Scientific paper

Shielding of Peroxidase Heme Vinyl Groups from Autocatalytically Generated Electrophilic Metabolites

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Abstract

The heme vinyl substituents of horseradish peroxidase (HRP) are converted to halohydrin and vinyl halide substituents by the HOX generated on halide (X^-) oxidation. To determine the role of active site residues in shielding the vinyl groups, we have investigated the L37M, F41M, S151M, and F152M mutants. A methionine was employed to simultaneously test for possible crosslinking to the vinyl group, but crosslinking was not observed. Kinetic analysis reveals that the F41M mutant is particularly altered, its compound I forming more slowly but decaying more rapidly than that of native HRP. The F41M mutant also has the highest halide oxidation activity. Modification of the heme vinyl groups of all the mutants by catalytically-generated HOBr and HOCl gives epoxides in addition to the previously observed halohydrins and vinyl halides. Individual replacement of residues near the vinyl groups with a methionine increases the formation of bromohydrins at the expense of vinyl halides. The results indicate that the normal active site residues partially protect the vinyl groups and restrict water access. The lower polarity of the heme cavity in HRP than myeloperoxidase is consistent with the formation of heme vinyl halides and may help explain the absence of heme vinyl-methionine crosslinking.

Keywords: Horseradish peroxidase, myeloperoxidase, heme modification, hypobromide, hypochloride, heme crosslinking

1. Introduction

In their normal function, the heme peroxidases react with H_2O_2 to generate compound I, in which a ferryl species (Fe^{IV}=O) is coupled to either a porphyrin or a protein radical cation.[1] In conventional catalytic turnover, an electron from the substrate reduces this intermediate to compound II, which only retains the ferryl species. Finally, the ferryl of compound II is reduced by a second substrate-derived electron to the resting ferric state. Under high peroxide conditions, compound III, which corresponds to a ferrous dioxygen complex (Fe^{II}–O_2), can be formed. However, in addition to their ability to catalyze one electron oxidations, the peroxidases have a variable ability to catalyze the two electron oxidation of halide and pseudohalide ions.[2–6] In this activity, it is thought that compound I directly transfers the ferryl oxygen to the halide, producing HOCl from chloride, HOBr from bromide, and HOSCN from thiocyanide.

The oxidation of halides is not a relevant biological function for most plant and fungal peroxidases, but it is a critical physiological function of the mammalian peroxidases.[1,2] The most powerful of these enzymes is myeloperoxidase (MPO), which readily oxidizes chloride ions to the antimicrobial agent HOCl. The analogous oxidation of bromide, iodide, and thiocyanide to HOBr, HOI, and HOSCN, respectively, is similarly a key function of eosinophil peroxidase, lactoperoxidase (LPO), and thyroid peroxidase. Interestingly, a unique feature of all of these mammalian peroxidases is the presence of two, or in the case of MPO three, covalent bonds between the prosthetic heme group and the protein.[7–9] No such covalent bonds are found in any of the known plant and fungal peroxidases. Two of these covalent bonds involving

ester links between heme methyl groups and aspartate or glutamate carboxylic acid groups are shared by all the mammalian peroxidases. However, the third bond in which a methionine sulfur atom adds to a heme vinyl to produce a vinyl sulfonium link, is only present in MPO and is related to its singular ability to oxidize chloride ions.[7] A valine is located at the corresponding position in the sequence of thyroid peroxidase,[10] a threonine in eosinophil peroxidase,[11] and a glutamine in bovine lactoperoxidase.[12]

We have recently demonstrated that native horseradish peroxidase (HRP), under acidic conditions, is able to oxidize not only bromide to HOBr but also chloride to HOCl.[13–15] Furthermore, these hypohalides (HOX), which can be quantitated in solution by reaction with monochlorodimedone, react with the prosthetic heme vinyl groups, converting them into halohydrin [–CH(OH)CH_2X] and vinyl halide (–CH=CHX) substituents. A third reaction that occurrs on oxidation of chloride, but not bromide, is substitution of the halide atom for a *meso*-hydrogen of the heme.[13,16] Similar reactions occur when exogenous HOCl or HOSCN is added to HRP and other peroxidases.[14,16] These modifications of the prosthetic heme group attenuate or inactivate the peroxidase activity of the enzyme.

In parallel studies, we have introduced a carboxylic acid residue into the active site of HRP by mutagenesis and have shown that the resulting F41E mutant, when exposed to H_2O_2, autocatalytically forms a covalent bond between the 3-methyl and the carboxyl group of Glu41.[17] Related studies with lactoperoxidase have established that the covalent links are also autocatalytically formed in this mammalian enzyme.[18] Most interestingly, formation of the covalent link in F41E HRP virtually suppresses reaction of the heme vinyl groups with the HOCl and HOBr generated in the oxidation of chloride and bromide, respectively.[19] These results provide strong support for the conclusion that one function of the covalent ester bonds between the heme methyl groups and acidic protein residues in the mammalian peroxidases is to protect the heme vinyl groups.

It is likely that the third covalent link in MPO, the sulfonium link between a heme vinyl and a methionine sulfur atom, is also formed autocatalytically. Support for this inference is provided by the demonstration using ascorbate peroxidase that replacement of Ser160 by a methionine leads to formation of a heme vinyl-methionine crosslink, although the structure of the crosslink differs from that in MPO by the presence of an additional hydroxyl on the sidechain.[20]

In the present study, we have mutated several active site residues in HRP to explore their role in modulating the reactivity of the vinyl groups with hypohalides. The four residues we have mutated, Leu37, Phe41, Ser151, and Phe152 (Figure 1), were selected because of their proximity to one or the other of the heme vinyl groups in the HRP crystal structure (pdb: 1HRP). The residues were mutated to methionines for two reasons: (a) methionine is a smaller residue with a non-polar sidechain and is thus useful as a probe for steric effects, and (b) it can be used to simultaneously test for possible crosslinking of the methionine to a vinyl group, as in MPO. Native HRP already contains two methionine residues near the 4-vinyl group: Met284, with the sulfur 5.38 Å from the β carbon of the vinyl group, and Met281, with the sulfur 4.58 Å from the β carbon (Figure 1). These distances were estimated using Swiss-PDB viewer v.3.7. The distances between the appropriate vinyl β carbon and the methionine sulfur atom in the mutants, assuming the HRP crystal structure is not altered by mutagenesis, were estimated to be: L37M, 4.38 Å; F41M, 2.13 Å; S151M, 5.8 Å; and F152M, 5.44 Å.

