−1	675
10	630	630[c]	−	−	−1	630
11	693	630[c]	46 (1)	17 (1)	−	693
12	675	630[c]	46 (1)	−	−1	675
13	630	630[c]	−	−	−	630
14	624	624[d]	−	−	−	624
15	706	616	92 (2)	-	−2	706

[a] The molecular ion is accompanied by a strong ion 32 mass units higher due to the methanol-complexed iron porphyrin. [b] The molecular mass of NO_2 is 46. [c] The mass of 2-cyclopropylheme and 4-cyclopropylheme is 630. [d] The mass of mesoheme-d_4 is 624.

The most polar product **1** has −OH and $-NO_2$ groups added to the former 2-vinyl group and one $-NO_2$ on the 4-vinyl group, whereas **3** only has the OH- and NO_2-modified 2-vinyl group. Compound **4** is a regioisomer of **3** in that it has the same modification as in **3** but at the 4-vinyl group. Compound **6** is a major product of heme modification by $NO_2^{\bullet}$ and is formed in about 95% excess compared to its isomer **5** with the modification on the 2-vinyl group. At 10 mM nitrite and 2 mM H_2O_2, about 50% of the heme (**7**) remains unreacted. At very high nitrite concentration (i.e., 0.8 M) a new product **15** (Figure 3) containing an NO_2 group at each of the two vinyl groups is formed in a yield about 5%. This product was not detected in the chromatogram of HRP incubated with a low concentration of nitrite in the presence of H_2O_2.

HRP reacts relatively slowly with *tert*-BuOOH, presumably because its binding to the protein is impaired by its size, to give a compound I species[37] in a reaction that also produces the *tert*-$BuOO^{\bullet}$ and *tert*-$BuO^{\bullet}$ radicals.[38] However, no heme modified products were observed when we incubated HRP with low concentrations of *tert*-BuOOH (data not shown). We therefore incubated *tert*-BuOOH at both pH 4 and 8 with the F152M mutant in which the vinyl groups are more sterically accessible (Figure 7). A modified heme (**16**) and unreacted heme (**7**) were found in these incubations at pH 8 but not at pH 4. This product has a retention time of 15 min in our HPLC assay (Figure 7), in contrast to a retention time of 20.5 min for unmodified heme. Because formation of this product was pH dependent, we examined its formation over the range of pH from 4 to 10 (Figure 8A). A plot of the areas of the signals corresponding to the modified heme and heme itself as a function of pH clearly shows that the yield of the modified heme was maximal at pH 8.5. Because high pH may cause protein denaturation, we chose to carry out further studies at pH 8, as similar results are obtained at this pH.

Having optimized the pH for modified heme formation, we examined the time course required for complete conversion of the heme to the modified heme. With 10 equiv of *tert*-BuOOH only ∼60% conversion to the modified heme is observed even after 16 h (result not shown), but at the same pH with 40 equiv of *tert*-BuOOH complete heme modification is obtained within 3.5 h (Figure 8B). After this time, the modified heme was slowly degraded to unidentified products. In further experiments we therefore used pH 8, an incubation time of 5 h, and 40 equiv of *tert*-BuOOH to ensure maximum conversion of the heme to the modified heme.

The molecular mass of modified heme **16** is 680.2 (heme 616 + 64), but the mass increment does not uniquely define its

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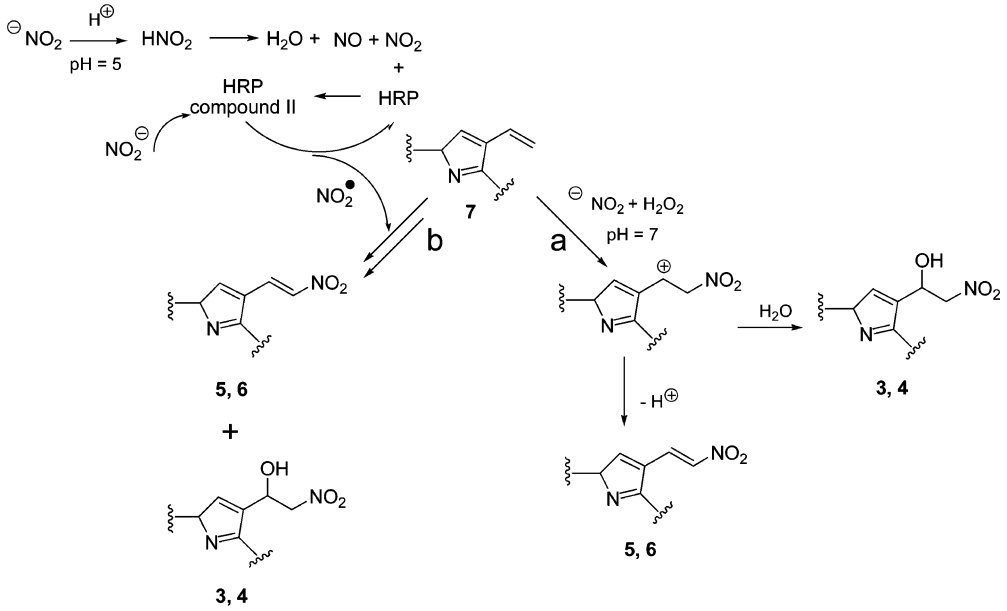

Figure 6. Proposed pathways for modification of the heme prosthetic group in the reaction of HRP with nitrite at (a) a low concentration of nitrite with H_2O_2, (b) a high concentration of nitrite without H_2O_2. Only the heme pyrrole ring B bearing the 4-vinyl is shown.

