Horseradish Peroxidase-catalyzed Two-electron Oxidations

OXIDATION OF IODIDE, THIOANISOLEs, AND PHENOLS AT DISTINCT SITES*

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Horseradish peroxidase, a prototypical hemoprotein peroxidase (1-3), catalyzes the one-electron oxidation of a large variety of substrates. Mechanistic studies have established that the ferric enzyme reacts with H2O2 to give compound I, the oxidized ferryl porphyrin radical cation (P**FeVI = O) (1-3). In classical peroxidase reactions, sequential electron abstraction from two substrate molecules reduces the porphyrin radical cation first to the ferryl porphyrin (PFeIV = O) species known as compound II, and subsequently to the ferric resting state. Normally, the rate of the first electron abstraction is approximately 10 times faster than that of the second, so that the chromophore of compound II is usually observed during steady-state catalytic turnover (4, 5). Despite the structural similarity of the ferryl complex of compound I to the proposed catalytic species of cytochrome P-450 and to well-characterized ferryl metalloporphyrin complexes (6, 7), horseradish peroxidase does not generally transfer the ferryl oxygen to its substrates. It does not, for example, catalyze the epoxidation of styrene (8) or butadiene (9), activated olefins that are readily oxidized by cytochrome P-450 monooxygenases and metalloporphyrin monooxygenase models (4, 5). To explain this divergence in catalytic function of nominally related catalytic species, we have proposed that substrates interact with the &omicron;meso edge of the heme group of horseradish peroxidase rather than with the ferryl oxygen itself (10). This proposal derives much of its support from the observation that catalytic oxidation of several substrates by horseradish peroxidase results in alkylation of the &omicron;meso carbon rather than, as in cytochrome P-450, the iron or the porphyrin nitrogens of the prosthetic heme group (10-13). Furthermore, horseradish peroxidase reconstituted with a &omicron;meso-ethylheme group appears to react with H2O2 but has no peroxidase activity (13). An active site in which peroxidase substrates bind near the &omicron;meso edge but are prevented by a protein barrier from interacting with the ferryl oxygen has been postulated to rationalize the &omicron;meso edge sensitivity of the enzyme.

Horseradish peroxidase has been reported to catalyze a few two-electron substrate oxidations that, at face value, involve transfer of the ferryl oxygen to the substrate. Some of these reactions have been shown by mechanistic studies to be normal peroxidative processes (8, 14, 15), but the evidence on the sulfoxidation of thioanisole and other sulfur compounds (16-19) and the oxidation of iodide (20, 21) suggests that these horseradish peroxidase-catalyzed reactions may truly be two-electron transfer processes. Strong evidence in favor of ferryl oxygen transfer in the sulfoxidation of thioanisole is provided by the finding that the oxygen incorporated into the product derives primarily from the peroxide (16, 19), although the reported formation of racemic products favors a solution rather than active site oxidation (17, 22). Although the oxidation is a net two-electron transformation, oxygen transfer may involve one-electron abstraction followed by combination of the resulting sulfur radical cation with the ferryl oxygen.

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‡ HPLC analysis of the guaiacol oxidation products fails to reveal the presence of either catechol or ring-hydroxylated derivatives of guaiacol (J. DeVoss and P. R. Ortiz de Montellano, unpublished results).

1 The abbreviations used are: heme, iron protoporphyrin IX regardless of oxidation or ligation state; HPLC, high pressure liquid chromatography.
Support for such a mechanism is provided by correlation of the oxidation potentials with the rates of oxidation of substituted thioanisoles (16, 18) and, more importantly, by observation of compound I as a transient intermediate in the sulfonation reaction (23, 24).

The primary evidence for direct two-electron oxidation of iodide by horseradish peroxidase is provided by the stoichiometry of the reaction (H₂O₂ + 2II⁻ → 2H⁺ + I₂ + 2H₂O) and the failure to observe compound II in the reaction (21). The direct reduction of compound I to the ferric enzyme could be achieved by ferryl oxygen transfer to I⁻ to give HOI (25) or by an alternative mechanism that results in the formation of I⁺ or its equivalent. NMR studies with ferric horseradish peroxidase suggest that iodide binds within 10 Å of the 1- and 8-methyls of the prosthetic heme group, and therefore near the β-meso heme edge (26). This places the iodide-binding site near the site of classical peroxidase substrates such as resorcinol (27) and benzhydroxamic acid (28), which have been shown by nuclear Overhauser NMR studies to bind near the 1- and 8-methyls of the prosthetic heme group. The possibility therefore exists that iodide is oxidized by two one-electron steps, the second of which is not significantly slower than the first.

The possible catalysis by horseradish peroxidase of two-electron oxidations, and particularly of ferryl oxygen transfer reactions, must be reconciled with the hypothesis that horseradish peroxidase promotes peroxidase reactions by limiting access of the substrate to the ferryl oxygen. We report here an examination of the sites of thioanisole and iodide oxidation and their relationship to the guaiacol oxidation site that sheds some light on the question of peroxidative versus peroxynogenetic mechanisms in horseradish peroxidase catalysis.