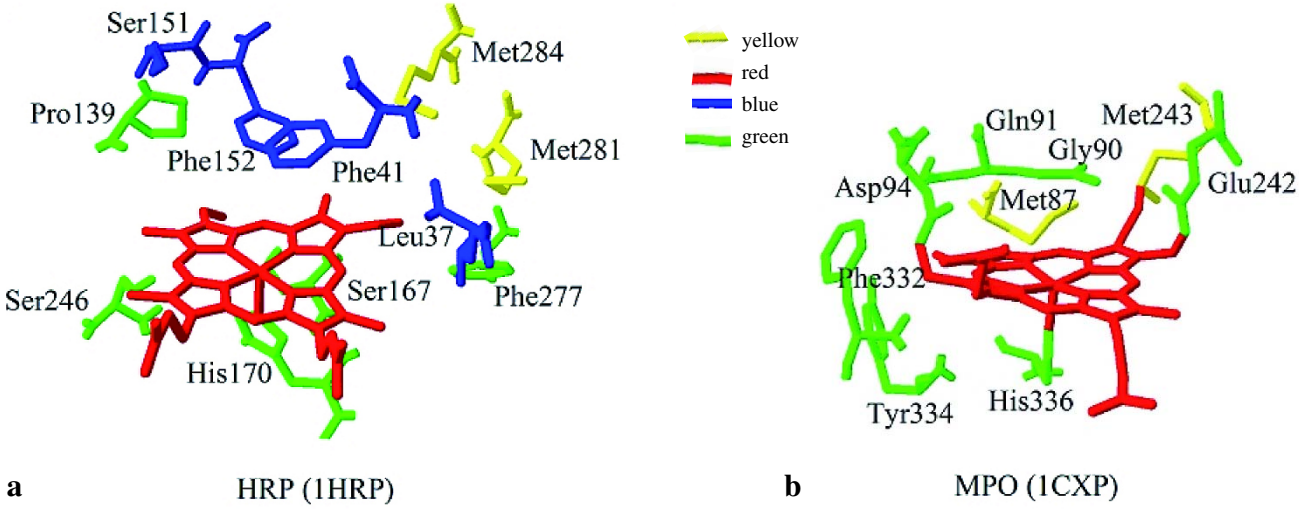


Figure 1: The structures of the HRP and MPO active sites near the heme vinyl groups. Methionine residues already present in the native proteins are colored yellow, residues that have been mutated to methionines are in blue. Other important residues are in green.

2. Materials and Methods

2. 1. Materials

Restriction enzymes were purchased from New England Biolabs. All other chemicals, including buffer components and HPLC solvents, were from Fisher Scientific. Buffer was prepared from citric acid titrated to pH 4 with 1 M KOH. Water was double distilled prior to use. Native HRP was purchased from Roche. High-Five™ cells were grown in supplemented Express Five SFM™ medium, both purchased from Invitrogen. The Sf9 cells (Invitrogen) were grown in Excell 420™ medium purchased from JRH Biosciences (Lenexa, KS). Penicillin/streptomycin and Fungizone were purchased from the UCSF Cell Culture Facility. Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using λ_{402} = 102,000 M^{-1} cm^{-1} and a molecular weight of 44,000.[21] The H_2O_2 concentration was standardized spectrophotometrically at 240 nm by using the molar extinction coefficient ε = 43.6 $M^{-1}cm^{-1}$.[22] 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 C_{18} column (2.1 × 50 mm, 3.5 μm). Modeling of the mutants on the basis of the native HRP crystal structure was carried out with Swiss-PDB Viewer 3.7.

2. 2. Cloning and Expression

The starting construct was the previously prepared 6-His-tag HRP subcloned into the *BamH*I site of pUC19.[17] Primers were designed for the mutagenesis with the nucleotide substitutions in the center of the sequences: L37M, GG ATC GCT GCT TCA ATA ATG CGT CTG CAC TTC CAT GAC; F41M, ATA TTA CGT CTG CAC ATG CAT GAC TGC TTC GTG; S151M, CCC CAG CTG AAG GAT ATG TTT AGA AAC GTG GGT; F152M, CAG CTG AAG GAT AGC ATG AGA AAC GTG GGT CTG; S167M, GAC CTT GTG GCT CTG ATG GGA GGA CAC ACA TTT. Mutagenesis was undertaken with the QuikChange™ kit (Stratagene, La Jolla, CA). After transformation in DH-5α cells, DNA was isolated from positive clones with the Promega Wizard Plus SV Miniprep™ kit and the mutated genes, isolated from their pUC19 plasmids through a *BamH*I digestion, were purified from agarose gels with the QiaEX II™ kit (Qiagen, Valencia, CA). Ligation was accomplished at 4 °C using a Quick ligation Kit (New England Biolabs) into a pAcGP67B plasmid (Pharmingen, BD Biosciences) preliminarily digested by *BamH*I, dephosphorylated, and purified with the Promega Wizard DNA clean-up™ kit. The plasmids were transformed into DH5α™ cells and their sequences were verified. Transfection in Sf9 cells was done with the Baculogold transfection kit (Pharmingen, BD Biosciences). A plaque assay on the transfection supernatants allowed for the isolation of recombinant virus that was subsequently amplified to a titer of 1 × 10^8 plaque-forming units/ml using standard baculovirus protocols.

2. 3. Expression and Purification

High-FiveTM cells at a density of 2.2 × 10^6 cells/ml were supplemented with hemin (final concentration 6 μM), 1 x penicillin/streptomycin, and 1 x Fungizone before being infected at a multiplicity of infection of 10. At 90 h after infection, the cultures were harvested and centrifuged at 4 °C and 10,000 *g* for 15 min. The resulting supernatant was then filtered successively through 1.2 μm, 0.64 μm, and 0.44 μm regenerated cellulose filters to remove the remaining cell debris. The clarified liquid was then concentrated to 100 ml by ultrafiltration over a spiral-wound regenerated cellulose membrane (molecular mass cut-off 10 kDa, Millipore) connected to an Amicon TCF10 peristaltic pump equipped with a 2l RA2000S reservoir. The resulting solution was then made 1.5 M with solid NaCl and 20 mM with KH_2PO_4/K_2HPO_4 at pH 8, and ultracentrifuged for 30 min at 4 °C and 100,000 *g*. The supernatant was loaded onto a Ni^{2+} column (nickel nitrilotriacetic acid-agarose, Qiagen) pre-equilibrated with 20 mM KH_2PO_4/K_2HPO_4, pH 8, containing 1.5 M NaCl. The resin was then washed with 10 volumes of this buffer, then with 5 volumes of the same buffer containing 5 mM imidazole. Elution was achieved by increasing the imidazole concentration to 100 mM at pH 8. The colored fractions were pooled and dialyzed against 20 mM KH_2PO_4/K_2HPO_4, pH 8 buffer for at least 36 h with at least three bath changes. The dialyzed fractions were then concentrated to 3 ml by ultrafiltration over an Amicon YM10 membrane, and passed through a pre-equilibrated QFF-Sepharose (Amersham Biosciences) column. The colored fractions were concentrated again to 1–2 ml over an Amicon Ultra-4TM centrifugal filter device (10 kDa). The concentrations of expressed proteins were determined using the hemochromogen method. The L37M, F41M, S151M, and F152M mutants were expressed at levels that ranged from 4.6 to 13.5 mg/L (Table 1), whereas the S167M mutant was not expressed at all even with different MOI values and incubation times.