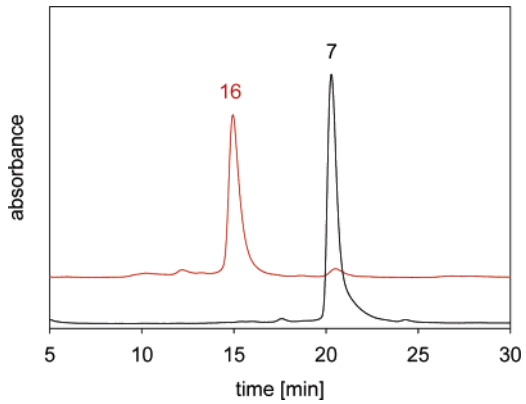

Figure 7. Chromatogram of F152M HRP (black) and HRP F152M incubated with 40 equiv of *tert*-BuOOH at pH 8 (red). Heme is **7**, and modified heme is **16**.

structure (Figure 9, Table 2). To pinpoint the site of modification in product **16** we reconstituted F152M with mesoheme, in which the two heme vinyl groups are replaced by ethyl groups, and with the 2- and 4-cyclopropylhemes. All three reconstituted proteins were incubated with 40 equiv of *tert*-BuOOH for 5 h at pH 8, and the incubations were then analyzed by LC/MS (Figure 10). In the case of mesoheme-reconstituted F152M no product other than mesoheme **18**, which appears at ~19 min (m/z = 620), was observed.

This result implies that heme modification occurs at one or the other of the two heme vinyl groups. In the reactions with the 2- and 4-cyclopropylheme-reconstituted F152M mutant, no product other than the parent iron porphyrin was observed with 2-cyclopropylheme **10**, but a new peak (**17**) in addition to 4-cyclopropylheme **13** (m/z = 630) was observed in the reaction of the enzyme reconstituted with the latter prosthetic group. The increment in the molecular ion of **17** (m/z = 694) indicates that the same modification, which increased the mass by 64 units, occurred with 4-cyclopropylheme as with heme itself. Clearly, the site of the modification is the 2-vinyl group.

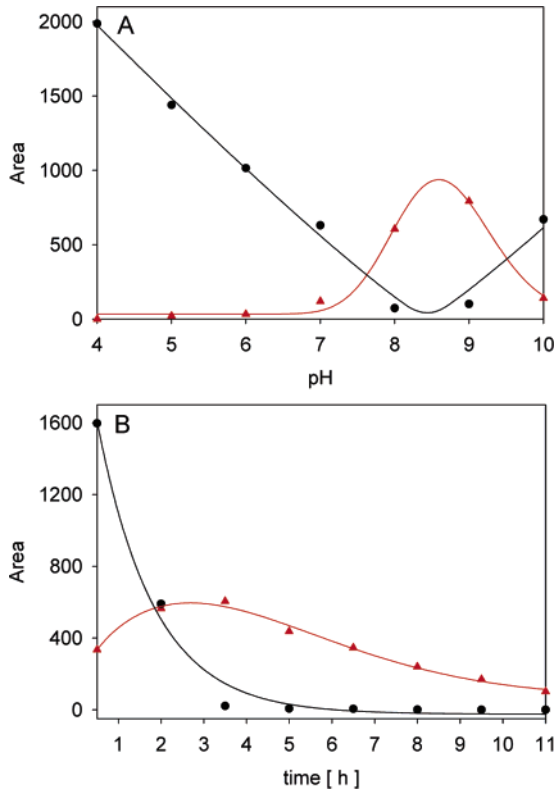

Figure 8. (A) The pH dependence of modified heme formation from HRP F152M treated with 40 equiv of *tert*-BuOOH: heme (●); modified heme (▲); (B) Time-dependence of modified heme formation with 40 equiv of *tert*-BuOOH at pH 8: heme (●); modified heme (▲).

The increment in the molecular ion of the product suggests two possible structures: heme (616) + OH (17) + SCH_3 (32 + 15) or heme (616) + OH (17) + $OOCH_3$ (16 + 16 + 15). To determine if an OH was present in the structure, we incubated the F152M mutant with *tert*-BuOOH in [^{18}O]H_2O (Figure 11).

LC/MS analysis of the products from the incubation in [^{18}O]-H_2O shows that the modified heme is formed in a lower than

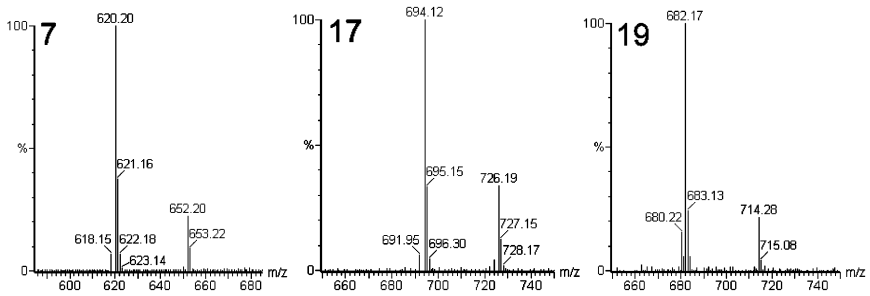

Figure 9. Mass spectra of modified heme **16**, modified 4-cyclopropylheme **17**, and ^{18}OH-labeled modified heme **19**.

