Role of Heme-Protein Covalent Bonds in Mammalian Peroxidases

PROTECTION OF THE HEME BY A SINGLE ENGINEERED HEME-PROTEIN LINK IN HORSEADISH PEROXIDASE

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

From the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Oxidation of SCN\(^-\), Br\(^-\), and Cl\(^-\) (X\(^-\)) by horseradish peroxidase (HRP) and other plant and fungal peroxidases results in the addition of HOX to the heme vinyl group. This reaction is not observed with lactoperoxidase (LPO), in which the heme is covalently bound to the protein via two ester bonds between carboxylic side chains and heme methyl groups. To test the hypothesis that the heme of LPO and other mammalian peroxidases is protected from vinyl group modification by the heme-protein covalent bonds, we prepared the F41E mutant of HRP in which the heme is attached to the protein via a covalent bond between Glu\(^41\) and the heme 3-methyl. We also examined the E375D mutant of LPO in which only one of the two normal covalent heme links is retained. The prosthetic heme groups of F41E HRP and E375D LPO are essentially not modified by the HOBr produced by these enzymes. The double E375D/D225E mutant of LPO that can form no covalent bonds is inactive and could not be examined. These results unambiguously demonstrate that a single heme-protein link is sufficient to protect the heme from vinyl group modification even in a protein (HRP) that is normally highly susceptible to this reaction. The results directly establish that one function of the covalent heme-protein bonds in mammalian peroxidases is to protect their prosthetic group from their highly reactive metabolic products.

The mammalian enzymes lactoperoxidase (LPO), myeloperoxidase (MPO), and eosinophil peroxidase efficiently oxidize iodide, bromide, thiocyanate, and, at least in the case of myeloperoxidase, chloride ions (1–3). Indeed, the antimicrobial and other roles of the mammalian peroxidases depend on their oxidation of halide and/or pseudohalide ions. In contrast, the substrates of most plant and fungal peroxidases are low oxidation potential compounds such as phenols, but the enzymes can also oxidize iodide, thiocyanate, bromide, and, albeit very poorly, chloride ions (4–6). Apart from these differences in their normal substrates, the most notable difference between the mammalian and plant/fungal peroxidases is the presence, in the mammalian enzymes, of two (or in MPO three) covalent bonds between the heme group and active site residues. In LPO, Glu\(^375\) and Asp\(^225\) form covalent ester bonds with the 1- and 5-methyl groups, respectively, of the heme (7–9). In MPO, in addition to the two ester bonds common to all the mammalian peroxidases, the 2-vinyl is attached via an unusual vinyl sulfonium link to Met\(^243\) (10, 11). No such covalent links have been detected in native plant or fungal peroxidases.

We have demonstrated that HRP can oxidize thiocyanate, bromide, and chloride ions and that these reactions result in the addition of HOX (where X = SCN, Br, or Cl) to the prosthetic heme 2- and/or 4-vinyl groups (6, 12). These results were recently extended to the Arthromyces ramosus and soybean peroxidases (13), confirming that modification of the heme vinyl groups is a common property in the oxidation of halides and pseudohalides by plant and fungal peroxidases. Reconstitution of apo-HRP with the modified hemes shows that vinyl group modification greatly attenuates the catalytic activity of the enzyme (6). In contrast, the heme group in LPO has been shown to be impervious to modification by the products it forms when it oxidizes thiocyanate, bromide, or chloride (12, 13). This is fortunate, because the physiological roles of the mammalian enzymes require the oxidation of precisely these substrates.

Based on these correlative results we have hypothesized that the role of the covalent bonds between the heme 1- and 5-methyl groups and protein carboxyl residues is, in part, to protect the prosthetic group from autocatalytic heme modification during oxidation of the normal halide and pseudohalide substrates of these enzymes and thus to prolong their lifetime despite their production of highly reactive metabolites (12, 13).

