Published in final edited form as:

J Am Chem Soc. 2007 February 14; 129(6): 1663–1672. doi:10.1021/ja067067s.

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 regioselectivity of these reactions, however, are unclear. We report here modification of the heme of HRP by autocatalytically generated, low energy $\text{NO}_2^\bullet$ and $\text{CH}_3\text{OO}^\bullet$ radicals. The $\text{NO}_2^\bullet$ 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, but 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 [$-\text{CH}(\text{OH})\text{CH}_2\text{OOCH}_3$]. [18O]-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., $\text{CH}_3^\bullet$) add to the *meso*-carbon. Less energetic radicals, including $\text{NO}_2^\bullet$ and $\text{CH}_3\text{OO}^\bullet$, 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 chloride¹ radicals at the δ -*meso* carbon of the heme. The regioselectivity 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 alkyl⁵ radicals to the γ -*meso* carbon, aryl radicals to the iron and porphyrin nitrogens,⁹ and haloalkyl and NO₂[•]¹² radicals to the vinyls¹³ 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 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 (NO₂[•]) and alkylperoxy (ROO[•]) 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 sidechain is replaced by the smaller methionine sidechain. 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, provides general insights into the intrinsic and protein-dependent factors that control the regioselectivity of additions to the heme group in hemoproteins. The results have implications, for example, for the catalytic mechanism of heme oxygenase.

Results

Nitrite NO₂⁻ 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 NO₂⁻ 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} NO₂⁻ does not accumulate in organisms because it is rapidly oxidized to NO₃⁻ by oxyhemoglobin²⁰ and oxymyoglobin.²¹ Myeloperoxidase also oxidizes NO₂⁻ to NO₂[•] radicals and thereby nitrates tyrosine residues.^{22–26} In LPO (lactoperoxidase) an iron bound peroxynitrite is proposed to be the nitrating agent instead of NO₂[•]^{27–29} because nitrite is oxidized to nitrate NO₃⁻ in an extremely fast reaction ($\sim 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) by the compound I of LPO.³⁰ Eosinophil recruitment and enhanced production of NO are characteristic features of asthma,³¹ and eosinophil peroxidase is at least four-fold more effective than myeloperoxidase at nitration of tyrosine-containing peptides, a reaction that involves the NO₂[•] radical.^{32,33} Horseradish peroxidase (HRP) also produces NO₂[•] 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 NO₂ moiety upon incubation with nitrite,¹¹ but no information is available on possible nitrite-dependent modifications of the heme in plant or fungal peroxidases.

LCMS analysis indicates that six modified hemes are formed on incubation of HRP with H₂O₂ 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 NO₂[•] 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 H₂O₂ 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 NaNO₂ and 2 mM H₂O₂. However, at high ($\sim 0.8 \text{ M}$) nitrite concentrations at which H₂O₂ addition is not required for formation of modified heme **6**, heme modification occurs best at pH = 5 (Figure 1, 3, 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 H₂O₂, whereas a longer time is required at pH = 7 with 10 mM nitrite and 2 mM H₂O₂. 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 NO₂[•]. At high nitrite concentrations and pH = ~7, heme modification is more effective, with ~95% heme modification compared to the system with 10 mM NaNO₂ and H₂O₂, 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 H₂O₂, the heme is probably modified by NO_x products of HNO₂ decomposition, whereas when H₂O₂ is present NO₂[•] 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 H₂O₂ is required. Using higher or lower concentrations of NO₂[•] than 10 mM at pH = 7 in the presence of 2 mM H₂O₂ decreases the yield of heme products. HNO₂, which is formed at pH 5 (the pK_a of HNO₂ is 3.15³⁵ or 3.36³⁶), or its decomposition products (NO₂ or other unidentified NO_x) oxidize 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), known as ABTS, (without H₂O₂ and peroxidase addition) and also form compound II of HRP that can be further reduced by NO₂[•], forming NO₂[•]. 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 H₂O₂ 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 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 conditions.

To determine the site of heme modification, we reconstituted HRP with d₄-*meso* heme (**14**). No heme modification occurred on incubation of this reconstituted enzyme with nitrite and H₂O₂ 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 H₂O₂ 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 H₂O₂. Incubation of 2-cyclopropyl heme/HRP gives **8** with m/z = 693 [2-cyclopropyl heme (630) + NO₂ (46) + OH (17)] and **9** with m/z = 675 [2-cyclopropyl heme (630) + NO₂ (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, Figure 1 and 5) were formed.