Table 2. Summary of the Mass Spectrometric Data for Modified Heme Products

cmpd	M$^+$ ion (m/z)[a]	heme	$OOCH_3$ [b] (#)	OH (#)	H	CH_3	molecular mass
16	680	616	47 (1)	17 (1)	–	–	680
17	694	630[c]	47 (1)	17 (1)	–	–	694
18	620	620[d]	–	–	–	–	620
19	682	616	47 (1)	19 (1)[e]	–	–	682
20	648	616	–	17 (1)	–	15 (1)	648
21	662	616	–	17 (1)	−1	30 (2)	662
22	630	616	–	–	−1	15 (1)	630
23	644	630[c]	–	–	−1	15 (1)	644
24	684	616	51 (1)[f]	1 (17)	–	–	684
25	651	616	–	34 (2)	–	–	650

[a] The molecular ion is accompanied by a strong ion 32 mass units higher due to the methanol-complexed iron porphyrin. This signal is absent in the case of **25**. [b] The molecular mass of $OOCH_3$ is 47. [c] The mass of both 2- and 4-cyclopropylhemes is 630. [d] The mass of mesoheme is 620. [e] The mass of ^{18}OH is 19. [f] The mass of $^{18}O^{18}OCH_3$ is 51.

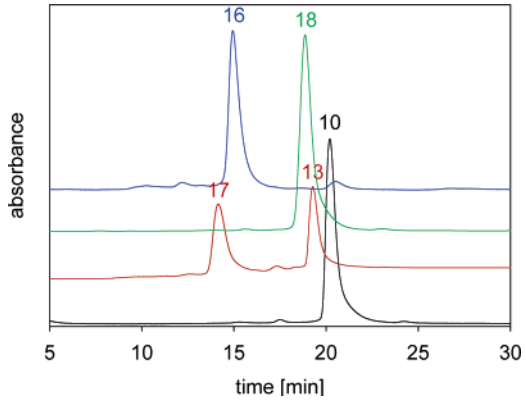

Figure 10. LC/MS chromatograms of the F152M mutant reconstituted with 2-cyclopropylheme, black; 4-cyclopropylheme, red; mesoheme, green; and for comparison, F152M containing heme itself, blue. The samples were incubated with 40 equiv of *tert*-BuOOH for 5 h at pH 8.

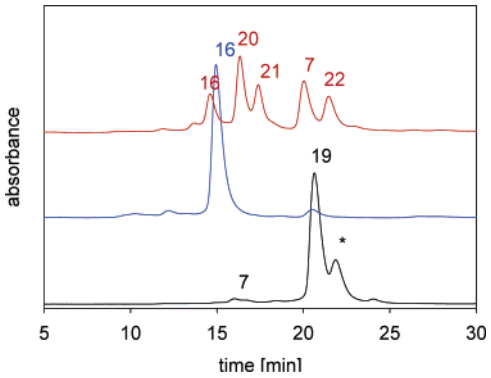

Figure 11. LC/MS chromatograms of the F152M mutant incubated in [^{18}O]-H_2O with *tert*-BuOOH, (black); F152M incubated in [^{16}O]H_2O and *tert*-BuOOH (blue); and F152M incubated in [^{16}O]H_2O and *tert*-BuOOH under anaerobic conditions (red). A signal marked with (∗) corresponds to an unknown product with m/z = 646.

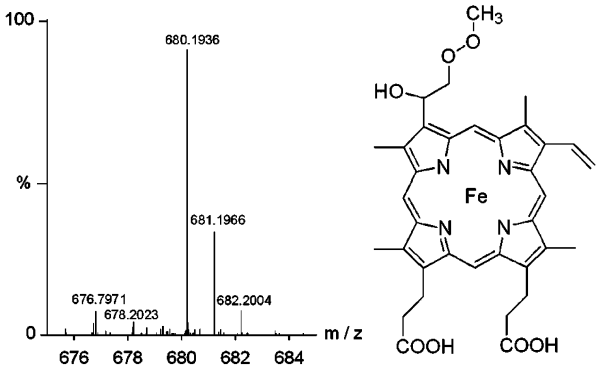

Figure 12. High-resolution mass spectrum and proposed structure of the modified heme (**16**) formed during incubation of F152M with 40 equiv of *tert*-BuOOH, pH 8, for 5 h.

normal yield, presumably as the result of a solvent isotope effect because control experiments with HRP F152M carried out by exactly the same protocol but in non-isotopically enriched water gave a normal high yield of the modified heme. The LC/MS of the modified heme from the [^{18}O]H_2O incubation gave a mass two units higher than that obtained in normal [^{16}O]H_2O (m/z 682.2 versus 680.2) (Figure 9), confirming both the presence of a hydroxyl group in the modified heme and its derivation from water (i.e., as in **19**). The remaining increase in the molecular mass of the modified heme (i.e., m/z = 680 − 616 = 47) can be explained by incorporation of either an $-SCH_3$ or an $-OOCH_3$ group.