Part of these results have been communicated in abstract form (29, 30).

**EXPERIMENTAL PROCEDURES**

**Materials**—Thioanisole, 4-methylthioanisole, 4-methoxythioanisole, 4-nitrothioanisole, thioanisole sulfide, hydrogen peroxide, phenylhydrazine, HCl, and m-chloroperbenzoic acid were purchased from Aldrich. Horseradish peroxidase (type IV), hemoglobin, heme, and guaiacol were from Sigma. Authentic samples of 4-methylthioanisole sulfide and 4-methoxythioanisole sulfide were kindly provided by Julia Fruegel. Sigma horseradish peroxidase was used to generate β-meso-alkylheme groups but was not used for kinetic studies because isozyme C is contaminated with significant amounts of another isozyme. Kinetic studies were carried out with horseradish peroxidase isozyme C from Boehringer Mannheim. Ethylhydrazine was from Fluka (Konokoma, NY) and methylhydrazine from American Tokyo Kasei Inc. (Portland, OR). 2-Mercaptoethanol was from Bio-Rad. [30]H₂O₂ from ICON was shown to be 95 atom % [30]O by gas chromatography-mass spectrometry of the menadione epoxide produced by reaction of the peroxide with menadione (31). Buffers were made with deionized, glass-distilled water that had been stirred overnight with 5 g/liter Chelex 100 beads (Bio-Rad).

**Synthesis of 4-Nitrothioanisole Sulfide—m-Chloroperbenzoic acid (57 mg, 0.50 mmol) in 10 ml CH₂Cl₂ was added to a solution of 4-nitrothioanisole (100 mg, 0.52 mmol) and KF (32 mg, 0.55 mmol) in 10 ml of dry CH₂Cl₂ and the mixture was stirred at 25 °C for 1 h. An additional 32 mg of KF was then added and the reaction was stirred another hour. The solution was filtered to remove potassium m-chlorobenzoate and the filtrate was washed once with 20 ml of 1 M NaOH and once with 20 ml of brine. The CH₂Cl₂ was then evaporated under a stream of argon and the residual solid redissolved for HPLC analysis.

**Modified Horseradish Peroxidase—β-meso-Methylheme and β-meso-ethylheme were reconstituted with these prosthetic groups (13), as previously reported. Phenylhydrazine modified horseradish peroxidase was obtained by adding phenylhydrazine and H₂O₂ (2 mM final concentration of each) over a 5-min period to a solution of horseradish peroxidase (50 µM) in 5 ml of 50 mM sodium phosphate buffer (pH 7.0). The final mixture was stirred 10 min at room temperature and was then passed through a Sephadex G-25 column equilibrated in 50 mM phosphate buffer (pH 7.0). The enzyme thus obtained was reincubated in a similar manner with phenylhydrazine and H₂O₂ to insure complete loss of guaiacol activity. After 10 min, 20 µl of a 10 mM solution of sodium ascorbate was added to reduce the heme iron to the ferric state, and the mixture was again filtered through a G-25 Sephadex column. The modified enzyme was then dialyzed in the same buffer containing 0.1 M sodium phosphate buffer (pH 7.0) to remove the 2-mercaptoethanol and any iron that may have been released.

**Thioanisole Sulfonation**—To a solution of horseradish peroxidase or modified horseradish peroxidase (25 µM) in 2 ml of sodium phosphate buffer (pH 7.0) was added to 10 µl of 1 M solution of thioanisole in methanol, giving a nominal 5 mM thioanisole concentration. This quantity of thioanisole did not dissolve completely in the buffer. H₂O₂ (100 µl of a 40 mM solution) was added over 1 h, giving a nominal 2 mM peroxide concentration. Acetophenone (25 µl of a 10 mM solution in methanol) was added as an internal standard, and the solution was extracted with 3 ml of CH₂Cl₂. When necessary, low speed centrifugation was used to separate the layers. The organic layer was concentrated nearly to dryness under a stream of nitrogen and the residue was redissolved in 80% hexane, 20% isopropl alcohol for HPLC analysis. Essentially identical incubations were performed with other thioanisole substituted thioanisoles.

The sulfoxides from thioanisole, p-methylthioanisole, and p-methoxythioanisole were analyzed by isocratic HPLC on a Diacel chiral column eluted with 80% hexane, 20% isopropl alcohol at a flow rate of 0.5 ml/min. A Hewlett Packard model 1040A system equipped with a diode array detector and a Varian 9010 solvent pump system was used for this purpose. The solvent was changed to 60% hexane, 40% isopropl alcohol in experiments with p-nitrothioanisole. The HPLC eluent was monitored at 254 nm with a bandwidth of 6 nm. A thioanisole sulfoxide standard curve was constructed from the peak area ratios obtained after analogous workup of known sulfoxide-acceptor aminohexane mixtures.