The mass spectra of products **8** and **11** as well as **9** and **12** (supporting materials) show that they correspond to regioisomeric structures. This confirms that both the 2- and 4-vinyl groups can be modified by NO₂[•] 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 NO₂ moiety.

$\text{NO}_2^\bullet$ thus only reacts with the vinyl groups. Compound **9**, the major product of the reaction, is formed by $\text{NO}_2^\bullet$ 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 feryl 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.

The most polar product **1** has $-\text{OH}$ and $-\text{NO}_2$ groups added to the former 2-vinyl group and one $-\text{NO}_2$ 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 $\text{NO}_2^\bullet$ 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 species³⁷ 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 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 eq 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 eq 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 eq of *tert*-BuOOH to insure 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 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-cyclopropyl hemes. All three reconstituted proteins were incubated with 40 eq of *tert*-BuOOH for 5 h at pH 8 and the incubations were then analyzed by LCMS (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-cyclopropyl heme-reconstituted F152M mutant, no product other than the parent iron porphyrin was observed with 2-cyclopropyl heme **10**, but a new peak (**17**) in addition to 4-cyclopropyl heme **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.

The increment in the molecular ion of the product suggests two possible structures: heme (616) + OH(17) + SCH₃(32+15) or heme (616) + OH(17) + OCH₃ (16+16+15). To determine if an OH was present in the structure, we incubated the F152M mutant with *tert*-BuOOH in [¹⁸O]H₂O (Figure 11).

LCMS analysis of the products from the incubation in [¹⁸O]H₂O shows that the modified heme is formed in a lower than 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 LCMS of the modified heme from the [¹⁸O]H₂O incubation gave a mass two units higher than that obtained in normal [16O]H₂O (*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 –SCH₃ or an –OCH₃ group.

Three experiments confirm that the moiety added to the vinyl is, in fact, an –OCH₃ 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 CH₃OO group (theoretical value *m/z* = 680.1933) but not a CH₃S group (theoretical value *m/z* = 680.1756).

Second, incubation of HRP F152M with *tert*-BuOOH in a solution saturated with ¹⁸O₂ 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 ¹⁸O atoms (**24**, Figure 13). Finally, reduction of the peroxy function in the modified heme by NaBH₄ led to formation of a diol with *m/z* = 651, a molecular *M*+1 ion that corresponds to the ferric porphyrin (**25**, Figure 13).

Which carbon of the original vinyl group bears the –OH and which the –OCH₃? Incubation of F152M with *tert*-BuOOH in the presence of the radical trap DMPO (5,5-dimethyl-1-pyrroline N-oxide)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 CH₃OO• 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-cyclopropylheme-reconstituted F152M mutant is **17** (Figure 14).

Formation of the CH₃OO• radical is readily rationalized by radical chain decomposition of *tert*-BuOOH, via a *tert*-BuO• radical, to give a CH₃• radical that combines with O₂ before adding to the heme (Figure 15).³⁹ This mechanism is consistent with the ¹⁸O-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 ¹⁸O₂, molecular oxygen is essential for the formation of CH₃OO• from the CH₃• produced in the decomposition of *tert*-BuO• (Figure 15).³⁹ Formation of the methylperoxy adduct **16** should therefore be suppressed in the absence of O₂. 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, LCMS in supporting materials) 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 18, 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 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 ($-\text{CH}=\text{CHOOCH}_3$) 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 $\text{CH}_3^\bullet$ radical to add to the heme δ -*meso*-position is confirmed by the observation that under anaerobic conditions the F152M mutant reconstituted with 2-cyclopropyl heme 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 $\text{CH}_3\text{OO}^\bullet$ 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 regioselectivity 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 $\text{CH}_3\text{OO}^\bullet$ and not the *tert*- $\text{BuOO}^\bullet$ radical that adds to the vinyl group.

Discussion

The regioselectivity 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 thiocyno,² and acetoxy⁷ 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 $\text{NO}_2^\bullet$ or $\text{CH}_3\text{OO}^\bullet$ 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 $\text{CH}_3\text{OO}^\bullet$ radical results in addition of the $\text{CH}_3^\bullet$ radical to both the δ -*meso* and 2-vinyl positions. Thus, the $\text{CH}_3\text{OO}^\bullet$ radical, although larger, adds regioselectively to the vinyl group, whereas the $\text{CH}_3^\bullet$ 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 $\text{CH}_3\text{OO}^\bullet$ 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.