Three experiments confirm that the moiety added to the vinyl is, in fact, an $-OOCH_3$ group. First, high-resolution mass spectrometric analysis gave an exact mass of m/z = 680.1936 (Figure 12), a value in excellent agreement with addition of a hydroxyl and a CH_3OO group (theoretical value m/z = 680.1933) but not a CH_3S group (theoretical value m/z = 680.1756).

Second, incubation of HRP F152M with *tert*-BuOOH in a solution saturated with $^{18}O_2$ gas gave a modified heme with a molecular ion four units higher (i.e., m/z = 684) than that of the unlabeled heme product, indicating the incorporation of two ^{18}O atoms (**24**, Figures 13 and 14). Finally, reduction of the peroxy function in the modified heme by $NaBH_4$ led to formation of a diol with m/z = 651, a molecular M + 1 ion that corresponds to the ferric porphyrin (**25**, Figures 13 and 14).

Which carbon of the original vinyl group bears the −OH and which the $-OOCH_3$? Incubation of F152M with *tert*-BuOOH in the presence of the radical trap 5,5-dimethyl-1-pyrroline

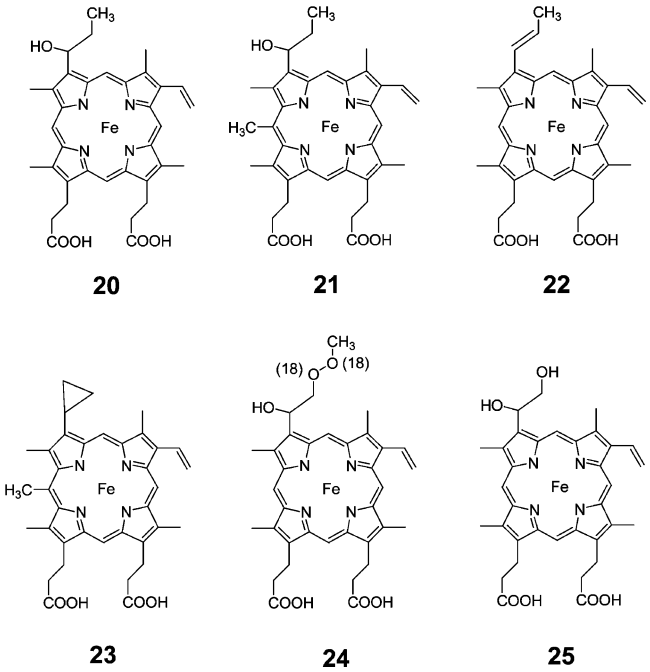

Figure 13. Structures of the modified hemes formed in the anaerobic reaction of *tert*-BuOOH with HRP F152M.

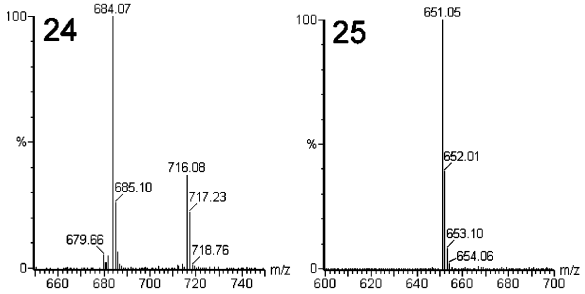

Figure 14. LC/MS spectra of modified heme **24** labeled with $^{18}O_2$ and the heme diol **25** obtained by reduction of the methylperoxy function.

N-oxide (DMPO)[39] suppresses formation of the modified product **16**, a finding that argues for a free radical mechanism (results not shown). This supports the inference that the reactive species that adds to the vinyl is the $CH_3OO^{\bullet}$ radical. Addition of the radical to the β-carbon of the vinyl group would place the unpaired electron on the α-carbon, from which it can be readily delocalized into the porphyrin ring. Transfer of the unpaired electron to the iron generates a carbocation at the α-carbon that can be trapped by water to give **16**. The corresponding chemical structure of the modified heme formed from the 4-cyclopropyl-heme-reconstituted F152M mutant is **17** (Figure 15).

Formation of the $CH_3OO^{\bullet}$ radical is readily rationalized by radical chain decomposition of *tert*-BuOOH, via a *tert*-$BuO^{\bullet}$ radical, to give a $CH_3^{\bullet}$ radical that combines with O_2 before adding to the heme (Figure 16).[39] This mechanism is consistent with the ^{18}O-labeling studies which established the origin of the two oxygen atoms in the peroxo moiety in the adduct.

As implied by incorporation of two labeled oxygen atoms into the modified heme in incubations carried out with $^{18}O_2$, molecular oxygen is essential for the formation of $CH_3OO^{\bullet}$ from the $CH_3^{\bullet}$ produced in the decomposition of *tert*-$BuO^{\bullet}$ (Figure 16).[39] Formation of the methylperoxy adduct **16** should therefore be suppressed in the absence of O_2. Indeed, when F152M was incubated with *tert*-BuOOH under anaerobic conditions (Figure 11), we observed only a trace of the normal peroxy adduct but found three new heme-derived products, **20** (m/z = 648), **21** (m/z = 662), and **22** (m/z = 630). The structures assigned to these products (Figure 13, LC/MS in Supporting Information) are based on their mass spectra and the reactivity patterns observed here for the heme group in the F152M mutant.

