On the Mechanism of the Peroxidase-Catalyzed Oxygen-Transfer Reaction†

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ABSTRACT: We reported evidence that horseradish peroxidase (HRP) and chloroperoxidase (CPO) catalyze oxygen transfer from H_2O_2 to thioanisoles [Kobayashi, S., Nakano, M., Goto, T., Kimura, T., & Schaap, A. P. (1986) *Biochem. Biophys. Res. Commun. 135*, 166–171]. In the present paper, the reaction mechanism of this oxygen transfer is discussed. The oxidation of para-substituted thioanisoles by HRP compound II showed a large negative ρ value of −1.46 vs. the σ^+ parameter in a Hammett plot. These results are in accord with the formation of a cation radical intermediate in the rate-determining step. Hammett treatments for HRP- and CPO-dependent S-oxygenations did not provide unequivocal proofs to judge the reaction mechanism, because of the poor correlations for σ^+ and σ_p parameters. Different behavior was found in kinetics and stereoselectivity between the two enzymes. Results in the present study and recent studies strongly suggested the formation of a cation radical intermediate. The oxygen atom would transfer by reaction of compound II and the cation radical intermediate. Although involvement of the cation radical was not confirmed in the CPO system, a similar mechanism was proposed for CPO.

Peroxidases are heme proteins that catalyze the oxidation of a wide variety of organic molecules at the expense of peroxides. The catalytic cycle involves two intermediate forms of the enzyme, compounds I and II. The electronic structures of these compounds have been well characterized by various physical methods. These active species are able to abstract one electron from a substrate to produce a free radical (one-electron-transfer oxidation) which undergoes coupling, disproportionation, and/or reaction with molecular oxygen (Yamazaki, 1977). Under certain conditions, compound I is considered to be reduced to the ferric form without producing compound II (two-electron-transfer oxidation). The involvement of one- and two-electron-transfer oxidations varies, depending on the substrate (Ohtaki et al., 1977; Nakamura et al., 1985).

Compound I (Groves et al., 1982) and compound II (White & Coon, 1980) like species have been proposed as the active forms of cytochrome P-450. It is reasonable to postulate that an active species of heme enzyme that is structurally similar to P-450 would be able to catalyze reactions typical of P-450. For example, N- and O-dealkylations are known to be assisted by both peroxidases and P-450, although different rate-determining steps were indicated from studies of deuterium isotope effects (Miwa et al., 1984a,b; Kedderis & Hollenberg, 1984). However, the question of whether a monooxygenated heme donates its oxygen atom to a substrate has been noted as a distinctively different catalytic function of P-450 compared to peroxidases. We have recently reported that oxygenation of sulfides to sulfoxides can be effected by peroxidases as well as P-450 (Kobayashi et al., 1986). In this paper, we provide evidence, from ^{18}O-labeling studies, that direct oxygen atom transfer to sulfides occurs during both HRP[1]- and CPO-dependent oxygenation.

Two mechanisms are considered for this reaction. One is the "one-step oxygen-transfer mechanism" with the oxygen atom concertedly moving from compound I to thioanisoles. Another is the "two-step oxygen-transfer mechanism", also referred to as the "rebound mechanism", involving a cation radical intermediate. The conformation of the sulfide and charge development on the sulfur atom would be quite different in the transition state between eq 2 and 3.

one-step oxygen-transfer mechanism

$$Fe^{3+} + H_2O_2 \xrightarrow{k_1} (Fe{=}O)^{3+} + H_2O \quad (1)$$

$$(Fe{=}O)^{3+} + S \xrightarrow{k_2} Fe^{3+} + SO \quad (2)$$

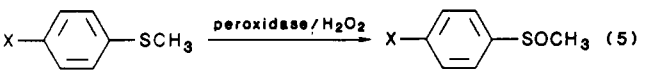
two-step oxygen-transfer (rebound) mechanism

$$Fe^{3+} + H_2O_2 \xrightarrow{k_1} (Fe{=}O)^{3+} + H_2O \quad (1)$$

$$(Fe{=}O)^{3+} + S \xrightarrow{k_3} (Fe{=}O)^{2+}{\cdot}S^{\bullet+} \quad (3)$$

$$(Fe{=}O)^{2+}{\cdot}S^{\bullet+} \xrightarrow{k_4} Fe^{3+} + SO \quad (4)$$

In this paper, we have conducted investigations of stereochemistry and substituent effect on rates of oxygenation (eq 5). Linear free energy relationships will allow us to distinguish these mechanisms.