**Source of the Oxygen in Thioanisole Sulfonation—Horseradish peroxidase and β-meso-ethylheme reconstituted horseradish peroxidase were incubated with thioanisole as described above except that the peroxide was replaced with [30]O₂H₂O₂. The products were extracted into CH₂Cl₂ and were analyzed by electron impact gas-liquid chromatography-mass spectrometry.**

**Spectroscopic Binding Studies—Difference spectra were recorded on an Aminco DW-2000 spectrophotometer. To each cuvette of a matched pair was added 1 ml of a 10 µM solution of horseradish peroxidase in 50 mM potassium phosphate (pH 7.0) or 50 mM sodium acetate (pH 5.25) buffer containing 5 mg/ml of bovine serum albumin (BSA) as the sole added protein.**

**Guaiacol and Iodide Oxidation**—The kinetics of the oxidation of guaiacol and iodide by native and β-meso-silylhexene-reconstituted horseradish peroxidase were determined on an Applied Photophysics Stoped Flow model SF.17MV instrument because the β-meso-silylhexene-reconstituted enzyme is particularly sensitive to degradation. A 10-nm path length was used. In the case of guaiacol, 1.1 mM H₂O₂ in 50 mM sodium phosphate buffer (pH 7.0) was mixed with an equal volume (125 µl) of the same buffer containing 10 mM sodium phosphate buffer (pH 7.0) monitored at 500 nm. The reaction rate of guaiacol oxidation was determined from the data obtained in the first 3 s. For iodide oxidation, 52.8 µM H₂O₂ in 125 µl of water was mixed with 125 µl of 100 mM sodium acetate (pH 5.25) buffer containing 20 µM horseradish peroxidase and 4.4 mM KI. The formation of I⁻ was monitored at 535 nm, and the reaction rate was determined from the increase in this absorbance between 1 and 10 s.

**Inhibition of Guaiacol Oxidation by Iodide**—The oxidation of guia-
col in the presence of increasing amounts of iodide was assayed at 470 nm on a Hewlett Packard 8452A diode array spectrophotometer. In a typical assay, horseradish peroxidase (5 pmol) was added to 1 ml of 50 mM sodium acetate buffer (pH 5.25) containing guaiacol (2, 4, 8, 10, 20 mM), potassium iodide (0, 100, 200, 300 mM), and sufficient potassium nitrate (300, 200, 100, 0 mM) to maintain a constant ionic strength. Horseradish peroxidase was added to the other components of the mixture just before adding H2O2 (440 nM) to initiate the reaction because the enzyme is inactivated with time if allowed to stand in the assay mixture. The reaction was monitored at 25 °C for 20 s. H2O2-dependent tri-iodide formation also contributed to the change in absorption at 470 nm, so this contribution was independently measured in incubations without the guaiacol and the resulting velocities were subtracted from those for the complete system. Each of the incubations was performed in triplicate and the relative velocities thus obtained were fit to the Michaelis-Menten equation ($v = \frac{V_{max} \times S}{K_m + S}$) by the program KinetAssay® (Copyright 1988, James G. Robertson) to obtain $V_{max}$ and $K_m$ values.

**Inhibition of Iodide Oxidation by Thioanisole**—Horseradish peroxidase-catalyzed iodide oxidation was monitored in the presence of varying amounts of thioanisole. In a typical reaction, 1 pmol of horseradish peroxidase, thioanisole (0, 0.5, 1.0, 1.5 mM), potassium iodide (5, 10, 15, 20, 40, 80 mM), and sufficient potassium nitrate (75, 70, 65, 60, 40, 0 mM) to maintain a constant ionic strength in 1 ml of 50 mM sodium acetate buffer (pH 5.25) buffer were vigorously stirred to insure dissolution of the thioanisole. H2O2 (110 mM final concentration) was added to initiate the reaction. Iodide oxidation at 25 °C was monitored at 354 nm for 20 s on a Hewlett Packard 8450A spectrophotometer. Enzyme-independent oxidation of iodide was measured in control experiments, and the velocities obtained were subtracted from velocities for the enzyme-catalyzed reaction. Data points were obtained in triplicate and were analyzed as described above.

**Inhibition of Guaiacol Oxidation by Thioanisole**—Solutions of horseradish peroxidase (7.5 nM), guaiacol (0.1-10 mM), and thioanisole (0, 0.5, 1.0, or 1.5 mM) in 1 ml of phosphate buffer (pH 7.0) were allowed to mix for at least 10 min before they were transferred to cuvettes and guaiacol oxidation was initiated by adding 6 μl of 0.1 M H2O2 (600 μM final concentration). Guaiacol oxidation was monitored on a Hewlett Packard 8450A spectrophotometer at 470 nm for 20 s.