The anaerobic formation of these products is readily explained by suppression of the methylperoxy radical reaction (Figure 17, path a) in favor of direct methyl radical attack (Figure 17, path b). Thus, addition of the methyl radical at the vinyl β-carbon generates the α-carbon radical that, after conversion to the cation by electron transfer to the iron, is quenched by water to give **20**. If deprotonation of the β-carbon in the cation occurs before

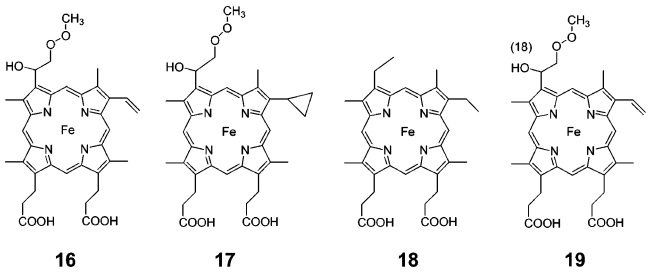

Figure 15. Structures of the modified hemes formed in the aerobic reactions of *tert*-BuOOH with HRP F152M and HRP F152M reconstituted with alternative hemes.

$$\text{Por(Fe}^{\text{III}}\text{)} + \textit{tert}\text{-BuOOH} \longrightarrow \text{Por}^{+\cdot}\text{(Fe}^{\text{IV}}\text{=O)} \quad (1)$$

$$\text{Por}^{+\cdot}\text{(Fe}^{\text{IV}}\text{=O)} + \textit{tert}\text{-BuOOH} \longrightarrow \text{Por(Fe}^{\text{IV}}\text{=O)} + \text{tert-BuOO}^{\bullet} \quad (2)$$

$$\text{tert-BuOO}^{\bullet} + \text{tert-BuOO}^{\bullet} \longrightarrow 2\ \textit{tert}\text{-BuO}^{\bullet} + O_2 \quad (3)$$

$$\textit{tert}\text{-BuO}^{\bullet} \longrightarrow CH_3^{\bullet} + CH_3COCH_3 \quad (4)$$

$$CH_3^{\bullet} + O_2 \longrightarrow CH_3OO^{\bullet} \quad (5)$$

$$\text{Por(Fe}^{\text{III}}\text{)} + \textit{tert}\text{-BuOOH} \longrightarrow \text{Por(Fe}^{\text{IV}}\text{=O)} + \textit{tert}\text{-BuO}^{\bullet} \quad (6)$$

Figure 16. Generation of $CH_3^{\bullet}$ and $CH_3OO^{\bullet}$ radicals in the reaction of *tert*-BuOOH with F152M HRP. Ferric heme is abbreviated as Por(Fe^{III}), where Por stands for porphyrin. Equation 6 shows a second route to the *tert*-$BuO^{\bullet}$ than that provided by eqs 1−3.

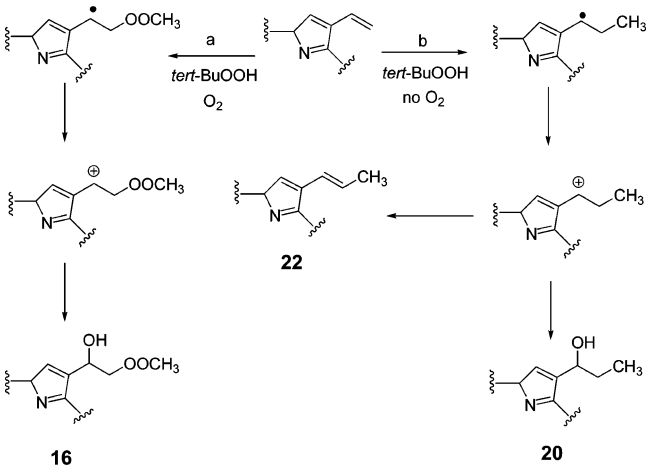

Figure 17. Proposed pathways for modification of the heme prosthetic group in the reaction of HRP F152M with *tert*-BuOOH under (a) aerobic and (b) anaerobic conditions. Only pyrrole ring A of the heme bearing the 2-vinyl is shown.

(39) Lee, C. H.; Lang, J.; Yen, C. W.; Shih, P. C.; Lin, T. S.; Mou, C. Y. *J. Phys. Chem. B* **2005**, *109*, 12277−12286.

it is trapped by water, the methylvinyl product **22** is generated. Secondary reaction of **20** with another methyl radical forms **21**. The corresponding methylperoxy vinyl product (−CH═CHOOCH$_3$) is not detected in the peroxy radical pathway, possibly because the peroxy oxygen can stabilize the adjacent cation by forming a bridged oxonium cation. The ability of the $CH_3^•$ radical to add to the heme *δ-meso* position is confirmed by the observation that under anaerobic conditions the F152M mutant reconstituted with 2-cyclopropylheme produces a heme adduct (**23**) bearing an additional methyl group, whereas no adduct is formed under aerobic conditions. Clearly, the methyl radical has the properties required for reaction at both the vinyl group and the *δ-meso* position, whereas the $CH_3OO^•$ radical can only react at the vinyl group.