Table 1: Yields of expressed proteins and their RZ values, Soret maxima, and Soret absorbance coefficients **ε**.

Enzyme	RZ	yield mg/L	soret (ε) nm (mM^{-1} cm^{-1})
HRP	3.0	–	402 (102)
L37M	2.4	13.5	404 (84.8)
F41M	3.4	13.5	406 (129)
S151M	2.8	4.6	402 (88.9)
F152M	2.7	7.7	402 (88.2)

2. 4. Analysis of Possible Covalent Heme Binding of HRP Mutants with H_2O_2 and Bromide

All the solutions were prepared in phosphate buffer (0.05 M, pH 8). A 50 µl aliquot of a 30 µM protein solution was mixed with 50 µl of phosphate buffer containing 1.2 M KBr and 50 µl of 300 µM H_2O_2 (10 eq). Product separation was performed by HPLC on a Vydac C-4 column (4.6 × 50 mm, 3.5 µm), where solvent A was water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.085% formic acid. The gradient program consisted of linear segments with 33% B (0–3 min), from 33 to 65% B (3–60 min), 95% B (60.1–65 min), and finally 33% B (65.1–80 min), all at a flow rate of 1 ml/min.

2. 5. HRP Heme Adducts

All the solutions were prepared in citrate buffer (0.5 M, pH 5). A 50 µl aliquot of a 20 µM protein solution was mixed with 50 µl of citrate buffer (containing either 1.2 M NaCl or KBr) and a total of 50 µl of 200 µM H_2O_2 was added in five equal parts separated by 5 min time intervals. Product separation was performed using LC-MS (2.1 × 50 mm, 3.5 µm). Solvent A was water containing 0.5% formic acid, and solvent B methanol containing 0.5% formic acid. The gradient program consisted of linear segments with 40% B (0–2 min), from 40 to 70% B (2–17 min), from 70 to 95% B (17–22 min), 95% B (22.1–25 min), and finally 40% B (25.1–28 min), all at a flow rate of 0.1 ml/min. The eluent was monitored at 280 and 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.

2. 6. Stopped-Flow Kinetic Studies

Stopped flow UV-visible spectrophotometric experiments were performed on a Hi-Tech SF-61 DX2 double mixing stopped-flow system employing a diode array spectrophotometer and were carried out at 25 °C in 20 mM phosphate buffer (pH 8). The initial (pre-mixing) concentrations were as follows: HRP, L37M, F41M, S151M, or F152M, 15 µM; H_2O_2 15 µM, 150 µM and 400 µM. Data were collected (300 scans) over 0.6, 6, 60, and 600 s using the KinetAsyst software package (Hi-Tech) and analyzed as pseudo-first order reactions using the Specfit Global Analysis System software package (Spectrum Software Associates).

2. 7. Chloride and Bromide Oxidation Assay

The assay was performed in 50 mM citrate buffer, pH 5. The amounts of compounds and their concentrations were as follow: monochlorodimedone 2.8 µl (1 mM), KBr or NaCl 3.5 µl (2 M), enzyme 3.5 µl (5 µM except F41M which was 0.5 µM), H_2O_2 in phosphate buffer 60.2 µl (0.5 mM H_2O_2). The decrease of the monochlorodimedone band intensity was monitored at 292 nm. The initial rate (V_0) was calculated and expressed as moles of monochlorodimedone (ε = 20000) oxidized per min per mole of HRP.

3. Results

3. 1. Spectroscopic Properties of the HRP Mutants

The Soret absorbance maxima of the S151M and F152M mutants are the same as that of native HRP, but the F41M absorbance maximum is red-shifted by 4 nm and that of the L37M mutant by 2 nm (Table 1). The F41M mutation, which also increases the Soret absorption coefficient by approximately 20% relative to that of native HRP, is thus the only one that appears to significantly alter the active site environment. The increase in the absorbance value suggests that the F41M mutant is hexacoordinated.[23] Furthermore, the UV-vis spectrum of the F41M mutant does not have the shoulder on the Soret band at 406 nm that is typical of five coordinated hemes (not shown).[23] Previous resonance Raman studies have shown that mutations of Phe41 give rise to increased hexacoordinated high spin species.[24] From these results, it appears likely that the iron in the F41M mutant is hexacoordinated, possibly with a water as a distal ligand. The other five mutants, like native HRP, appear to be pentacoordinated.

3. 2. Kinetic Constants for the HRP Mutants

Stopped-flow experiments (Table 2) reveal that the formation of compound I (k_1) is at least 10-fold slower for all the HRP mutants than for native HRP, with the F41M mutant being the slowest. In contrast, the rates for reduction of compound I to compound II (k_2) are comparable for the

Table 2: Rate constants for formation of compound I (k_1), compound II (k_2), resting enzyme (k_3), and compound III (k_4).

Enzyme	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})
HRP	$93.4e^6 \pm 1.57\ e^5$	$1.4e^3 \pm 6.73$	$3.37e^2 \pm 2.14$	$1.48e^5 \pm 1.4e^3$
L37M	$5.35e^6 \pm 1.7e^5$	$1.83e^3 \pm 11.9$	$4.03e^2 \pm 2.84$	$1.16e^5 \pm 1.8e^3$
F41M	$0.77e^6 \pm 0.05e3$	$864e^3 \pm 7490$	$110e^2 \pm 467$	$2.34e^5 \pm 3.8e^3$
S151M	$5.31e^6 \pm 0.96e^5$	$2.82e^3 \pm 26.4$	$3.41e^2 \pm 2.77$	$1.63e^5 \pm 1.66e^3$
F152M	$4.76e^6 \pm 1.46e^5$	$1.99e^3 \pm 14.6$	$3.37e^2 \pm 2.29$	$1.47e^5 \pm 1.76e^3$

mutants and native HRP with the exception of the F41M mutant, for which k_2 is more than 300 times faster. The rate of reduction of compound II to the resting ferric state (k_3) exhibits a similar pattern, in that the rates of the mutants and native HRP are comparable with the single exception of the F41M mutant, for which k_3 is approximately 25 times faster. Finally, the rates of formation of compound III (k_4), which only occurs at high H_2O_2 concentrations, are similar without exception for the mutants and the native enzyme.

3. 3. Chloride and Bromide Oxidation

We recently reported that HRP, contrary to earlier reports, is able to oxidize chloride ions, albeit at acidic pH values.[13] In general, the HRP mutants studied here, as judged by the monochlorodimedone assay, oxidize chloride and bromide ions at rates comparable to the native enzyme (Table 3). The salient exception is again the F41M mutant, which oxidizes chloride ion approximately 8-times faster and bromide ion 6-times faster than native HRP. Minor differences are also seen with the L37M mutant, which has a slightly higher bromide oxidizing activity, and the F152M mutant, which oxidizes chloride somewhat more slowly, than native HRP.

Table 3: The chloride and bromide ion oxidizing activity of HRP and its L37M, F41M, S151M and F152M mutants.