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 ramosus* peroxidase with chloride and H₂O₂, 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 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 be 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 $\epsilon_{402} = 102,000 \text{ M}^{-1} \text{ cm}^{-1}$ and a molecular weight of 44,000.⁵⁹ The H₂O₂ concentration was standardized spectrophotometrically at 240 nm by using the molar extinction coefficient $\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$.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 XTera MS C₁₈ column (2.1 $\times$ 50 mm, 3.5 μm) protected with a guard column (XTera MS C₁₈, 2.1 $\times$ 10 mm, 3.5 μm). Solvent A was water containing 0.1% formic acid, and solvent B 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 NaNO₂ (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 NaNO₂ in the same buffer. The samples were incubated in the dark at room temperature for 1 h and analyzed by LCMS.

Incubation of HRP with NaNO₂ (low concentration) and H₂O₂

All solutions were prepared freshly in phosphate buffer (100 mM, pH 7). A 50 μ L aliquot of 30 μ M protein solution was mixed with 50 μ L of the 6 mM H₂O₂ (final concentration of H₂O₂ in sample was 2 mM, 200 eq) in phosphate buffer pH = 7 and 50 μ L of 30 mM of NaNO₂ (final concentration in sample was 10 mM, 1000 eq) in the same buffer. The samples were incubated in the dark at room temperature for 1 h and analyzed by LCMS.

HRP and F152M HRP apoenzyme preparation and reconstitution with substituted hemes

This work was made according to previously published metod.¹

Incubation of HRP with NaNO₂ (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 μ M. The sample was incubated for 1 h and was then analyzed by LCMS.

Incubation of HRP with peroxynitrite

Peroxynitrite was synthesized according to a known procedure.⁶¹ Briefly, a 2.5 mL aliquot of 30% H₂O₂ was added to 10 ml of ice cold water, giving a final concentration of 2 M H₂O₂. 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 (pewashed with 6 $\times$ 2 vol of water) was added and mixture was stirred for 2 h at room temperature before it was extracted with 6 $\times$ 25 ml of CH₂Cl₂. A 1.5 $\times$ 10 cm column filled with 25 g of granular MnO₂ 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 H₂O₂. To minimize the dilution of peroxynitrite, the first few ml of eluent from the column was discarded. The concentration of the stock solution of peroxynitrite was estimated to be 0.3216 M using $\epsilon_{302} = 1660 \text{ M}^{-1} \text{ cm}^{-1}$ in 0.1 M NaOH.⁶¹

All solutions were prepared freshly in phosphate buffer (100 mM, pH 5 and pH =7). A 50 μ L aliquot of a 30 μ M protein solution was mixed with 50 μ L of the appropriate buffer and 50 μ L 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 LCMS.

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 μ L of 30 μ M protein and 100 μ L of buffer pH =5 and as well as 7 μ L of NOC-9 was added. The sample was incubated in the dark at room temperature for 30 min and analyzed by LCMS.

Cloning, expression and purification of F152M HRP

The starting construct was a 6-His-tagged HRP gene subcloned into the *Bam*HI 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 co-transfection and amplification was made according previously published method with increased expression time to 90 h.⁶² The F152M mutant was expressed in yield of 7.7 mg per liter of culture.

Incubation of HRP mutants with *tert*-BuOOH and H₂O₂

All solutions were prepared freshly in phosphate buffer (100 mM, pH 8). A 50 μ L aliquot of 30 μ M protein solution was mixed with 50 μ L of the appropriate buffer and 50 μ L of 1.2 mM (40 eq) of *tert*-BuOOH or 50 μ L 0.3 mM (10 eq) of H₂O₂ in the same buffer. The samples were incubated in the dark at room temperature for 5 h (*tert*-BuOOH) or 0.5 h (H₂O₂) and analyzed by LCMS.

Labeling of modified heme with [¹⁸O]H₂O

A 4.47 μ L aliquot of 335.44 μ M F152M was dissolved in 45.53 μ L of [¹⁸O]H₂O to give 50 μ L of 30 μ M 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 μ L of [¹⁸O]H₂O. A 10 μ L aliquot of 1 M phosphate buffer was lyophilized separately and the resulting solid was dissolved in 50 μ L of [¹⁸O]H₂O. A 5 μ L aliquot of a 1.2 mM stock solution of *tert*-BuOOH was diluted to 50 μ L in [¹⁸O]H₂O. 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 μ L aliquot of the solution was analyzed by LCMS under the standard conditions given above.