Surprisingly, addition occurs at the 2-vinyl but not 4-vinyl of the heme even though we have shown that electrophilic additions of HOBr, HOCl, and HOSCN to the heme in wild-type HRP occur at both the 2- and 4-vinyl groups.[1,2] This unexpected regiospecificity of the vinyl group addition presumably arises from decreased shielding of the 2- but not 4-vinyl by the F152M mutation. The fact that the heme modification does not occur in the reactions of *tert*-BuOOH with wild-type HRP gives a measure of the stringency of the steric requirements for the addition reaction, as does the fact that even in the F152M mutant, it is the $CH_3OO^•$ and not the *tert*-BuOO$^•$ radical that adds to the vinyl group.

Discussion

The regiospecificity of heme modification is unusual and highly informative. As already noted, previous studies of substrate-mediated HRP heme modifications, including those of alkyl,[4,42] azidyl,[6] nitroalkyl,[43] chloro,[1] thiocyano,[2] and acetoxy[7] radicals, indicate that free radicals react primarily or exclusively with the *δ-meso* carbon of the heme group in native HRP. The addition occurs at the *δ-meso* carbon and not at the other *meso* positions because it is the only exposed edge of the heme in HRP.[44] The finding that alkyl radicals add to the *γ-meso* position in myoglobin because it is the solvent-exposed heme edge in that protein clearly confirms that the *δ-meso* heme carbon is not unusually reactive.[5] It is therefore of interest that no trace of the *δ-meso* heme adduct was observed in aerobic reactions of HRP or its F152M mutant with either the $NO_2^•$ or $CH_3OO^•$ radicals, both of which add readily to the heme vinyl groups. In contrast, reaction of the F152M mutant under anaerobic conditions that suppress formation of the $CH_3OO^•$ radical results in addition of the $CH_3^•$ radical to both the *δ-meso* and 2-vinyl positions. Thus, the $CH_3OO^•$ radical, although larger, adds regiospecifically to the vinyl group, whereas the $CH_3^•$ radical adds to both the *δ-meso* carbon and the 2-vinyl group. Analysis of the X−H bond dissociation energies for the generation of radicals known to add to the heme group offers a general solution to this dichotomy. As shown in Table 3, the radicals that add to the *δ-meso* carbon (and vinyl groups if sterically allowed) have higher energies, as indicated by higher bond dissociation energies, than those that add exclusively to the vinyl group. The $CH_3OO^•$ radical is stabilized by conjugation to the adjacent oxygen atom, has a low bond dissociation energy (88 ± 1 kcal/ mol),[41] and is relatively unreactive. The BDE of the nitrite radical is also low (79.1 ± 0.2 kcal/ mol).[40] This provides an explanation for why these two radicals add to the vinyl but not the *δ-meso* carbon even though the crystal structure and modification experiments indicate that the *δ-meso* carbon is the most exposed region of the heme.[44,45] The methyl radical, on the other hand, is high in energy and can add at both the *δ-meso* carbon and the vinyl group. The correlation indicates that a bond dissociation energy above 88 ± 1 kcal/mol is required for addition to the *δ-meso* carbon.

Table 3. Molecular Bond Dissociation Energies for RH → R$^•$ + H$^•$ at 298 K; the Heme Position in HRP That Is Substituted by the Corresponding Radical Is Indicated

	DH_{298} (kcal/mol)	position of addition	ref to DH_{298}	ref to position of addition
HCN	126.3 ± 0.2	*δ-meso*	46	47
PhH	112.9 ± 0.5	*δ-meso*	46	3
CH_3COOH	112 ± 3	*δ-meso*	40	7
CH_4	104.99 ± 0.03	*δ-meso*, vinyl	48	this work
HCl	103.15 ± 0.03	*δ-meso*	49	1
CH_3CH_3	101.1 ± 0.4	*δ-meso*	50	4
HNCS	96 ± 6	*δ-meso*	51	2
HN_3	92 ± 5	*δ-meso*	52	6
CH_3OOH	88 ± 1	vinyl	41	this work
HBr	87.54 ± 0.05	vinyl	49	1
tert-BuOOH	84 ± 2	none	41	*a*
HNO_2	79.1 ± 0.2	vinyl	53	this work

[a] No modification of heme is observed, presumably because of the steric bulk of the *tert*-butyl group.

Like the relatively unreactive radicals, electrophilic agents such as HOBr, HOCl, and HOSCN add to the vinyl groups but not to the *meso* carbon[1,2] Although low yields of a *δ-meso* chloro adduct are formed on incubation of the *Arthromyces rhamosus* peroxidase with chloride and H_2O_2, we have recently demonstrated that this product, unlike the vinyl adducts, arises from reaction with a chloride radical rather than with HOCl (Liusheng Huang, unpublished results). It is remarkable that in no instance has a *meso* adduct been found that results from addition of a cationic species to the *meso* carbon of the prosthetic group of a hemoprotein.

Heme oxygenases catalyze the intramolecular hydroxylation of one of the *meso* carbons in the first step of heme catabolism.[54,55] The human and rat enzymes oxidize the α-*meso* carbon but this α-regiospecificity is not universal.[54] Two mechanisms have been postulated for this reaction, both involving the ferric

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hydroperoxide (Fe(III)-OOH as the immediate reaction precursor. In one mechanism, the ferric hydroperoxide reacts with the heme edge essentially as in an electrophilic aromatic substitution.[56] In an alternative mechanism proposed on the basis of theoretical calculations, the hydroxylation involves initial homolytic cleavage of the ferric hydroperoxide to give a hydroxyl radical that remains hydrogen bound to the residual iron-bound oxygen and through that association is specifically delivered to the *meso* carbon that is oxidized.[57] The fact that energetic radical species add to the *meso* carbon but electrophilic species do not, argues that the latter, "captive" hydroxyl radical mechanism may the correct one.