Enzyme	chloride (mole min^{-1} mole^{-1})	bromide (mole min^{-1} mole^{-1})
HRP	2.86 ± 0.04	78.52 ± 0.12
L37M	2.34 ± 0.08	117.08 ± 0.5
F41M	23.9 ± 0.42	498 ± 0.35
S151M	1.54 ± 0.02	69.72 ± 0.08
F152M	0.6 ± 0.08	60.07 ± 0.04

3. 4. Heme-Methionine Crosslinking

We previously established that HPLC on a C-4 column, which readily separates the HRP and lactoperoxidase apoenzymes from non-covalently bound heme, is a convenient method for detecting covalent heme-protein links.[17] In proteins in which an ester bond covalently links the heme and the protein, both components co-elute, as revealed by the coincidence of peaks with absorption maxima at 278 nm (protein) and 400 nm (heme). We therefore examined the possible formation of covalent links between the heme and the protein in incubations of HRP and its methionine mutants with H_2O_2.

HPLC analysis of native HRP before and after incubation with H_2O_2, as previously shown, results in complete separation of the heme and the protein. The same is observed for all of the HRP mutants investigated in this study (not shown). In all cases we observe intact heme at 9.2 min and a broad band of the apoenzyme at about 17.5 min. Clearly, in the case of the HRP mutants, a sulfonium ion link to the heme is not formed even though the nominal distance between the β carbon of the vinyl group and the methionine sulfur is not prohibitive of bond formation.

3. 5. Heme Modifications in the Oxidation of Bromide by HRP Mutants

We recently demonstrated that HRP oxidizes bromide to HOBr and showed that this metabolite modifies the prosthetic heme group by reacting with the heme vinyl groups.[13] These modifications of the heme result in deactivation of the enzyme.[13] In the case of native HRP, the number of modified heme products and their structures depend on the identity of the halide ion that is oxidized and the concentration of H_2O_2. We have therefore compared the patterns of modified heme products obtained with native HRP and with the four methionine mutants.

The oxidation of bromide by HRP and its four mutants produced five modified heme products (Figures 2 and 3, Table 4). The mass spectrometric data for the heme products is provided in Table S1, Fig. S1. Under the incubation conditions employed, the major products

Table 4: Heme modified products in relative percent after incubation of HRP and its mutants with H_2O_2 and Br^-.

Enzyme	1	2	3	4	5	6	other
			(%)				
HRP	17	7	7	44	20	–	5
L37M	37	19	26	10	–	–	8
F41M	84	2	6.7	4	–	–	3.5
S151M	45.1	4.5	32.6	2	3	12	0.8
F152M	3.6	5.6	75	–	11.7	4.1	–

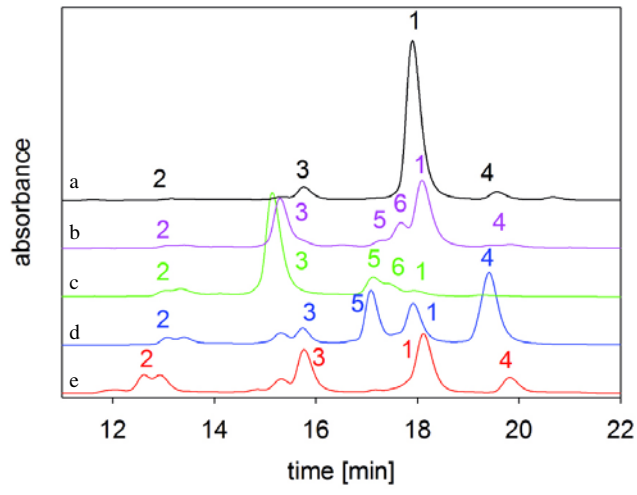


Figure 2: HPLC chromatograms of products from co-incubations of Br^- and H_2O_2 with native HRP (d) and its L37M (e), F152M (c), S151M (b), and F41M (a) mutants.

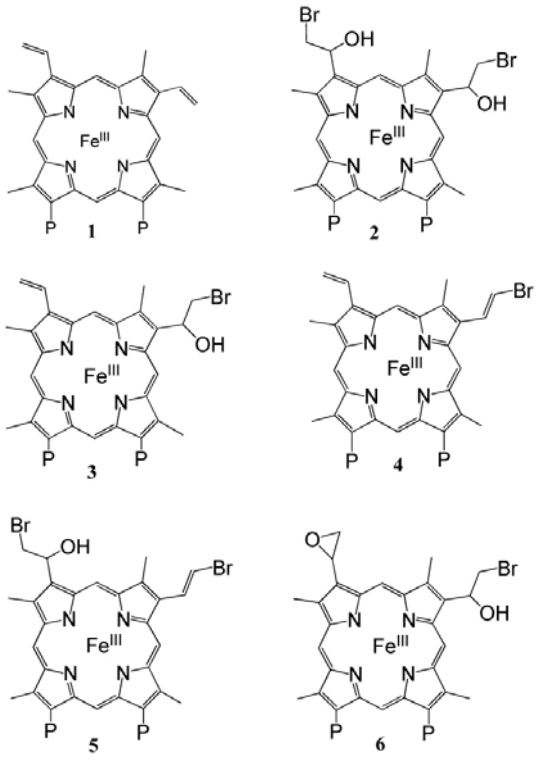


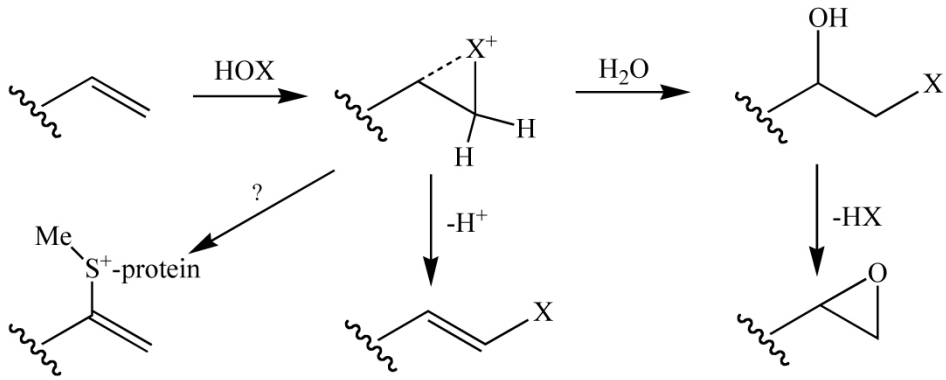
Figure 3: Structures of the products obtained in the reaction of HRP with bromide and H_2O_2.

with native HRP are unreacted heme **1** (17%), vinyl bromide **4** (44%), and the bromohydrin vinyl bromide **5** (20%). Interestingly, the formation of **5** is virtually suppressed in all the HRP mutants except for the F152M mutant, where it accounts for 12% of the heme products. The formation of the doubly modified heme with one vinyl as a bromohydrin and the other as an epoxide (**6**) is also influenced by the active site mutations, as it is only detected with the S151M (12%) and F152M (4%) mutants. The structure of this product is based on its mass spectrum and is secure except for the assignment of which former vinyl group is the epoxide and which the bromohydrin. As judged from the amounts of unmodified heme that are recovered, the heme is more resistant to modification in the L37M (37%), F41M (84%), and S151M (45%) mutants than it is in native HRP (17%) (Table 4). Monobromohydrin **3** is the dominant species in the F152M mutant (75%), but it is formed to some extent in all the proteins. Again, although the bromohydrin is shown at position 4 in this structure, it is not clear which of the two vinyl groups is actually converted to the bromohydrin. Overall, the data shows that the indicated active site residues contribute to protection of the heme vinyls from reaction with autocatalytically generated HOBr and influence whether the vinyl modification produces a bromohydrin, a vinyl bromide, or an epoxide substituent.