Incubation of F152M with *tert*-BuOOH in the presence of a radical trap

A 50 μ L aliquot of a 30 μ M solution of HRP 152M was mixed with 50 μ L of 100 mM phosphate buffer, pH 8, containing 40 mM DMPO (5,5-dimethyl-1-pyrroline N-oxide) and 50 μ L of 1.2 mM (40 eq) *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 LCMS.

Incubation of F152M and F152M/2-cyclopropyl heme 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 pyrrogallol oxygen trap (30 g of pyrogallol in 200 mL of 50% aqueous KOH) to remove traces of oxygen. A 50 μ L aliquot of 30 μ M HRP F152M mixed with 50 μ L 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 μ L of 1.2 mM *tert*-BuOOH in buffer was transferred to F152M (or F152M/2-cyclopropyl heme) 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 LCMS.

Incubation of F152M with *tert*-BuOOH in the presence of ¹⁸O₂ 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 ¹⁸O₂ 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 ¹⁸O₂ atmosphere.

Reduction of the hydroxyl-methylperoxy heme with NaBH₄

A 100 μ L sample of 30 μ M F152M solution was mixed with 100 μ L of 100 mM phosphate buffer (pH 8) and 100 μ L of 1.2 mM (40 eq) *tert*-BuOOH in the same buffer. After 5 h of incubation the reaction was quenched by addition of 50 μ L of 40 mM DMPO (5,5-dimethyl-1-pyrroline N-oxide), concentrated to ~20 μ L using Millipore filters (10 MWCO) and washed

twice with 300 μ L of water (buffer exchange). After dilution of the sample to 2 mL, 30 μ L 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 NaBH₄ 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 μ L 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 re-dissolved in 100 μ L of methanol and 50 μ L of water. A 100 μ L aliquot was analyzed by LCMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by National Institutes of Health Grant GM32488. We thank David Maltby of the Mass Spectrometry Facility, UCSF, for the high resolution mass spectrum of heme adduct **16**.

References

1. Huang L, Wojciechowski G, Ortiz de Montellano PR. J Am Chem Soc 2005;127:5345–5353. [PubMed: 15826172]
2. Wojciechowski G, Huang L, Ortiz de Montellano PR. J Am Chem Soc 2005;127:15871–15879. [PubMed: 16277530]
3. Ator M, Ortiz de Montellano PR. J Biol Chem 1987;262:1542–1551. [PubMed: 3805041]
4. Ator M, David SK, Ortiz de Montellano PR. J Biol Chem 1987;262:14954–14960. [PubMed: 3667617]
5. Choe YS, Ortiz de Montellano PR. J Biol Chem 1991;266:8523–8530. [PubMed: 1850746]
6. Ortiz de Montellano PR, David SK, Ator MA, Tew D. Biochemistry 1988;27:5470–5476. [PubMed: 3179265]
7. Huang L, Colas C, Ortiz de Montellano PR. J Am Chem Soc 2004;126:12865–12873. [PubMed: 15469283]
8. Ortiz de Montellano PR. Biochimie 1995;77:581–593. [PubMed: 8589069]
9. Ringe D, Petsko GA, Kerr DE, Ortiz de Montellano PR. Biochemistry 1984;23:2–4. [PubMed: 6691963]
10. Raag R, Swanson BS, Poulos TL, Ortiz de Montellano PR. Biochemistry 1990;29:8119–8126. [PubMed: 2261467]
11. Chen YR, Deterding LJ, Tomer KB, Mason RP. Biochemistry 2000;39:4415–4422. [PubMed: 10757991]
12. Bondoc LL, Timkovich R. J Biol Chem 1989;264:6134–6145. [PubMed: 2703482]
13. Osawa Y, Hight RJ, Murphy CM, Cotter RJ, Pohl LR. J Am Chem Soc 1989;111:4462–4467.
14. Knowles RG, Moncada S. Biochem J 1994;298(Pt 2):249–258. [PubMed: 7510950]
15. Ischiropoulos H, Zhu L, Beckman JS. Arch Biochem Biophys 1992;298:446–451. [PubMed: 1329657]
16. Torre D, Ferrario G, Speranza F, Orani A, Fiori GP, Zeroli C. J Clin Pathol 1996;49:574–576. [PubMed: 8813957]
17. Farrell AJ, Blake DR, Palmer RM, Moncada S. Ann Rheum Dis 1992;51:1219–1222. [PubMed: 1466599]
18. Moncada S, Palmer RM, Higgs EA. Pharmacol Rev 1991;43:109–142. [PubMed: 1852778]
19. Moncada S, Higgs EA. Eur J Clin Invest 1991;21:361–374. [PubMed: 1718757]
20. Martin H, Huisman TH. Nature 1963;200:898–899. [PubMed: 14096079]
21. Doyle MP, Herman JG, Dykstra RL. J Free Radic Biol Med 1985;1:145–153. [PubMed: 3836241]
22. Huie RE. Toxicology 1994;89:193–216. [PubMed: 8023329]