Experimental Section

Materials. Native HRP (HRP_{com}) was purchased from Roche. 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (NOC-9) was from Sigma. All other chemicals, including buffer components and HPLC solvents, were from Fisher Scientific. 2-Cyclopropylheme and 4-cyclopropylheme were synthesized from protoporphyrin IX dimethyl ester by a modification of a published procedure.[58] The phosphate buffer was 100 mM, pH 7, and citric buffer was 100 mM, pH 5. Water was double distilled prior to use. Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using the absorption value $\epsilon_{402} = 102{,}000\ M^{-1}\ cm^{-1}$ and a molecular weight of 44,000.[59] The H_2O_2 concentration was standardized spectrophotometrically at 240 nm by using the molar extinction coefficient $\epsilon = 43.6\ M^{-1}\ cm^{-1}$.[60] 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 a Waters XTerra MS C_{18} column (2.1 mm × 50 mm, 3.5 μm) protected with a guard column (XTerra MS $C_{18,}$ 2.1 mm × 10 mm, 3.5 μm). Solvent A was water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The gradient program consisted of linear segments with 40% B (0−2 min), from 40 to 70% B (2−22 min), 70% B (22−26 min), 70 to 95% B (26−27 min), 95% B (27−30 min), 40% B (30.1−40 min) at a flow rate of 0.2 mL/min. The eluent was monitored at 400 nm. 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.

Incubation of HRP with $NaNO_2$ (High Concentration). All solutions were prepared freshly in citric buffer (100 mM, pH 5). A 50 μL aliquot of 30 μM protein solution was mixed with 50 μL of the appropriate buffer and 50 μL of 2.4 M of $NaNO_2$ in the same buffer. The samples were incubated in the dark at room temperature for 1 h and analyzed by LC/MS.

Incubation of HRP with $NaNO_2$ (Low Concentration) and H_2O_2. All solutions were prepared freshly in phosphate buffer (100 mM, pH 7). A 50 μL aliquot of the 30 μM protein solution was mixed with 50 μL of the 6 mM H_2O_2 (final concentration of H_2O_2 in sample was 2 mM, 200 equiv) in phosphate buffer pH = 7 and 50 μL of 30 mM of $NaNO_2$ (final concentration in sample was 10 mM, 1000 equiv) in the same buffer. The samples were incubated in the dark at room temperature for 1 h and analyzed by LC/MS.

HRP and F152M HRP Apoenzyme Preparation and Reconstitution with Substituted Hemes. This work was done according to previously published methods.[1]

Incubation of HRP with $NaNO_2$ (High Concentration) in the Presence of Catalase. The sample was prepared under the same conditions as reported above. Both nitrite solution in buffer pH = 5 and HRP in the same buffer contained catalase at a concentration of 1 μM. The sample was incubated for 1 h and was then analyzed by LC/MS.

Incubation of HRP with Peroxynitrite. Peroxynitrite was synthesized according to a known procedure.[61] Briefly, a 2.5 mL aliquot of 30% H_2O_2 was added to 10 mL of ice cold water, giving a final concentration of 2 M H_2O_2. This solution was added to 1.5 mL of 40 mM DTPA (diethylenetriaminepentaacetic acid) in 200 mM NaOH in a 60 mL Erlenmeyer flask. A 5 mL portion of 5 M NaOH was added to this solution, giving a pH of ∼12.8−13. Next, 5 mL of water was added, and the solution was stirred vigorously. An equimolar amount of isoamyl nitrite (prewashed with 6 × 2 vol of water) was added, and mixture was stirred for 2 h at room temperature before it was extracted with 6 × 25 mL of CH_2Cl_2. A 1.5 × 10 cm column filled with 25 g of granular MnO_2 was washed with 20 mL of water and then with 0.5 M of NaOH. The organic extract was then passed through this column to remove the excess of H_2O_2. To minimize the dilution of peroxynitrite, the first few milliliters of eluent from the column were discarded. The concentration of the stock solution of peroxynitrite was estimated to be 0.3216 M using $\epsilon_{302} = 1660\ M^{-1}\ cm^{-1}$ in 0.1 M NaOH.[61]

All solutions were prepared freshly in phosphate buffer (100 mM, pH 5 and pH = 7). A 50 μL aliquot of a 30 μM protein solution was mixed with 50 μL of the appropriate buffer and 50 μL of 3 mM peroxynitrite in the same buffer. The samples were incubated in the dark at room temperature for 1 h and were then analyzed by LC/MS.

Incubation of HRP with Nitrogen Monoxide. Nitrogen monoxide (NO) was generated in situ using 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (NOC-9). To a mixture of 50 μL of 30 μM protein and 100 μL of buffer pH = 5 and as well as 7 a 1 mg of NOC-9 was added. The sample was incubated in the dark at room temperature for 30 min and analyzed by LC/MS.