Here we report the first direct and unambiguous evidence that the covalent links between the protein and the heme do, in fact, protect the heme from modification by HOX. HRP was engineered to introduce a covalent ester bond between the protein and the 3-methyl of the heme by mutating Phe\(^31\) to a glutamic acid and pretreating the enzyme with low amounts of peroxide (14). The heme covalent bond is formed in essentially quantitative yield by this procedure. The oxidation of Br\(^-\) by the HRP F41E mutant with its single covalent heme bond produced HOBr as efficiently as native HRP but in contrast to the reaction of native HRP resulted in little modification of the heme vinyl.
groups. Moreover, the LPO E375D mutant, in which one of the two covalent heme bonds has been eliminated, not only oxidizes halides normally but remains as impervious to the hypohalide product as the wild-type enzyme. Thus, a single covalent bond between a heme methyl and the protein clearly suffices to protect the heme vinyl groups from reaction with the catalytically generated electrophilic HOX species. This protective effect was postulated earlier from correlative studies, but this is the first direct demonstration of its validity.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—**Sf9 cells (Invitrogen) were grown in Excell 420™ (JRH Biosciences), and High Five™ cells were grown in Express Five™ (Invitrogen) supplemented with glutamine (2.7 g/liter of medium). Both cell lines were kept in suspension at 28 °C and maintained at densities between 0.5 × 10⁶ and 2 × 10⁶ cells/ml. All of the experiments were performed at room temperature unless otherwise stated. Spectrophotometric measurements were performed on an Agilent 8453 diode array spectrophotometer. HPLC was performed on a Hewlett-Packard 1090 Series II instrument equipped with a photodiode array detector. LC/MS was performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system (2695 separations module, Waters 2487 Dual λ Absorbance Detector) employing an Xterra® MS C₁₈ column (2.1 × 50 mm, 3.5 μm).

**HPLC Analysis—**Unless otherwise mentioned, HPLC analysis was performed on a Vydac protein C₂ column (4.6 × 150 mm, 5 μm) fitted with a precolumn filter. Solvent A was water containing 0.1% trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% trifluoroacetic acid. The gradient program consisted of linear segments with 15% B (0–3 min), 15–40% B (3–53 min), 95% B (54–57 min), and 15% B (58–60 min) at a flow rate of 1 ml/min. The eluent was monitored at 278 and 400 nm.

**Expression and Purification of the HRP F41E Mutant—**The HRP F41E mutant was expressed and purified following modifications of the previously reported procedure (14). Viral stock was generated and amplified from single virus populations. High Five™ cells were infected at a density of ~2 × 10⁶ cells/ml using a multiplicity of infection of ~5, and hemin was added (10 mg/liter cells). After 3.5 days, cell suspensions (7 liters) were centrifuged at 4 °C at 5000 rpm for 20 min. The supernatant was concentrated to ~200 ml by ultrafiltration over a spiral-wound regenerated cellulose membrane (molecular mass cut-off, 10 kDa; final volume, 300 μl; Aₛₒₜ = 0.713 after 4-fold dilution; yield, ~23%). An aliquot of the final HRP F41E solution (100 μl, 17 μM) was incubated with KBr (60 μl, 2 mM) and H₂O₂ (40 μl, 1.26 mM, 30 equivalents) for 30 min. The solution was then centrifuged and exchanged into bis-tris propane buffer (100 mM, pH 8.2), giving a final volume of 150 μl with a Soret band absorption at λₘₐₓ = 409 nm. EDTA (100 mM, 3 μl) was then added followed, after 15 min, by trypsin (1 mg/ml, 5 μl). The resulting solution was incubated at ~20 °C overnight. On the second day, protease K (10 mg/ml, 10 μl) was added, the resulting solution was incubated at ~20 °C overnight, and the digested solution was analyzed by LC/MS. The LC conditions were as follows where one solvent (A) is water plus 0.1% formic acid, and the other solvent (C) is methanol containing 0.1% formic acid: solvent C was maintained at 40% for 2 min, followed by a linear gradient from 40 to 70% over the following 20 min. The MS settings were: mode, ES⁺; capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 300 °C; and source temperature, 120 °C.

A control experiment was performed with HRP as follows. HRP (100 μl, 40 μM) in citrate buffer (0.5 M, pH 4.4) was mixed with KBr (60 μl, 2 mM) and H₂O₂ (40 μl, 3 mM, 30 equivalents). After 30 min of incubation, the solution was exchanged into bis-tris propane buffer (0.1 M, pH 8.2) at a final volume of 150 μl. The Soret absorption band of the protein at this point was at λₘₐₓ = 395 nm. The solution was digested with trypsin and protease K and was analyzed by LC/MS following the procedure described above.