**Incubation of β-meso-Ethylheme-reconstituted Horseradish Peroxidase with Phenylhydrazine**—Methylphenylhydrazine carboxylate azo ester (4 μl) was hydrolyzed to phenylhydrazine carboxylate in 400 μl of argon-saturated 0.1 M NaOH. A 2-μl aliquot of this solution was added to 1 ml of a solution of 6-meso-ethylheme-reconstituted horseradish peroxidase (10 μM) in phosphate buffer (pH 7.0). Three additional aliquots of phenylhydrazine were added every 5 min while the absorption spectrum of the solution was monitored.

**RESULTS**

**Enantioselectivity of Sulfoxide Formation**—Thioanisole, p-meththioanisole, p-ethoxythioanisole, and p-nitrothioanisole are oxidized by horseradish peroxidase to the corresponding sulfoxides (Table I), as expected from previous reports of horseradish peroxidase-catalyzed thioanisole oxidation (16-18). However, in contrast to reports that horseradish peroxidase-catalyzed sulfoxidations yield racemic products (17, 22), we have observed considerable stereoselectivity in the oxidation of thioanisoles (Table I). The lowest stereoselectivity was observed with p-methoxythioanisole, and the highest with thioanisole, but the enantioselectivities are similar for all four compounds. The identities of the products were established by HPLC comparisons with authentic sulfoxide standards, and their stereochemistries by HPLC on a chiral column that affords excellent resolution of the sulfoxide enantiomers (Fig. 1). Control experiments without horseradish peroxidase indicate that the racemic sulfoxides are formed at up to 4% of the enzymatic rate by direct reaction with H2O2. To circumvent this problem, the incubation time was held to 60 min and, when necessary, the amounts of achiral products formed in parallel incubations without horseradish peroxidase were subtracted from the total product before determining the enantiomeric excess. The same enantiomer ratios were obtained at pH 4.5 and 7.0 (not shown). Colonna et al. (22) probably failed in their original work to detect the enantioselectivity of the reaction due to the long incubation times (1-8 days), but in studies reported as this paper was in preparation also observed chiral product formation (32). Long incubation times and the use of optical rotation rather than a more specific analytical method presumably explain why Kobayashi et al. (17) failed to detect chiral product formation.

**Oxidation of Thioanisole and Iodide by β-meso-Alkylheme-reconstituted Horseradish Peroxidase**—Earlier work demonstrated that the reaction of horseradish peroxidase with ethylhydrazine, which adds an ethyl group to the β-meso carbon of the prosthetic heme group, inactivates the enzyme toward guaiacol oxidation (11). The specific inhibitory effect of the β-meso-ethyl substituent is confirmed by the fact that horseradish peroxidase reconstituted with β-meso-ethylheme is inactive toward guaiacol oxidation despite the fact that it appears to react with H2O2. A β-meso-ethyl group also virtually blocks iodide oxidation but actually increases sulfoxygenase activity (Table II). Thus, β-meso-ethylheme-reconstituted horseradish peroxidase with less than 5% of the guaiacol oxidizing activity of native horseradish peroxidase exhibited 180% of the thioanisole sulfoxidation activity. The enantiomeric excess of thioanisole sulfoxide produced by β-meso-ethylheme-reconstituted horseradish peroxidase decreased from 70 to 28%, indicating that the reconstituted enzyme is more active but less stereoselective (Table II). Reconstitution of the enzyme with hemin did not result in loss of stereoselectivity, so that the loss observed with β-meso-ethylheme is unlikely to be an artifact of the reconstitution process. Control experiments demonstrated that heat-denatured horseradish peroxidase retained 3% of its ability to stimulate guaiacol oxidation and 15% of its ability to catalyze sulfoxygenation, but the resulting sulfoxide product was racemic. Control in-

**TABLE I**

<table>
<thead>
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<tr>
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</tr>
<tr>
<td>OMe</td>
<td>60</td>
</tr>
<tr>
<td>NO2</td>
<td>62</td>
</tr>
</tbody>
</table>

**FIG. 1.** A typical chromatograph from a thioanisole peroxidase incubation. The peak at 21 min is acetophenone, the peaks at 28 and 46 min are the sulfoxide ((S)-(-) and (R)-(+) respectively) enantiomers. The HPLC conditions are given in the text.
cubations with no enzyme did not support guaiacol peroxidase activity but did, as already noted, support slow oxidation of thioanisole to racemic sulfoxide.

Earlier work showed that the oxygen incorporated into the sulfoxide in the horseradish peroxidase-catalyzed oxidation of thioanisoles derives primarily (90%) from the peroxide (16, 19). In agreement with the earlier results, we found 93% incorporation of oxygen from H218O2 into the sulfoxide. Exactly the same value was obtained for the incorporation of peroxide oxygen into the sulfoxide when the reaction was catalyzed by \( \delta \)-meso-ethylheme-reconstituted horseradish peroxidase. This provides clear evidence that the mechanisms of the sulfoxidation reactions catalyzed by native and \( \delta \)-meso-ethylheme-reconstituted horseradish peroxidase are the same.