Cloning, Expression, and Purification of F152M HRP. The starting construct was a 6-His-tagged HRP gene subcloned into the *BamH*I site of pUC19. F152M primer was designed for the mutagenesis with the nucleotide substitutions in the center of the sequence: CAG CTG AAG GAT AGC **ATG** AGA AAC GTG GGT CTG. Mutagenesis, transformation, baculovirus cotransfection, and amplification were made according to previously published methods with expression time increased to 90 h.[62] The F152M mutant was expressed in yield of 7.7 mg/L of culture.

Incubation of HRP Mutants with *tert*-BuOOH and H_2O_2. All solutions were prepared freshly in phosphate buffer (100 mM, pH 8). A 50 μL aliquot of 30 μM protein solution was mixed with 50 μL of the appropriate buffer and 50 μL of 1.2 mM (40 equiv) of *tert*-BuOOH or 50 μL 0.3 mM (10 equiv) of H_2O_2 in the same buffer. The samples were incubated in the dark at room temperature for 5 h (*tert*-BuOOH) or 0.5 h (H_2O_2) and analyzed by LC/MS.

Labeling of Modified Heme with $[^{18}O]H_2O$. A 4.47 μL aliquot of 335.44 μM F152M was dissolved in 45.53 μL of $[^{18}O]H_2O$ to give 50 μL of 30 μL F152M solution, and the solution was incubated for 3 h at room temperature. The resulting solution was lyophilized, and the resulting F152M residue was redissolved in 50 μL of $[^{18}O]H_2O$. A 10 μL aliquot of 1 M phosphate buffer was lyophilized separately, and the resulting solid was dissolved in 50 μL of $[^{18}O]H_2O$. A 5 μL aliquot of a 1.2 mM stock solution of *tert*-BuOOH was diluted to 50 μL in $[^{18}O]H_2O$. The F152M solution was then combined with buffer and after addition of the *tert*-BuOOH sample was incubated for 5 h at room temperature. A 100 μL aliquot of the solution was analyzed by LC/MS under the standard conditions given above.

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Incubation of F152M with *tert*-BuOOH in the Presence of a Radical Trap. A 50 μL aliquot of a 30 μM solution of HRP 152M was mixed with 50 μL of 100 mM phosphate buffer, pH 8, containing 40 mM DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), and 50 μL of 1.2 mM (40 equiv) *tert*-BuOOH in phosphate buffer was added. The solution was incubated in the dark at room temperature for 5 h and was then analyzed by LC/MS.

Incubation of F152M and F152M/2-Cyclopropylheme with *tert*-BuOOH under Anaerobic Conditions. The reaction was carried using a Schlenk line under argon prepurified by passage through a Ridex Oxygen Scavenger and a secondary pyrrogalol oxygen trap (30 g of pyrogallol in 200 mL of 50% aqueous KOH) to remove traces of oxygen. A 50 μL aliquot of 30 μM HRP F152M mixed with 50 μL of phosphate buffer (pH 8) was degassed under vacuum for 5 min. The solution was then saturated with argon by passing the gas very slowly above the surface of the solution for 15 min. This procedure (vacuum/argon saturation) was repeated three more times with the final exposure to argon lasting 30 min. The same procedure was employed for the preparation of a 1.2 mM solution of *tert*-BuOOH in buffer. Using a syringe flushed with argon 50 μL of 1.2 mM *tert*-BuOOH in buffer was transferred to F152M (or F152M/2-cyclopropylheme) solution by injection via a septum. The sample was incubated as usual in the dark at room temperature for 5 h and was then analyzed by LC/MS.

Incubation of F152M with *tert*-BuOOH in the Presence of $^{18}O_2$ Gas. The sample was prepared in the same way as was done for the anaerobic incubation. After degassing separately the F152M and *tert*-BuOOH solutions in septum-capped vials, 4 mL of $^{18}O_2$ was passed for 30 min over the solutions with magnetic stirring to saturate them. After combination of the two solutions, the mixture was incubated at room temperature for 5 h under an $^{18}O_2$ atmosphere.

Reduction of the Hydroxylmethylperoxy Heme with $NaBH_4$. A 100 μL sample of 30 μM F152M solution was mixed with 100 μL of 100 mM phosphate buffer (pH 8) and 100 μL of 1.2 mM (40 equiv) *tert*-BuOOH in the same buffer. After 5 h of incubation the reaction was quenched by addition of 50 μL of 40 mM DMPO, concentrated to $\sim$20 μL using Millipore filters (10 MWCO) and washed twice with 300 μL of water (buffer exchange). After dilution of the sample to 2 mL, 30 μL of 3 M HCl was added before the sample was extracted twice with 0.5 mL of 2-butanone. The butanone was evaporated, and the sample was dissolved in 1 mL of dry methanol. A total of 5 mg of $NaBH_4$ was added with stirring in small portions over 10 min, and the final mixture was stirred for a further 10 min at room temperature. To quench the reaction, 3 mL of water containing 30 μL of 3 M HCl was added. The solution was saturated with NaCl and was then extracted with butanone (2 $\times$ 1 mL). The butanone was evaporated, and the residue was redissolved in 100 μL of methanol and 50 μL of water. A 100 μL aliquot was analyzed by LC/MS.

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Supporting Information Available: The mass spectra of compounds **1**−**25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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