3. 6. Heme Modifications in the Oxidation of Chloride by HRP Mutants

As already noted, we have demonstrated that HRP oxidizes not only bromide but also chloride to the corresponding hypohalide.[13] We have furthermore established that the prosthetic heme vinyl groups are modified by reaction with catalytically generated (or exogenously added) HOCl.[13] The structures of unmodified **1** and the vinyl-modified products **11**, **12**, and **14** (Figure 4) were established in our earlier work and are assigned here by comparison with the earlier results. In our earlier work, reconstitution of HRP with 2- and 4-cyclopropylheme was used to define the regiochemistry of the heme vinyl modifications. Thus, in 2-cyclopropylheme-reconstituted HRP, the carbocation formed by addition of a halonium ion to the 4-vinyl group is primarily trapped by water to give a halohydrin (Scheme 1). In contrast, in 4-cyclopropylheme-reconstituted HRP, halonium ion addition is preferentially followed by proton elimination to give a 2-halovinyl group. Indeed, vinyl halide formation appeared to only occur in reactions of the 4-vinyl. We therefore tentatively attribute the 4-position as the site of the vinyl halide in compound **15** (Figure 4). The only other ambiguity among the related new structures is compound **13**, which has a *meso*-chloro substituent and one vinyl as a chlorohydrin. Again, based on the structure of **12**, it is likely that the chlorohydrin, as shown, is on the 4-vinyl. The epoxide-containing structures (i.e., **6**, **7**, **8**, **9**) were not previously characterized and the structures attributed here rest on their absorption and mass spectra and the vinyl substitution patterns observed with the structurally established products. We are not able to assign which of the two vinyl groups is involved in epoxide formation in compounds **6**, **7**, and **8**, but there is no ambiguity in the structure of diepoxide **3**. The relevant mass spectrometric data is provided in Table S2, Fig. S1).

A key difference between bromide and chloride is that the oxidation of chloride (but not bromide) produces not only the hypohalide, but also a radical-like species that adds to a *meso*-carbon of the heme.[13,16] As we have reported,[13,16] after reconstitution of both HRP and *Arthromyces rhamosus* peroxidase with mesoheme-d4 a single modified heme product with a molecular ion at m/z = [624 (mesoheme d4) + 35 (chloride) – 2 (deuterium)] is obtained. The loss of two mass units on addition of the chloride, together with the absence of vinyl groups in mesoheme, confirms the *meso*-addition. The demonstration that monochlorodimedone prevents vinyl group but not *meso*-chlorination shows that a different chlorinating agent is involved in the *meso*-reaction.[16] We have postulated that this alternative species is a chloride radical. The literature on the *meso*-addition of radicals to the heme in HRP indicates that the reactions occur at the δ-*meso*-carbon.[25–29] These findings allow us to assign structure **10** to the compound eluting at ~18.3 min in Figure 5. This unique chloride-related reaction, and the fact that more than one mo-



Scheme 1: The reaction manifold that produces halohydrins, vinyl halides, epoxides, and potentially heme-vinyl sulfonium crosslinks in reactions of the heme vinyl groups with hypohalides (HOX).

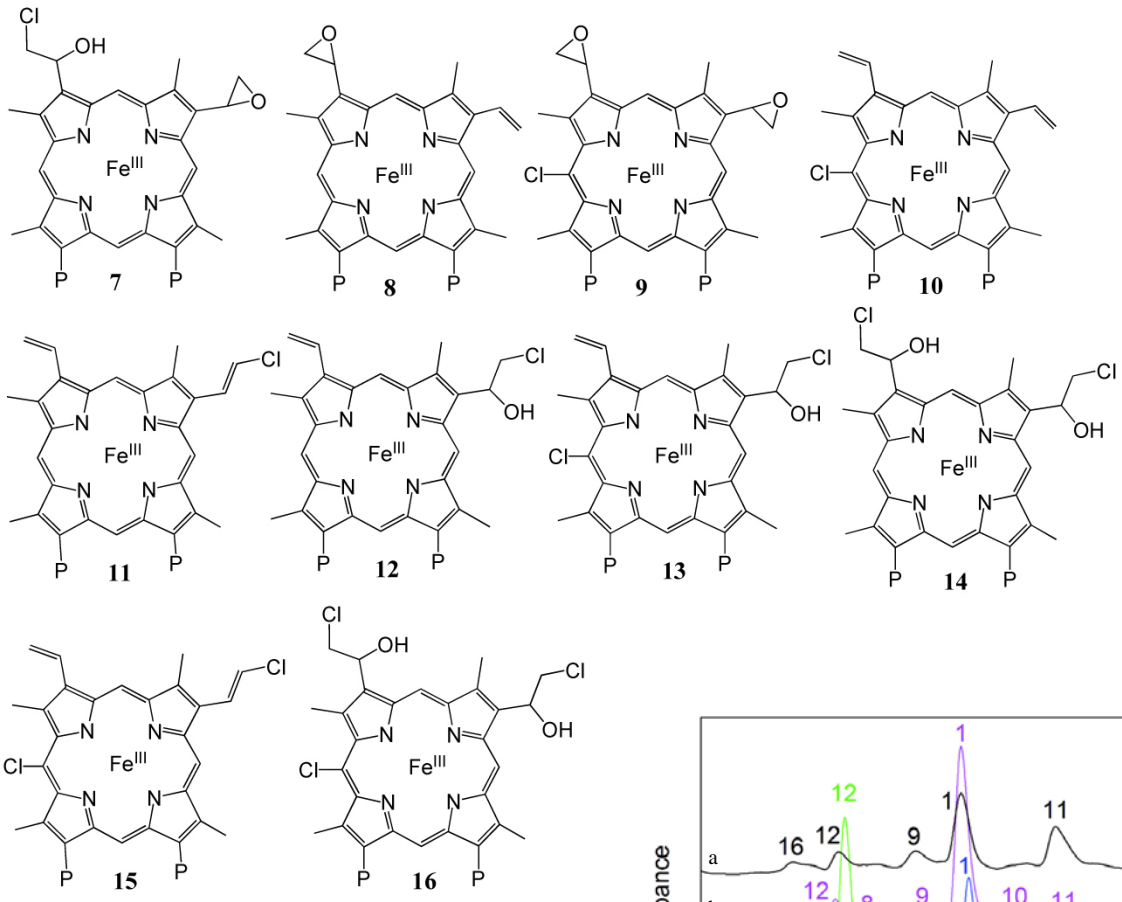


Figure 4: Structures of the products obtained in the reaction of HRP with chloride and H_2O_2.