\( \delta \)-meso-Methylheme-reconstituted horseradish peroxidase retains guaiacol oxidizing activity but is more sensitive to H2O2-dependent inactivation (13). Retention of guaiacol oxidizing activity with a \( \delta \)-meso-methyl but not \( \delta \)-meso-ethyl group is consistent with the hypothesis that loss of peroxidase activity is due to the steric bulk of the \( \delta \)-meso-alkyl group. In contrast to guaiacol oxidation, which is slightly stimulated by a \( \delta \)-meso-methyl substituent, iodide oxidation, and thioanisole sulfoxidation are partially inhibited (Table II). The sulfoxidation enantioselectivity, however, is decreased by the \( \delta \)-meso-methyl as well as \( \delta \)-meso-ethyl groups.

Reaction of \( \delta \)-meso-Ethylheme-reconstituted Horseradish Peroxidase with Phenylhydrazine—The increased sulfoxidation activity and decreased enantioselectivity caused by \( \delta \)-meso-ethyl substitution could reflect a perturbation of the substrate binding site that makes the heme iron atom more accessible. Earlier work has shown that all heme proteins with an accessible iron atom react with phenylhydrazine to form a spectroscopically detectable phenyl-iron complex (33-36). The fact that horseradish peroxidase does not form such a complex is one of the pieces of evidence that argues that its iron atom is not accessible to substrates (10). Spectroscopic examination of the reaction of \( \delta \)-meso-ethylheme-reconstituted horseradish peroxidase with a large excess of phenylhydrazine shows that the modified enzyme also does not form a phenyl-iron complex (not shown). The \( \delta \)-meso substituent therefore does not perturb the active site structure sufficiently to make the heme iron available to phenylhydrazine.

Oxidation of Thioanisole and Iodide by Phenylhydrazine-modified Horseradish Peroxidase—The reaction of phenylhydrazine (or its oxidation product phenylidyazine) with horseradish peroxidase results in covalent binding of 2 phenyl residues to the protein and loss of guaiacol oxidizing activity (10). Full inactivation of the enzyme requires incubation with phenylhydrazine, filtration through a Sephadex G-25 column to remove an unidentified inhibitor of the inactivation reaction, and reincubation with phenylhydrazine (10). Although the sites to which the phenyl residues are bound have not been identified, it is known that loss of guaiacol oxidizing activity correlates with protein modification rather than with the minor degree of \( \delta \)-meso-phenyl heme substitution that also occurs. Phenylhydrazine-modified horseradish peroxidase is also inactive toward the oxidation of iodide but, surprisingly, is considerably more active, albeit less enantiopure, toward thioanisole sulfoxidation than native horseradish peroxidase (Table II).

**Inhibition of Guaiacol and Iodide Oxidation by Thioanisole**—To determine whether thioanisole, guaiacol, and iodide bind to the same or different sites, we investigated the inhibition of guaiacol and iodide oxidation by thioanisole. Inhibition of the oxidation of both substrates is characterized by alteration of both \( K_m \) and \( V_{max} \) and therefore involves something other than competitive kinetics (Figs. 2 and 3). In particular, the change in \( V_{max} \) for guaiacol oxidation indicates that thioanisole and guaiacol bind in different sites. Fitting the data to the classical scheme for mixed kinetics yields \( K_m = 1.2 \text{ mM} \) and \( k_{cat} = 112 \text{ s}^{-1} \) for the horseradish peroxidase-catalyzed oxidation of guaiacol. This gives a value of \( k_{cat}/K_m (9.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \) in reasonable agreement with the value of \( 3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) reported for the second order rate constant for the oxidation of guaiacol by horseradish peroxidase compound II (37). The \( K_i \) value for thioanisole was found to be 0.41 mM (Fig. 2), a value close to the \( K_m \) value of 0.56 mM determined by Doerge (18) for the oxidation of thioanisole by horseradish peroxidase. Inhibition is unlikely to result from consumption of the oxidizing species by thioanisole because the maximum rate of sulfide oxidation in these incubations is \( 6.6 \times 10^4 \text{ M} \text{s}^{-1} \), whereas the guaiacol oxidation rate is 100 times faster (6.5 \( \times 10^7 \text{ M}^{-1} \text{s}^{-1} \)). The \( K_i \) for inhibition of iodide oxidation by thioanisole is 13.0 mM. This value is subject to considerable error because concentrations of thioanisole above the \( K_i \) value could not be examined due to the limited solubility of thioanisole.