**Expression and Purification of the LPO Mutants—**The LPO E375D mutant and D225E/E375D double mutant were expressed and purified according to the previously reported procedure (8). Viral stock was generated and amplified from single virus populations. High Five™ cells were infected at a density of ~2 × 10⁶ cells/ml using a multiplicity of infection of ~4, and hemin was added (10 mg/liter of cells). After 3 days, cell suspensions were centrifuged at 4 °C at 5000 rpm for 20 min. The supernatant was diluted with an equal volume of deionized water, followed by adding phenylmethanesulfonyl fluoride (as a protease inhibitor) at a final concentration of 200 mg/liter. To this solution Amberlite CG50 cation exchange resin (pre-equilibrated in 50 mM Tris-HCl, pH 7.4) was added in three portions over 2 h (~20 ml buffer (20 mM, pH 8) for at least 14 h with at least three bath changes. The dialyzed fractions were then concentrated 12-fold by ultrafiltration over an Amicon YM10 membrane and passed through a pre-equilibrated QFF-Sepharose (Amersham Biosciences) column. The colored fractions were concentrated again over an Amicon Ultra-₄™ centrifugal filter device (molecular mass cut-off, 10 kDa) to 1–2 ml. The protein was tested by the ABTS assay (6) for peroxidase activity and by SDS-PAGE analysis for purity.

**Oxidation of Br⁻ by the HRP F41E Mutant—**The HRP F41E mutant (0.5 mM, 50 μl) in 400 μl of bis-tris propane buffer (0.1 M, pH 8.2) was treated with H₂O₂ (2 equivalents × 6) at 5-min intervals and was incubated for a further 10 min after the last H₂O₂ addition. A 50-μl aliquot was injected onto the HPLC for analysis. Most of the heme (>90%) co-eluted with the protein. The medium was then changed to citrate buffer (0.5 M, pH 5.0) with a Millipore ultrafilter centrifugal filter device (molecular mass cut-off, 10 kDa; final volume, 300 μl; Aₛₒₜ = 0.713 after 4-fold dilution; yield, ~23%). An aliquot of the final HRP F41E solution (100 μl, 17 μM) was incubated with KBr (60 μl, 2 mM) and H₂O₂ (40 μl, 1.26 mM, 30 equivalents) for 30 min. The solution was then centrifuged and exchanged into bis-tris propane buffer (100 mM, pH 8.2), giving a final volume of 150 μl with a Soret band absorption at λₘₐₓ = 409 nm. EDTA (100 mM, 3 μl) was then added followed, after 15 min, by trypsin (1 mg/ml, 5 μl). The resulting solution was incubated at ~20 °C overnight. On the second day, protease K (10 mg/ml, 10 μl) was added, the resulting solution was incubated at ~20 °C overnight, and the digested solution was analyzed by LC/MS. The LC conditions were as follows where one solvent (A) is water plus 0.1% formic acid, and the other solvent (C) is methanol containing 0.1% formic acid: solvent C was maintained at 40% for 2 min, followed by a linear gradient from 40 to 70% over the following 20 min. The MS settings were: mode, ES⁺; capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 300 °C; and source temperature, 120 °C.
of settled resin/liter of supernatant). After decantation, the slurry was packed into a column, and the column was subsequently washed with ~5 volumes of 50 mM Tris·HCl and ~3 volumes of 150 mM Tris·HCl, pH 7.4. The protein was then eluted with 500 mM Tris·HCl at pH 7.4. The fractions with absorbance at 412 nm were pooled, concentrated, and exchanged into phosphate buffer. The final product was tested with the ABTS assay (2.5 μM/100 μL) and 412 nm were combined, concentrated, and exchanged into bis-tris propane buffer (0.1 M, pH 8.2), followed by exchange of the sample into citrate buffer (0.5 M, pH 4.4) with a Millipore ultrafree centrifugal filter device (molecular mass cut-off, 10 kDa). The final volume was 100 μL of ~18 μm protein (based on heme absorption). To this were added KBr (60 μL, 2 m) and H2O2 (40 μL, 1.81 mM, 40 equivalents), and the solution was incubated for 20 min before it was exchanged into bis-tris propane buffer (0.1 m, pH 8.2). The final volume was 110 μL of a solution with a Soret absorption band at λ max = 410 nm. CaCl2 (10 mM, 10 μL) was then added to this solution, followed after a few minutes by trypsin (1 mg/ml, 10 μL). The resulting solution was incubated at ~20 °C overnight. On the second day, CaCl2 (10 mM, 20 μL) was again added followed after a few minutes by protease K (10 mg/ml, 10 μL). The resulting solution was incubated at ~20 °C overnight. In a control experiment, the E375D mutant prior to incubation with KBr was also digested with trypsin/protease K. The digests were analyzed by LC/MS using the conditions already described above.