dification can occur on a given heme group, means that a larger number of heme products is generated in chloride than bromide oxidation. Thus, chloride substitution at the *meso*-position helps to rationalize the structures of the products **13**, **15**, and **16**. Interestingly, using different gradients in the HPLC system and varying the concentration of H_2O_2 has allowed us to detect an additional product (**9**) with m/z = 682. Based on its molecular mass, we identify

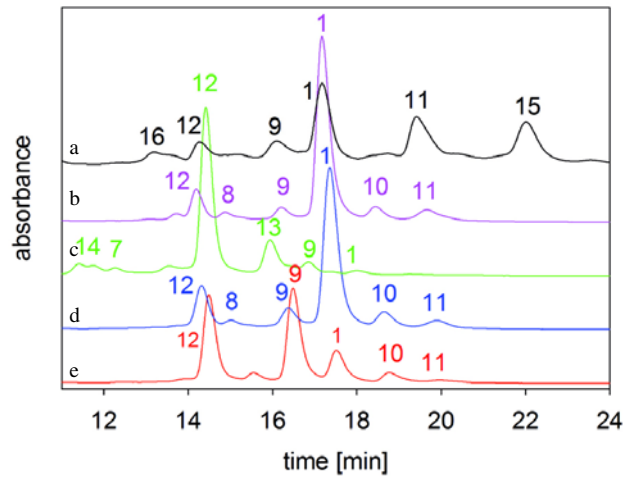


Figure 5: HPLC chromatograms of products from incubations of HRP and its mutants with Cl^- and H_2O_2: HRP (d), L37M HRP (e); F152M HRP (c); S151M HRP (b); and F41M HRP (a).

Table 5: Heme modified products ratios given as relative percentage values after incubation of HRP and its mutants with H_2O_2 and Cl^-.

Enzyme	1	7	8	9	10 (%)	11	12	13	14	15	16	other
HRP	58.5	–	4.4	9.1	8	5.6	14.4	–	–	–	–	–
L37M	15.5	–	–	38	6.2	2	33.3	–	–	–	–	5
F41M	24.6	19	–	9	–	18.1	6.9	–	–	21	10.2	13.2
S151M	63	–	3.1	6.3	6.7	8.5	12.4	–	–	–	–	–
F152M	1.8	–	–	5	–	–	65.7	14.5	7	–	–	6

this product as the diepoxide derivative of heme with an additional chlorine atom at the δ-*meso* position.

In comparison to native HRP, which under the present conditions gave chlorohydrin **12** (14.4%) as the major product (apart from unreacted heme), the L37M mutant gives the diepoxide **9** (38%) in addition to **12** (33.3%) as major products (Table 5). In contrast the F41M mutation favored production of the chlorohydrin epoxide **7** (19%), the vinyl chloride **11** (18%), and the *meso*-chloro vinyl chloride **15** (21%). The S151M mutation gave a pattern very similar to that of native HRP, but the F152M mutation not only led to an enhanced rate of modification, as evidenced by a low recovery of unmodified heme (1.8%), but also overwhelmingly led to the formation of chlorohydrin **12** (66%) and the *meso*-chloro chlorohydrin **13** (15%).

4. Discussion

The F41M mutant, the only one of the proteins examined here that may have a hexacoordinated iron atom, forms compound I about 120 times more slowly than native HRP (Table 2). However, this diminution in the rate is considerably smaller than that observed for compound I formation by metmyoglobin, which has a water distal ligand and which forms compound I several powers of ten more slowly than native HRP. In contrast, the compound I of all the mutants and of native HRP decays to compound II at approximately the same rate except for the F41M mutant, for which compound I is unstable and decays to compound II much more quickly.[30] Interestingly, the F41M compound II is also the only one among these proteins that decays much faster to the ferric state. This suggests that either the sulfur of Met41 can efficiently provide two electrons, thus reducing both compounds I and II, or that perturbation by the mutation of the hydrogen bonding interactions with the ferryl species promotes its faster decay. It is to be noted that among the residues mutated in this study, Phe41 is the closest to the heme vinyl groups and its mutation could therefore be expected to have a greater impact on the vinyl group environment (see below).

All of the mutants are able to oxidize bromide and chloride ions (Tables 3, 4, 5). The F41M mutant oxidized both halide ions substantially more rapidly than native HRP. The other mutants show activities that are either similar to the native enzyme or are somewhat lower, except for a modest increase observed with the L37M mutant. We showed earlier that monochlorodimedone, by trapping the resulting HOCl and HOBr metabolites, suppresses the heme modifications mediated by these reagents. The rates measured here for oxidation of these ions therefore represent the intrinsic catalytic rates without complications from differential rates of heme modification. Previous studies have shown that mutations of Phe41 to smaller residues increase the peroxidative activity of HRP.[17,31–33] These findings suggest that the higher activity of the F41M mutant can be attributed to the greater exposure of the ferryl species provided by replacement of the bulky Phe41 by a smaller methionine.

If faster reduction of the F41M compound I is due to electron transfer to the ferryl from Met41, the resulting sulfur radical cation could conceivably add to the vicinal heme vinyl group, resulting in covalent attachment of the heme to the protein. However, HPLC analysis after incubation of the various proteins with H_2O_2 established that this did not occur with F41M or any of the other mutants. The accelerated reduction of compound I therefore probably involves associated physicochemical changes in the active site rather than actual electron transfer from the methionine sulfur.

In view of the ability of both MPO and HRP to oxidize halide ions, we considered the possibility that covalent heme binding might depend on this specific catalytic activity. Thus, the cation formed by addition of Br^+ to the vinyl group, instead of being trapped by water to give a bromohydrin, could be trapped by the proximal methionine sulfur to give a crosslinked species (Scheme 1). Elimination of HBr from this adduct would produce the vinyl sulfonium link that is observed in MPO. However, incubation of our panel of HRP mutants with H_2O_2 and either bromide or chloride did not result in covalent attachment of the heme to the protein. Clearly, factors other than simple proximity to the vinyl group are required for the autocatalytic covalent binding of the heme to an active site methionine observed with MPO and the ascorbic peroxidase S160M mutant. Without crystal structures we cannot rule out the possibility that the methionine mutations distort the HRP active site and place the methionines at unfavorable crosslinking positions, although this would

seem unlikely for all the mutants. A comparison of the residues within close proximity (6 Å) of the vinyl groups in the active sites of HRP and MPO reveals that in MPO the vinyl groups are surrounded by polar residues such as Gln and Glu, whereas in HRP one finds Phe, Met and Ser residues, all of which define a less polar cavity. This difference may play an important role in formation of the methionine-vinyl link in MPO.