**Inhibition of Guaiacol Oxidation by Iodide**—The effects of iodide on guaiacol oxidation have been examined to determine if iodide and guaiacol bind to a common site. Iodide alters the \( V_{max} \) for the oxidation of guaiacol in a concentration-dependent manner and appears to alter the \( K_m \) value for guaiacol, although the error in the \( K_m \) measurements makes conclusions with regard to this parameter less definitive (Fig. 4). The \( V_{max} \) data clearly indicate that iodide does not inhibit guaiacol oxidation by competing for a common binding site because \( V_{max} \) should be independent of the iodide concentration for such a process. The \( K_i \) value for inhibition of guaiacol oxidation by iodide is high (~1000 mM), but it has not been possible to obtain an accurate value due to the variability in the \( K_m \) data.

**Binding of Substrates to the Enzyme**—The difference spectrum obtained when guaiacol binds to horseradish peroxidase (\( \lambda_{max} = 408 \text{ nm} \)) and the \( K_m \) value (11 mM) determined from the substrate concentration dependence of the amplitude of the difference spectrum, are essentially identical to those reported earlier (Fig. 5) (38). The difference spectrum caused by binding of thioanisole is distinct from that obtained for the binding of guaiacol but is similar to those reported for the binding of phenol, hydroquinone, aniline, \( p \)-cresol, and \( p \)-toluidine (38, 39). The spectrum obtained with thioanisole is

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ee</th>
<th>Thioanisole oxidation</th>
<th>Guaiacol oxidation</th>
<th>Iodide oxidation</th>
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<tbody>
<tr>
<td>Native horseradish peroxidase</td>
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<td>100*</td>
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<tr>
<td>( \delta )-Ethyl horseradish peroxidase</td>
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<td>180</td>
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<td>9</td>
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<tr>
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<td>35</td>
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<tr>
<td>No enzyme</td>
<td>0</td>
<td>4*</td>
<td>0</td>
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</table>

* The concentration of thioanisole sulfoxide formed was approximately 197 \( \mu \text{M} \).

* ND, not done.

* This control value has been subtracted from the enzyme-catalyzed values listed in the table.
Peroxidase Oxidation of Thioanisoles and Iodide

**Fig. 2.** Inhibition of guaiacol oxidation by thioanisole. The incubations contained 8 nM horseradish peroxidase, 600 μM H₂O₂, and from top to bottom, 0, 0.5, 1.0, and 1.5 mM thioanisole. The two subplots show the changes in $k_\text{on}$ and $K_a$ as functions of the thioanisole concentration. The incubation details are given under "Experimental Procedures."

**Fig. 3.** Dependence of the $V_{\text{max}}$ and $K_m$ for iodide oxidation on the concentration of thioanisole. The incubation details are given under "Experimental Procedures."

**Fig. 4.** Dependence of the $V_{\text{max}}$ and $K_m$ for guaiacol oxidation on the iodide concentration. The incubation details are given under "Experimental Procedures."

also similar to that reported for the binding of styrene (39).

A $K_a$ value could not be determined for the binding of thioanisole due to variability in the data, but a binding interaction between guaiacol and thioanisole is suggested by a shift in the $K_a$ value for guaiacol from 11 to 19 mM in the presence of 1.5 mM thioanisole. The binding of guaiacol to $\delta$-meso-ethylyme-
reconstituted horseradish peroxidase shifts the λ<sub>max</sub> value to approximately 390 nm and decreases the K<sub>d</sub> value to 4 mM (Fig. 5). An altered spectrum is also obtained when thioanisole binds to β-meso-ethylheme-reconstituted horseradish peroxidase (Fig. 5).

Spectroscopic studies of the binding of guaiacol and thioanisole to the enzyme in the presence of iodide were carried out at pH 5.25, the pH at which iodide oxidation is assayed. The binding of iodide (K<sub>d</sub> = 68 µM) gives a difference spectrum with a broad peak at approximately 380 nm and a trough with a minimum at 424 nm (Fig. 6). Binding of low concentrations (<0.5 mM) of guaiacol at pH 5.25 yields a difference spectrum nearly identical in shape to that of iodide from which the K<sub>d</sub> for guaiacol (0.4 µM) can be calculated. At guaiacol concentrations greater than 1 mM, however, a new peak with a maximum at approximately 407 nm is superimposed on the difference spectrum observed with low guaiacol concentrations (Fig. 6). The K<sub>d</sub> for the binding of guaiacol responsible for the latter change in the difference spectrum is ~1 mM. The difference spectrum observed with 0.4 µM guaiacol is virtually suppressed when iodide (340 µM) is added to both the sample and reference cuvettes (loss of broad absorption in Fig. 6), a result which suggests that guaiacol is displaced by iodide. The addition of saturating amounts of iodide do not, however, suppress the sharp peak at 407 nm observed when the initial guaiacol difference spectrum is obtained with a relatively high (10 mM) concentration of guaiacol (Fig. 6). Iodide thus does not seem to displace guaiacol from the latter site. At pH 5.25, the binding of thioanisole gives rise to a strong difference maximum at 440 nm (Fig. 6). The thioanisole difference spectrum is markedly altered but not suppressed on addition of saturating concentrations of iodide (Fig. 6). Analogous spectroscopic competition experiments between thioanisole and guaiacol give ambiguous results because the low solubility of thioanisole does not allow its concentration to be raised sufficiently above its binding constant.