**TABLE 1**

| Activity of the F41E HRP mutant | HRP | F41E |
|--------------------------------|-----|------|
| **MCD assay**                  | 52 ± 13 | 285 ± 32 | 68 ± 16 |
| (2.5 ± 0.5) × 10⁴             | (1.8 ± 0.1) × 10⁴ | (3.6 ± 0.7) × 10³ |
| **ABTS assay**                 | (2.5 ± 0.5) × 10⁴ | (1.8 ± 0.1) × 10⁴ | (3.6 ± 0.7) × 10³ |

**FIGURE 1.** LC/MS analysis of the reaction mixture after incubation of KBr and the HRP F41E mutant in the presence of H2O2. Upper panel, LC trace of the F41E reaction mixture after trypsin-protease K digestion. Lower panel, LC trace of the HRP reaction mixture after trypsin-protease K digestion. Insets are the mass spectra of the corresponding peaks. The molecular ion of the porphyrin is followed by a second molecular ion at m/z +32 caused by the complex of the iron porphyrin with methanol (M⁻ + 32).

**RESULTS**

**Preparation of the HR F41E Mutant with a Heme-Protein Cross-link—**As previously reported, the F41E mutant of HRP can be expressed in a baculovirus insect cell system as a protein with a noncovalently bound heme, but exposure of this protein to several equivalents of H2O2 results in autocatalytic cross-linking of the heme 3-methyl group to Glu41 via an ester link essentially identical to those found in the mammalian peroxidases (14). The F41E HRP mutant was therefore expressed and purified as previously reported. The resulting protein in bis-tris propane (0.1 M, pH 8.2) was incubated with H2O2 (2 equivalents × 6) over a period of 35 min, after which HPLC analysis indicated that the heme was quantitatively covalently bound. The Soret band of the heme shifted from 406 to 409 nm as a result of formation of the covalent heme bond.

The activities of native HRP, the F41E mutant without H2O2 pretreatment, and the final F41E mutant after exposure to H2O2 were determined and are shown in Table 1. Compared with the native enzyme, the F41E is ~5-fold better at oxidizing Br⁻ before preincubation with H2O2 but has roughly the same activity as the native enzyme after H2O2 pretreatment. The peroxidase activity of all three proteins is roughly comparable in the ABTS peroxidase assay, although native...
HRP is somewhat more active in this assay. The F41E mutant is therefore fully active both in the oxidation of Br\(^-\) and as a peroxidase, both before and after pretreatment with peroxide to form the heme-protein covalent bond.

**Oxidation of Br\(^-\) by H\(_2\)O\(_2\)-pretreated HRP F41E**—HRP F41E, after pretreatment with H\(_2\)O\(_2\) to promote heme-protein covalent binding, was incubated with 0.6 M Br\(^3\)-hydroxymethyl heme\(^3\) derivative (digested sample showed a major peak that was identified as the product expected from hydrolysis of the ester bond between the 3-methyl and Glu\(_{41}\) and is the same as is obtained if the incubation with Br\(^-\) is omitted. In addition to the major heme-derived product, several very minor heme-like peaks were detected, including a residual unmodified heme (2, \(t = 24.53\) min) and two possible products of further modification: 3 (\(t = 20.42\) min) and 4 (\(t = 10.64\) min).

Compound 3, with a molecular ion at \(m/z\) 710 and a 1:1 710:712 isotopic peak ratio, corresponds to the addition of one bromine atom. Based on our previous work with native HRP (6), this compound is identified as the 4-bromovinyl derivative of 3-hydroxymethyl heme.