We previously reported that autocatalytic formation of a covalent bond between Glu41 in the F41E mutant of HRP and the heme 3-methyl group greatly protects the heme vinyl groups from reactions with HOCl and HOBr.[19] In this study, we have investigated the role of active site residues close to the heme vinyls, in the absence of heme-protein covalent bonds, in preventing or modulating the reactions of the vinyl groups. As shown here, mutation of the relevant residues alters both the rate of heme modification and the number and structures of the modified heme products. In addition to the halohydrin and vinyl halide heme products characterized in our earlier studies, we have identified epoxide derivatives. Furthermore, we previously identified a *meso*-chloro adduct[13] and showed that the *meso*-reactive species is probably a chloride radical.[16] In this study, we have found products that combine this *meso*-modification with vinyl group alterations. All the vinyl group modifications conform to a reaction manifold that is initiated by electrophilic reaction of the vinyl group with HOBr or HOCl to give a transient cationic intermediate (Scheme 1). Trapping of the cation by water yields the halohydrin, whereas deprotonation of the cation produces a vinyl halide. Formation of the epoxides is readily explained by intramolecular halide elimination from the halohydrin, a well-known base-catalyzed chemical reaction.[34] The extent of epoxide formation within the enzyme active site is uncertain, as the epoxide could also be formed during isolation of the heme derivatives.

No obvious relationship exists between the rate of heme modification and the various methionine mutations. Despite the increase in the oxidation of both bromide and chloride caused by the F41M mutation (Table 3), the mutation increases heme modification (25% recovered heme versus 59% with native HRP) in chloride oxidation, but decreases it in bromide oxidation (84% recovered heme versus 17% with native HRP). Thus, replacement of Phe41 by a methionine protects or enhances the reactivity of the heme vinyl group relative to native HRP in a substrate-dependent manner. The basis for this differential effect on the reactions with HOBr and HOCl is unclear, particularly as the F152M mutation greatly enhances the reactivity of the heme vinyl groups in both the bromide and chloride oxidations, as might be expected from a decreased steric protection of the vinyl groups. The differential protection of the vinyl groups in the F41M mutant may stem from an alteration in the polar properties of the active site that differentially affect the reactions of HOBr and HOCl.

The distribution of the heme products in bromide oxidation shows that the mutations consistently increase the formation of bromohydrins at the expense of vinyl bromide derivatives. We have included the epoxides as bromohydrin products because they arise by halide elimination from the bromohydrins. Thus, for native HRP the bromohydrins **2** + **3** + **6** account for 14% of the total products, and the vinyl bromides **4** + **5** for 64%, whereas in the F152M mutant the bromohydrin products add up to 85% and the vinyl bromide products to only 12% (Table 4). In chloride oxidation, vinyl chloride products represent a very minor fraction of the modified hemes except for the F41M mutant, for which they add up to 39% (Table 5). Again, all the mutations enhance the formation of chlorohydrin products (**7** + **8** + **9** + **12** + **13** + **14** + **16**) at the expense of unreacted heme (**1**) or vinyl chloride products (**11** + **15**), culminating in the case of the F152M mutant in 93% of halohydrin or halohydrin-derived products. The additional presence of *meso*-chloro adducts alone and in combination with other alterations complicates the analysis, but the trend is consistent and clear. These results suggest that all the mutations increase the accessibility of the halonium cation intermediate to water. This is consistent with our earlier finding that alkylperoxo radicals have greater access to the heme vinyl groups in the F41M mutant.[35] In the absence of a water molecule, loss of a proton to give a vinyl halide is favored, as almost all protein residues have some degree of basicity that can be utilized for deprotonation of the halonium cation to give the vinyl halide.

In sum, the residues vicinal to the heme vinyl groups afford limited protection against reactive electrophilic agents and, by altering the physicochemical properties, particularly water access and polarity, influence the nature of the heme modification products.

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Povzetek

Vinilne substituente hema v encimu peroksidaze hrena (HRP) se pretvorijo v halohidrin in vinil halidne substituente HOX, ki nastanejo z oksidacijo halida (X^-) Da bi določili vlogo ostankov, ki so v aktivnem mestu odgovorni za senčenje vinilnih skupin, smo proučevali L37M, F41M, S151M in F152M mutante. Za simultano testiranje zamreženja z vinilno skupino smo uporabili metionin, vendar zamreženja nismo opazili. Kinetične študije so pokazale, da je mutanta F41M še posebej spremenjena, saj se je komponenta I tvorila počasneje in je razpadala hitreje kot pri nativnem HRP encimu. Mutanta F41M je imela tudi največjo aktivnost oksidacije halida. Sprememba vinilnih skupin na hemu je pri vseh mutantah s katalitsko tvorjenim HOBr ali HOCl vodila do nastanka epoksidov, poleg že prej opaženih halogeniranih hidrinov in vinil-halogenov. Zamenjava posameznih ostankov blizu vinilnih skupin z metioninom je povečala tvorbo bromo-hidrinov na račun vinil-halidov. Rezultati nakazujejo, da so v nativnem aktivnem mestu vinilne skupine delno zaščitene in da je dostop vode otežen. Nižja polarnost v hemski odprtini hrenove peroksidaze v primerjavi z mieloperoksidazo je skladna s tvorbo vinil-halidov hema in lahko razloži odsotnost zamreženja pri vinil-metioninu hema.

Table S1: Summary of the mass spectrometric data for the brominated heme products.

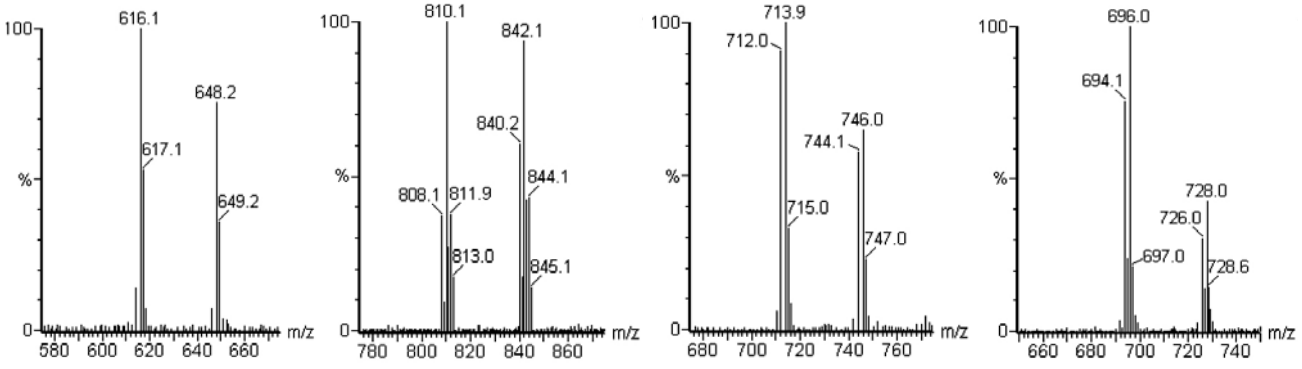
Compound	M^+ ion (m/z)[a]	heme	Br^- (#)	OH (#)	O (#) epoxide	H	Molecular mass
1	616	616	–	–	–	–	616
2	810	616	160 (2)	34 (2)	–	–	810
3	712	616	80 (1)	17 (1)	–	–	712
4	694	616	80 (1)	–	–	–	694
5	790	616	160 (2)	17 (1)	–	–1	790
6	726	616	80 (1)	17 (1)	16 (1)	–	726

[a] The molecular ion is usually accompanied by a strong ion 32 mass unit higher due to the methanol-complexed iron porphyrin.