23. Burner U, Furtmuller PG, Kettle AJ, Koppenol WH, Obinger C. *J Biol Chem* 2000;275:20597–20601. [PubMed: 10777476]
24. Sampson JB, Ye Y, Rosen H, Beckman JS. *Arch Biochem Biophys* 1998;356:207–213. [PubMed: 9705211]
25. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A. *Nature* 1998;391:393–407. [PubMed: 9450756]
26. van der Vliet A, Eiserich JP, Halliwell B, Cross CE. *J Biol Chem* 1997;272:7617–7625. [PubMed: 9065416]
27. Monzani E, Roncone R, Galliano M, Koppenol WH, Casella L. *Eur J Biochem* 2004;271:895–906. [PubMed: 15009202]
28. Sala A, Nicolis S, Roncone R, Casella L, Monzani E. *Eur J Biochem* 2004;271:2841–2852. [PubMed: 15206949]
29. Casella L, Monzani E, Roncone R, Nicolis S, Sala A, De Riso A. *Environ Health Perspect* 2002;110 (Suppl 5):709–711. [PubMed: 12426117]
30. Bruck TB, Fielding RJ, Symons MC, Harvey PJ. *Eur J Biochem* 2001;268:3214–3222. [PubMed: 11389723]
31. MacPherson JC, Comhair SA, Erzurum SC, Klein DF, Lipscomb MF, Kavuru MS, Samoszuk MK, Hazen SL. *J Immunol* 2001;166:5763–5772. [PubMed: 11313420]
32. van Dalen CJ, Winterbourn CC, Kettle AJ. *Biochem J* 2006;394:707–713. [PubMed: 16336215]
33. Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusi AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL. *J Biol Chem* 2002;277:17415–17427. [PubMed: 11877405]
34. Gebicka L. *Acta Biochim Pol* 1999;46:919–927. [PubMed: 10824860]
35. Tahon JP, Van Hoof D, Vinckier C, Witters R, De Ley M, Lontie R. *Biochem J* 1988;249:891–896. [PubMed: 2833232]
36. de Saint-Blanquat G. *Ann Nutr Aliment* 1980;34:827–864. [PubMed: 7020546]
37. Shintaku M, Matsuura K, Yoshioka S, Takahashi S, Ishimori K, Morishima I. *J Biol Chem* 2005;280:40934–40938. [PubMed: 16221678]
38. Davies MJ. *Biochim Biophys Acta* 1988;964:28–35. [PubMed: 2825818]
39. Lee CHLJ, Yen CW, Shih PC, Lin TS, Mou CY. *J Phys Chem B* 2005;109:12277–12286. [PubMed: 16852515]
40. Blanksby SJ, Ellison GB. *Acc Chem Res* 2003;36:255–263. [PubMed: 12693923]
41. Blanksby SJ, Ramond TM, Davico GE, Nimlos MR, Kato S, Bierbaum VM, Lineberger WC, Ellison GB, Okumura M. *J Am Chem Soc* 2001;123:9585–96. [PubMed: 11572679]
42. Ator MA, David SK, Ortiz de Montellano PR. *J Biol Chem* 1989;264:9250–9257. [PubMed: 2722829]
43. Porter DJ, Bright HJ. *J Biol Chem* 1983;258:9913–9924. [PubMed: 6885775]
44. Ortiz de Montellano PR. *Accts Chem Res* 1987;20:289–294.
45. Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL. *Nat Struct Biol* 1997;4:1032–1038. [PubMed: 9406554]
46. Ervin KM, De Turi VF. *J Phys Chem A* 2002;106:9947–9956.
47. Chen YR, Deterding LJ, Tomer KB, Mason RP. *Biochemistry* 2000;39:4415–4422. [PubMed: 10757991]
48. Ruscic B, Litorja M, Asher RL. *J Phys Chem A* 1999;103:8625–8633.
49. Berkowitz J, Ellison GB, Gutman D. *J Phys Chem* 1994;98:2744–2765.
50. Seakins PW, Pilling MJ, Niiranen JT, Gutman D, Krasnoperov LN. *J Phys Chem* 1992;96:9847–9855.
51. Bradforth SE, Kim EH, Arnold DW, Neumark DM. *J Chem Phys* 1993;98:800–810.
52. Pellerite MJ, Jackson RL, Brauman JI. *J Phys Chem* 1981;85:1624–1626.
53. Gurvich, LV.; Veyts, IV.; Alcock, CB.; Iorish, VS. *Thermodynamic Properties of Individual Substances*. 4. 2. Hemisphere; New York: 1991.
54. Colas C, Ortiz de Montellano PR. *Chem Rev* 2003;103:2305–2332. [PubMed: 12797831]