Compound 4 has a mass spectrometric molecular ion at \(m/z\) 666 and thus is obtained by the addition of two hydroxyl groups in addition to the hydroxyl group on the 3-methyl. In our earlier work with native HRP, the major products include the bromohydin derivatives formed by addition of the equivalents of HOB\(_2\) across either the 2- or 4-vinyl group of the heme (15). Bromohydrins readily eliminate bromide to give epoxides under basic conditions, and epoxides are readily hydrolyzed to diols by water addition, reactions that are to be expected given the prolonged digestion conditions (2 days at pH 8.2). Here we performed control experiments in which native HRP was incubated with Br\(^-\)/H\(_2\)O\(_2\) and then subjected to the same proteolytic conditions. In addition to the 2-(1\(^\prime\)-hydroxyl, 2\(^\prime\)-bromo)ethyl, 4-(2\(^\prime\)-bromo)vinyl heme (product 5), which was reported previously, another product (6, \(m/e = 728:730\), isotope ratio 1:1) was obtained. We further observed that the amount of product 6 increased concomitantly with a decrease in product 5 upon digestion at pH 8.2 for an additional day, indicating that product 6 was derived from 5. This observation in conjunction with the mass spectrum of product 6 identified it as 2-(1\(^\prime\), 2\(^\prime\)-dihydroxyethyl, 4-(2\(^\prime\)-bromo)vinyl heme. Therefore, in the incubations with the F41E mutant, product 4 was identified as the 1,2-dihydroxyethyl derivative of either the 2- or 4-vinyl group, a product analogous to product 6 obtained from native HRP.

Quantitative comparison of the extent of formation of vinyl group-modified products 3 and 4 in incubations of F41E HRP with Br\(^-\)/H\(_2\)O\(_2\) with the vinyl-modified products obtained in incubations of native HRP under the same conditions shows that a dramatic decrease in vinyl modified products is associated with introduction of the heme-protein bond in the F41E mutant (Fig. 1 and Table 2). In the F41E HRP mutant, only \(~\)10% of total heme signals correspond to vinyl-modified products (4% for 3, 4% for 4). The major peak is due to 3-hydroxymethyl heme (81%), the “unmodified” prosthetic group. In contrast, 100% of heme in native HRP was modified under the same conditions, and the two major vinyl-modified hemes accounted for \(~\)80% of the total heme products.

**Preparation of the Mature E375D Mutant of LPO**—Mature LPO has two covalent bonds, the first between Asp\(^{226}\) and the...
5-methyl and the second between Glu$^{375}$ and the 1-methyl (8, 9). Previous work has shown that mutation of Glu$^{375}$ to an Asp prevents formation of the covalent bond to that residue, although the 1-methyl is still oxidized to some extent to the 1-hydroxymethyl derivative (8). To determine whether the heme of LPO would be more susceptible to modification by HOBr if it were only attached to the protein through one covalent bond, we expressed and purified this enzyme as previously reported (8). As previously reported, we also preincubated this enzyme with H$_2$O$_2$ (1 equivalent × 4) to ensure complete autocatalytic formation of the covalent bond between Asp$^{225}$ and the 5-methyl (8). Activity assays established that the recombinant E375D LPO mutant had similar bromide oxidation and peroxidatic activities as the native enzyme before and after pretreatment with H$_2$O$_2$ (Table 3).

**Oxidation of Br$^-$ by the E375D LPO Mutant**—The mature (covalently cross-linked) E375D mutant was incubated with Br$^-$ in the presence of 40 equivalents of H$_2$O$_2$ for 20 min. The reaction solution was changed to bis-tris propane buffer (100 mM, pH 8.2) before the protein was digested as previously described, and the lysate was analyzed by LC/MS (Fig. 2). The two major heme-derived peaks at $t = 17.00$ and 10.19 min exhibited molecular ions at $m/z$ 632 and 648, respectively, and were identified as 5-hydroxymethyl heme (8) and 1,5-di(hydroxymethyl) heme (9) by their molecular masses and retention times. These same species were observed in control experiments in which the mature LPO E375D mutant was digested without first reacting it with Br$^-$ and H$_2$O$_2$. 5-Hydroxymethyl heme is the iron porphyrin expected hydrolytically from LPO E375D, which can still form the covalent bond between Asp$^{225}$ and the 5-methyl 1,5-(Dihydroxymethyl)heme arises from oxidation of the 1-methyl in the mutated protein in addition to hydrolysis of the bond to the 5-methyl. Most importantly, no bromo-heme adducts were detected in the lysates.

**Expression of the D225E/E375D LPO Double Mutant**—We have demonstrated above that introduction of one covalent bond into HRP protects the heme from modification by autocatalytically generated HOBr and have shown that an LPO with only one such bond is also fully protected. To complete the test set, we expressed the D225E/E375D double mutant of LPO, in which formation of all covalent bonds to the heme is disrupted (8). Unfortunately, we have confirmed that this double mutant binds heme poorly and has no measurable peroxidase activity. It has therefore not been possible to test whether the heme of LPO with no covalent heme bonds is susceptible to modification by catalytically generated HOBr.