Table S2: Summary of the mass spectrometric data on the chlorinated heme products.

Compound	M^+ ion (m/z)[a]	heme	Cl^- (#)	OH (#)	O (#) epoxide	H	Molecular mass
7	684	616	35 (1)	17 (1)	16 (1)	-	684
8	632	616	–	–	16 (1)	–	632
9	682	616	–	–	32 (2)	–	682
10	650	616	35 (1)	–	–	– 1	650
11	650	616	35 (1)	–	–	– 1	650
12	668	616	35 (1)	17 (1)	–	–	668
13	702	616	70 (2)	17 (1)	–	–1	702
14	720	616	70 (2)	34 (2)	–	–	720
15	684	616	70 (2)	–	–	–2	684
16	754	616	70 (2)	34 (2)	–	–	754

[a] The molecular ion is usually accompanied by a strong ion 32 mass unit higher due to the methanol-complexed iron porphyrin.



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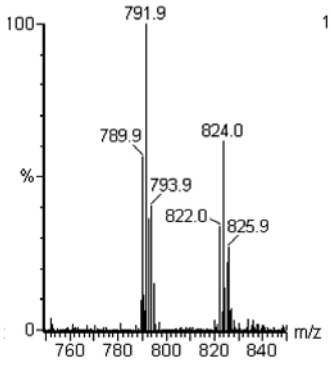

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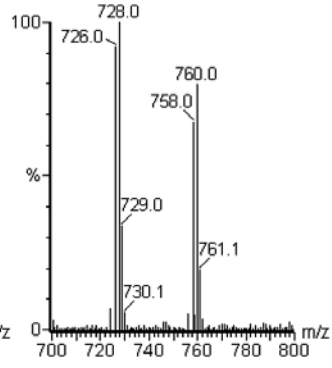

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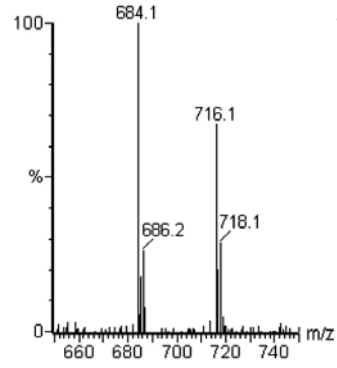

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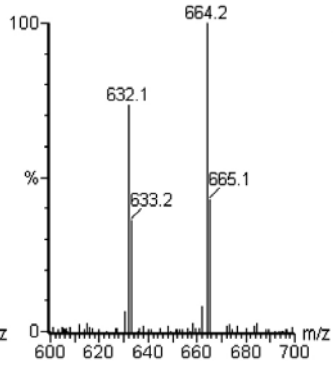

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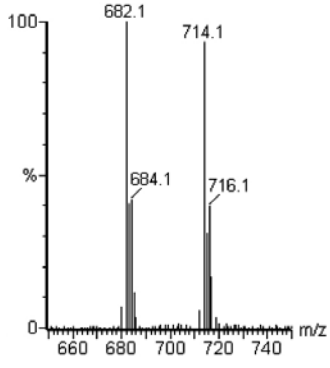

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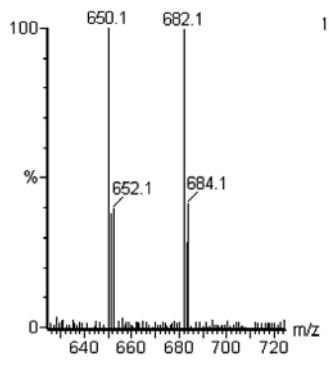

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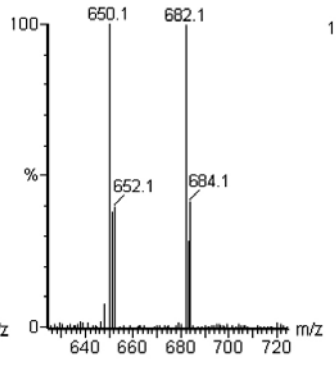

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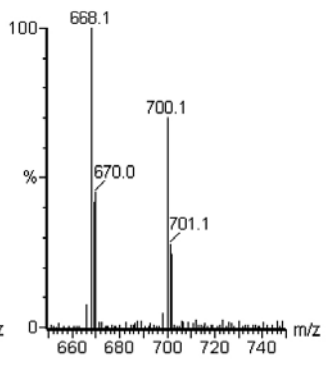

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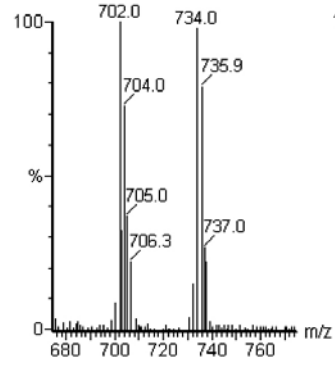

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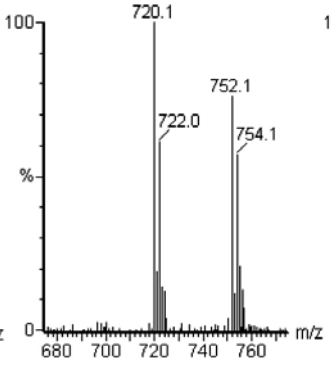

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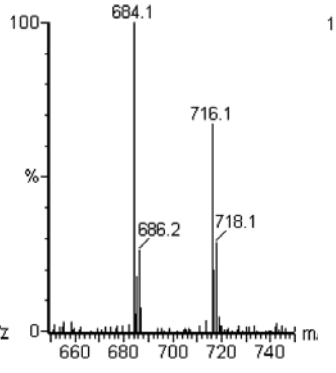

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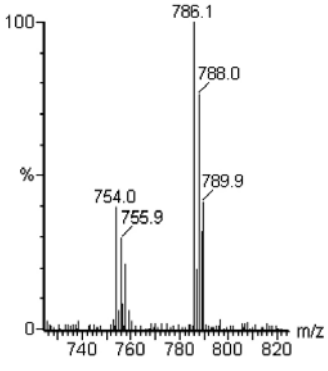

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