**DISCUSSION**

Horseradish peroxidase-catalyzed oxidation of thioanisoles to the corresponding sulfoxides proceeds, contrary to earlier reports (17, 22), with considerable enantioselectivity. Approximately 5 times more S than R sulfoxide is produced from thioanisole by horseradish peroxidase at both pH 7.0 and 4.5. Similar enantiomeric excesses are obtained in the oxidation of thioanisoles bearing electron donating (i.e. MeO) and withdrawing (i.e. NO<sub>2</sub>) substituents, indicating that the electronic properties of the substrate do not significantly contribute to the reaction enantioselectivity. This is consistent with the report that the enantioselectivity for the sulfoxidation of substituted thioanisoles by a chiral metalloloporphyrin, in contrast to the epoxidation of substituted styrenes, does not depend on the electronic properties of the substituent (40).

The mechanism of the sulfoxidation reaction appears to be the same in the native and β-meso-ethylheme-reconstituted enzymes because the sulfoxide oxygen in both instances derives primarily (>90%) from the peroxide. The small fraction of the sulfoxide oxygen not derived from the peroxide may
Peroxidase Oxidation of Thioanisoles and Iodide

FIG. 6. Difference spectra at pH 5.25 for the binding to horseradish peroxidase of 10, 20, 30, or 40 μM iodide (A); 1, 2, 5, 10, or 20 mM guaiacol (B); 10 mM guaiacol alone (a) or in the presence of 340 (b), 680 (c), or 1020 μM (d) iodide (C); and finally 1.5 mM thioanisole alone (a) or after the addition of 340 (b) or 680 μM (c) iodide (D).

arise by exchange of the ferryl oxygen with the medium at pH 7 (41) or by diffusion of the sulfur radical cation into the medium. Nevertheless, the enantioselectivity of δ-meso-methyl- or δ-meso-ethylheme-reconstituted or phenylhydrazine-treated horseradish peroxidase is lower than that of native enzyme. The δ-meso-alkyl substituent and protein modification by phenylhydrazine clearly decrease the specificity of the oxidation, probably by causing conformational changes that perturb either the thioanisole-binding site or the groups that sequester the ferryl species. The conformational changes, however, do not make the heme iron atom accessible to reaction with phenyldiazene. The possibility that the oxidation potential of the ferryl species is increased to the point that sulfoxidation involves a looser, more reactant-like transition state is unlikely in view of the absence of a correlation between the electronic properties of the thioanisole and the sulfoxidation enantioselectivity (Table I).

Guaiacol oxidation is blocked by a δ-meso-ethyl group on the heme even though the substituent does not prevent the binding of guaiacol (Fig. 5). In contrast, thioanisole sulfoxidation is not blocked and is actually accelerated by a δ-meso-ethyl substituent (Table II). Horseradish peroxidase reconstituted with δ-meso-methylheme retains both peroxidase and sulfoxidase activities, presumably because the smaller methyl group does not effectively interfere with either process, but the sulfoxidase activity is slightly lower than that of the native enzyme. The lower activity with the δ-meso-methylheme may be due to the lower stability of the enzyme reconstituted with this prosthetic group and the relatively long incubation times required for sulfoxidation (13). This explanation is supported by the fact that the chromophore of the δ-meso-methyl substituted enzyme is lost more rapidly than that of the δ-meso-ethyl substituted enzyme (not shown). A further divergence between guaiacol peroxidation and thioanisole sulfoxidation is provided by the fact that preincubation of horseradish peroxidase with phenylhydrazine completely inactivates the guaiacol activity but stimulates sulfoxidation (Table II). Sulfoxidation and guaiacol peroxidation are thus readily dissociated by heme and protein modifications. It is interesting, in this context, that phenylhydrazine treatment reportedly also does not inactivate the porphobilinogen oxygenase activity of horseradish peroxidase (42).