**DISCUSSION**

It is well established that the prosthetic heme group in mammalian peroxidases is covalently bound to protein, but the functional role of the covalent linkage remains unclear. We have reported that both plant/fungal and mammalian peroxidases oxidize bromide, and the resulting hypobromous acid modifies the prosthetic heme in plant and fungal peroxidases but not in mammalian peroxidases (13). We proposed a hypothesis that the heme of LPO and other mammalian peroxidases is protected from vinyl group modification by the heme-protein covalent bonds. In this study we provide direct evidence to support the hypothesis.

This laboratory previously reported that the HRP F41E mutant was able to form a heme-protein covalent link between the 3-methyl of the heme and Glu$^{41}$ of the protein (14). Here we expressed the same mutant and pretreated it with H$_2$O$_2$ to form the heme-protein link. The enzymatic activity of the final protein in the oxidation of Br$^-$ is roughly the same as that of native HRP. However, the Br$^-$-oxidizing activity of the F41E mutant is 5-fold higher before the covalent link is formed (Table 1). The basis for the enhanced bromide oxidation activity of the noncovalently linked F41E mutant is unclear but may reflect a change in the electrostatic properties of the active site caused by the negative charge of the unmasked carboxylic acid group. In any case, the prosthetic heme group of the cross-linked F41E mutant remains almost intact in the presence of Br$^-$ and up to 30 equivalents of H$_2$O$_2$. In contrast, the heme of native HRP is completely modified under identical conditions. Indeed, complete modification of the heme of native HRP is observed under even milder conditions (10–20 equivalents H$_2$O$_2$, 0.4 mM Br$^-$). When the H$_2$O$_2$ concentration is >50 times the concentration of the enzyme, the heme and heme-like signals are greatly diminished in the LC/MS analysis. This is due to both the bleaching effect of H$_2$O$_2$ and the degradation of the bromovinyl groups under the digestion (2 days, pH 8.2), as we observed secondary transformations of the bromohydrin heme derivatives during the digestion process. In the control experi-

---

**TABLE 2**

| LPO                | Modified hemes | Unmodified hemes |
|--------------------|---------------|-----------------|
| F41E HRP           | 3 (4%), 4 (4%)| 1 (81%), 2 (8%) |
| HRP                | 5 (49%), 6 (30%), 7 (4%) | not detectable |

**TABLE 3**

| LPO               | E375D | Before H$_2$O$_2$ treatment | After H$_2$O$_2$ treatment |
|-------------------|-------|-----------------------------|----------------------------|
| MCD assay         | (5.8 ± 1.2) × 10$^3$ | (6.4 ± 0.6) × 10$^3$ | 2.2 × 10$^3$ |
| ABTS assay        | (4.5 ± 1.6) × 10$^3$ | (2.6 ± 0.8) × 10$^3$ | 2.2 × 10$^3$ |
Role of Heme-Protein Links in Mammalian Peroxidases

ment with native HRP, one of the two major products (6) was not detected before the digestion but rather derived from 5 as a result of incubation under the digestion conditions. The conversion of 5 to 6 involves substitution of the Br in the bromohydrin by an OH group, possibly via intermediate formation of an epoxide. The corresponding product (4) was identified in the reaction of the F41E mutant. Overall, the majority of the heme in the F41E mutant remained intact, whereas the heme of HRP was completely modified during Br\textsuperscript{−} oxidation. This result clearly demonstrates that the heme–protein covalent bond protects the prosthetic heme from modification and thus protects the enzyme from inactivation.

A similar result was obtained with the LPO E375D mutant in which only the covalent bond between the 5-methyl of the heme and Asp\textsuperscript{225} is retained. We detected 5-hydroxymethyl heme (8) from this mutant after the heme was released by trypsin/protease K digestion. Most importantly, even after reaction with Br\textsuperscript{−} and H\textsubscript{2}O\textsubscript{2}, LC/MS analysis showed that the heme species that are present were the same as those obtained by direct digestion of the protein. One covalent bond thus suffices to protect the heme from modification by HOBr, in agreement with the results from the HRP F41E mutant. Efforts to prepare D225E/E375D double mutant and to test whether LPO without any covalent bond to its heme would become susceptible to modification by HOBr were unsuccessful because, as previously found, this double mutant is essentially inactive.