55. Auclair, K.; Ortiz de Montellano, PR. Porphyrin Handbook. Kadish, K.; Smith, K.; Guillard, R., editors. 12. Academic Press; New York: 2002. p. 183-210.
56. Wilks A, Torpey J, Ortiz de Montellano PR. J Biol Chem 1994;269:29553–29556. [PubMed: 7961940]
57. Kumar D, de Visser SP, Shaik S. J Am Chem Soc 2005;127:8204–8213. [PubMed: 15926850]
58. Kozyrev ANAJL, Robinson BC. Tetrahedron 2003;59:499–504.
59. Ohlsson PI, Paul KG. Biochim Biophys Acta 1973;315:293–305.
60. Hildebrandt AGRI, Tjoe M, Heinemeyer G. Methods Enzymol 1978;52:342–350. [PubMed: 672638]
61. Uppu RM, Pryor WA. Anal Biochem 1996;236:242–249.
62. Colas C, Ortiz de Montellano PR. J Biol Chem 2004;279:24131–24140. [PubMed: 15039425]

Figure 1.

LCMS 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; d_4 - *meso* heme 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.

Figure 2.

A) pH profile of the yield of modified heme after a 1 h incubation: (●) HRP with 0.8 M NaNO₂ without H₂O₂; (▲) HRP with 10 mM NaNO₂ and 2 mM H₂O₂; B) time course of the heme modification reaction (●) HRP with 0.8 M NaNO₂ without H₂O₂ at pH 5; (▲) HRP with 10 mM NaNO₂ and 2 mM H₂O₂ at pH = 7.

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

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

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

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 eq *tert*-BuOOH at pH 8 (red). Heme is **7** and modified heme is **16**.

Figure 8.

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

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

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

Figure 11.

LCMS 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 eq of *tert*-BuOOH, pH 8, for 5 h.

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

Figure 14.

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

Figure 15.

Generation of $\text{CH}_3^\bullet$ and $\text{CH}_3\text{OO}^\bullet$ radicals in the reaction of *tert*-BuOOH with F152M HRP. Ferric heme is abbreviated as $\text{Por}(\text{Fe}^{\text{III}})$, where Por stands for porphyrin. Equation (6) shows a second route to the *tert*-BuO $^\bullet$ than that provided by equations (1)-(3).

Figure 16.

LCMS spectra of modified heme **24** labeled with $^{18}\text{O}_2$ and the heme diol **25** obtained by reduction of the methylperoxy function.

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.

Table 1
Mass spectrometric data for the modified heme products

Compound	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

^aThe molecular ion is accompanied by a strong ion 32 mass unit higher due to the methanol-complexed iron porphyrin.

^bThe molecular mass of NO₂ is 46.

^cThe mass of 2-cyclopropyl heme and 4-cyclopropyl hemes is 630.

^dThe mass of d4-mesoheme is 624.

Table 2

Compound	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

^aThe molecular ion is accompanied by a strong ion 32 mass unit 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.

^cThe mass of both 2- and 4-cyclopropyl hemes is 630,

d The mass of mesoheme is 620.

^eThe mass of ^{18}OH is 19.

^fThe mass of $^{18}\text{O}^{18}\text{OCH}_3$ is 51.

Table 3

Molecular bond dissociation energies for $RH \rightarrow R^\bullet + H^\bullet$ at 298 K. The heme position in HRP that is substituted by the corresponding radical is indicated.

	DH_{298} (kcal/mol)	position of addition	Reference to DH_{298}	Reference to position of addition
HCN	126.3 ± 0.2	δ -meso	46	47
PhH	112.9 ± 0.5	δ -meso	46	3
CH ₃ COOH	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 ₃ CH 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 ₃ OOH	88 ± 1	vinyl	41	This work
HBr	87.54 ± 0.05	vinyl	49	1
tert-BuOOH	84 ± 2	none	41	*
HNO ₂	79.1 ± 0.2	vinyl	53	This work

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