Iodide oxidation responds to modifications of the heme group or protein like guaiacol rather than thioanisole oxidation. Thus, iodide oxidation is blocked by a δ-meso-ethyl heme substituent or preincubation with phenylhydrazine and is inhibited but not blocked by a δ-meso-methyl heme substituent (Table II). The differences in the sensitivities of horseradish peroxidase-catalyzed guaiacol, iodide, and thioanisole oxidation to δ-meso-alkyl substitution and phenylhydrazine-mediated protein modification suggest that thioanisole oxidation occurs at a different site, and/or by a different mechanism, than the other two reactions. In agreement with this, kinetic analyses indicate that thioanisole inhibits both guai-
col and iodide oxidation by other than competitive mechanisms (Figs. 2 and 3), implying that thioanisole binds to the enzyme at a different site than either guaiacol or iodide. Iodide and guaiacol also appear to bind at distinct sites because iodide inhibits guaiacol oxidation in a noncompetitive manner (Fig. 4). Furthermore, the difference spectra obtained with thioanisole or relatively high concentrations of guaiacol are not suppressed by the addition of iodide, although iodide does suppress the difference spectrum obtained with low concentrations of guaiacol. This suggests that iodide does not displace guaiacol or thioanisole from at least some of their binding sites. The classical peroxidative activity of horseradish peroxidase, measured here as guaiacol oxidation, thus appears to occur at a different site than peroxgenation of the thioanisole sulfur or oxidation of iodide. NMR experiments suggest that iodide and thiocyanate bind to a common site roughly equidistant from the 1 and 8 heme methyl groups and approximately 6–10 Å from the iron atom (26, 43). Nuclear Overhauser (43) and NMR relaxation (44–46) experiments suggest that phenols and other aromatic substrates bind near the 8-methyl of the heme with the aromatic protons 6–10 Å from the iron atom. Evidence for a transfer nuclear Overhauser effect between benzhydroxamic acid and both the 7-propionate α-CH₃ and 8-methyl protons has recently been obtained (47). The NMR studies thus place the binding sites for iodide and phenolic substrates in close proximity to each other near the 6-meso-heme edge, with the phenolic site displaced toward the 8-methyl group relative to the iodide site. It be noted that these assignments are based on NMR studies of the ferric enzyme rather than peroxgenation of the thioanisole sulfur or oxidation of iodide. NMR spectroscopy (48).

The binding of thioanisole and guaiacol at distinct sites rationalizes the fact that the ferryl oxygen is transferred to thioanisole whereas an electron is removed from guaiacol. The reason for binding of thioanisole and guaiacol, both of which are aromatic and of comparable size, to different sites remains unclear. Spectroscopic studies have shown that substrates can be divided into two classes on the basis of the difference spectra observed when they bind to horseradish peroxidase (38, 39). Thioanisole gives a type I spectrum and guaiacol a type II spectrum and therefore belong to the two different classes of substrates (Fig. 5). The physical basis for this spectroscopic classification is obscure, however, because thioanisole is in the same class as normal peroxidase substrates such as phenol, p-cresol, hydroquinone, and aniline (38, 39), and guaiacol is in the same class as resorcinol (39). The spectroscopic differences therefore probably reflect different hydrogen bonding interactions to residues that modulate the chromophore rather than binding to different sites. This interpretation is supported by NMR relaxation studies, which suggest that all the protons of p-cresol (44) and resorcinol (45), which give rise to type II and I difference spectra, respectively, are located within 5.8–6.3 Å of the heme iron atom.

Sequestration of the ferryl species within a protected site to which access is restricted is suggested by the failure to form a phenyl-iron complex with phenyldiazene, or to epoxidize styrene despite the fact that styrene binds to horseradish peroxidase (Kₐ = 4 mM) and causes spectroscopic changes similar to those caused by thioanisole (39). This inability to epoxidize styrene is not due to an inherently unreactive ferryl species because we have shown that styrene is epoxidized by cytochrome c peroxidase (49). Horseradish peroxidase, like all peroxidases so far examined, readily transfers the ferryl oxygen to thioanisole, but the steric and electronic requirements of sulfoxidation and epoxidation differ. Sulfoxidation requires a single orbital contact between the sulfur and the ferryl oxygen, whereas epoxidation, at minimum, requires contact between the two carbon atoms and the oxygen. This geometric difference, in conjunction with differences in the epoxidation and sulfoxidation mechanisms, presumably determines the different sensitivities of the two reactions to sequestration of the ferryl oxygen (23, 24). It is likely that the phenyl ring of thioanisole is bound in a fixed site and that the two enantiomers of the sulfoxide are produced by oxygen transfer to conformers in which the thiomethyl group is in different orientations. Replacement of the thiomethyl with a thioethyl moiety, however, slightly decreases rather than increases the S/R sulfoxide ratio (enantiomeric excess = 40) and therefore does not increase the enantioselectivity.

The present results indicate that phenols, thioanisoles, and iodide bind at distinct sites. The thioanisole site, which is not blocked by a 6-meso-ethyl group, must be adjacent to the ferryl species for oxygen transfer to occur. The aromatic phenol- and iodide-binding sites, in contrast, are suggested by NMR nuclear Overhauser interactions with the 1- and/or 8-methyls (26, 28, 43, 47), NMR relaxation distance measurements (44–46), and sensitivity to 6-meso-ethyl substitution (Table II), to be near the 6-meso heme edge with the aromatic site close to the 8-methyl and 7-propionate α-methylene group and the iodide site between the 1- and 8-methyls. The spatial relationships between the heme and the three binding sites are summarized schematically in Fig. 7, although the detailed placement and orientation of the substrate binding sites and their relationship to the protein structure remain to be defined. The placement of substrates relative to the ferryl oxidizing species is important because it is one of the factors that determines whether a one- or two-electron oxidation is observed.

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