It is somewhat surprising that the single ester bond in the F41E HRP mutant protects not one but both heme vinyl groups from reaction with electrophilic agents. Analysis of a model based on the crystal structure of HRP in which Glu\textsuperscript{41} and the heme 3-methyl are simply linked together suggests that the resulting ester bond can sterically shield the 4-vinyl, but simultaneous steric protection of the 2-vinyl does not appear likely without an active site conformational change. A shift in the positions of the active site residues is almost certain, because the ester bond in the model is only awkwardly formed if the protein residues and the heme are held in their crystallographic positions. LPO and the other mammalian peroxidases normally have two ester bonds, each of which could sterically protect one of the vinyl groups. However, even if one of these bonds is lost, as in the D225E mutant, both vinyls are still protected. One factor that may contribute to protection of the vinyl groups by the heme–protein links, both in the F41E HRP mutant and the mammalian peroxidases, is the rigidification of the active site associated with covalent attachment of the heme to the protein. Anchoring of the heme to the protein is expected to dampen the natural dynamic breathing motions of the active site and could facilitate protection of the vinyl groups by the active site residues. We have recently proposed that rigidification of the active site by the heme–protein cross-link in CYP4 cytochrome P-450 enzymes is responsible, at least in part, for their unique ability to catalyze ω-hydroxylation of fatty acid chains (16).

The covalent heme links in the mammalian peroxidases are likely to have roles in addition to protection of the heme. LPO and even more so MPO have much higher activities in the oxidation of halides than HRP. It is very possible that the heme–protein cross-links help to adjust the reactivity of the ferryl species for halide oxidation, although no specific evidence for this is provided by the present studies.

In summary, this study provides direct support for the hypothesis that covalent linkages between the prosthetic heme and the protein in mammalian peroxidases serve to protect the heme from modification by the reactive HOX species generated during the catalytic process. This is based on two key findings. First, the HRP F41E mutant, which forms one covalent bond between the heme and the protein, is resistant to modification by autocatalytically generated HOBr. In contrast, HOBr modified the heme of native HRP in which no heme–protein covalent bond exists. Second, the LPO E375D mutant, which only retained one of the two normal heme–protein covalent bonds, remained completely resistant to heme modification by autocatalytically generated HOBr.

Acknowledgment—We thank Christophe Colas for providing an initial sample of the F41E HRP mutant and for advice.

REFERENCES
1. Dunford H. B. (1999) in Heme Peroxidases, pp. 349–385, Wiley-VCH, New York
2. Van Dalen, C. J., and Kettle, A. J. (2001) Biochem. J. 358, 233–239
3. Furtmüller P. G., Burner U., and Obinger C. (1998) Biochemistry 37, 17923–17930
4. Shah M. M., and Aust S. D. (1993) Arch. Biochem. Biophys. 300, 253–257
5. Munir, I. Z., and Dordick, J. S. (2000) Enz. Microbial. Technol. 26, 337–341
6. Huang, L., Wojciechowski, G., and Ortiz de Montellano, P. R. (2005) J. Am. Chem. Soc. 127, 5345–5353
7. Colas, C., and Ortiz de Montellano, P. R. (2003) Chem. Rev. 103, 2305–2332
8. Colas, C., Kuo, J. M., and Ortiz de Montellano, P. R. (2002) J. Biol. Chem. 277, 7191–7200
9. Rae, T. D., and Goff, H. M. (1998) J. Biol. Chem. 273, 27968–27977
10. Kooter, I. M., Moguilevsky, N., Bollen, A., van der Veen, L. A., Otto, C., Dekker, H. L., and Wever, R. (1999) J. Biol. Chem. 274, 26794–26802
11. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) J. Biol. Chem. 275, 11964–11971
12. Wojciechowski, G., Huang, L., and Ortiz de Montellano, P. R. (2005) J. Am. Chem. Soc. 127, 15871–15879
13. Huang, L., and Ortiz de Montellano, P. R. (2006) Arch. Biochem. Biophys. 446, 77–83
14. Colas, C., and Ortiz de Montellano, P. R. (2004) J. Biol. Chem. 279, 24131–24140
15. Van Tamelen, E. E., Storni, A., Hessler, E. J., and Schwartz, M. (1963) J. Am. Chem. Soc. 85, 3295–3296
16. He, X., Cryle, M. J., De Voss, J. J., and Ortiz de Montellano, P. R. (2005) J. Biol. Chem. 280, 22697–22705