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a, X = $OCH(CH_3)_2$; **b**, X = OCH_3; **c**, X = $NHCOCH_3$; **d**, X = CH_3; **e**, X = H; **f**, X = Cl; **g**, X = $OCOCH_3$; **h**, X = CN; **i**, X = NO_2

Materials and Methods

Chemicals. Thioanisole and para-substituted thiophenols were obtained from the Aldrich Chemical Co. Para-substituted thioanisoles were prepared by reaction of the corresponding thiophenols with CH_3I in basic methanol solution (Wargner & Zook, 1965). Para-substituted phenyl methyl

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[1] Abbreviations: HRP, horseradish peroxidase; CPO, chloroperoxidase; Fe^{3+}, ferric form; $(Fe{=}O)^{3+}$, compound I; $(Fe{=}O)^{2+}$, compound II; HPLC, high-performance liquid chromatography; ee, enantiomeric excess.

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sulfoxides (**2a**–**2g**) were synthesized by photosensitized oxidation (methylene blue/methanol) of the corresponding thioanisoles. Other sulfoxides (**2h** and **2i**) were prepared by the reaction of **1h** and **1i** with $NaIO_4$ in aqueous methanol solution (Johnson & Keiser, 1966). All the compounds prepared were isolated by column chromatography on silica gel, and then crude materials were purified by distillation or recrystallization. The purity of these compounds was checked by HPLC, and they were identified by NMR spectrometry with a Varian S-60T (data not shown). The concentration of H_2O_2 was determined by titration with permanganate (Flascka et al., 1969).

Enzymes. HRP (type VI) and CPO (RZ 1.0) were purchased from Sigma Chemical Co. The concentrations of HRP and CPO were determined spectrophotometrically at 403 nm; molar extinction coefficients of 102 000 (Schonbaum & Lo, 1972) and 75 300 (Hager, 1970) M^{-1} cm^{-1}, respectively, were used.

Oxidation of Thioanisoles (**1**) *by HRP Compound II.* HRP compound II was prepared according to a modification of the method of Ohtaki et al. (1982). A 1-mL aliquot of 0.1 M acetate (pH 5.0) containing 1.21×10^{-9} mol of HRP was treated with 1.71×10^{-9} mol of H_2O_2 at 25 °C. The mixture was allowed to stand until the absorbance of 430 nm was no longer significantly changed. The reaction was initiated by the addition of sulfides and followed by the decrease of compound II at 430 nm with a Cary 219 spectrophotometer.

Kinetics. Kinetic experiments were carried out at pH 5.0 and 25 °C. The reaction mixtures contained 1.02×10^{-9} mol of HRP or 6.6×10^{-12} mol of CPO, $(3–90) \times 10^{-8}$ mol of thioanisole, 1.24×10^{-6} mol of H_2O_2, and 0.1 M acetate buffer in a total volume of 3 mL. Reaction was initiated by the addition of H_2O_2 and followed by the decrease of the absorption of the thioanisoles. Initial rates were calculated by using the difference of molar extinction coefficients ($\Delta\epsilon$) between the sulfides and the corresponding sulfoxides. $\Delta\epsilon$ and monitored wavelength were as follows [$\Delta\epsilon$ in M^{-1} cm^{-1} (λ in nm)]: **a**, −1680 (250); **b**, 2710 (252); **c**, 150 (266); **d**, 8450 (252); **e**, 7000 (250), **f**, 10 400 (256); **g**, 8480 (250); **h**, 11 960 (283); **i**, 11 910 (350). A negative value indicates a decrease in absorbance for conversion of sulfide to sulfoxide.

Stereochemical Studies. A mixture containing 30 mg of **1d** and 9 mg of HRP in 700 mL of 0.1 M acetate buffer (pH 5.0) was incubated at 27 °C. The reaction was initiated by the addition of 2 mL of 4.95×10^{-4} M H_2O_2. To the reaction mixture were added 10 mg of **1d**, 3 mg of HRP, and 2 mL of the H_2O_2 solution at 2-h intervals. Total amounts of **1d** and HRP used were 100 and 30 mg, respectively. The reaction was continued for 6 h after the final additions of **1d**, HRP, and the H_2O_2 solution. The products were extracted with $CHCl_3$ and dried over Na_2SO_4. Sulfoxide (**2d**) was isolated by column chromatography on silica gel. Further purification by HPLC on a reversed-phase column gave 28 mg of **2d**.

In the case of the CPO system, **2d** was prepared essentially by the same procedure as described above except that the total amounts of **1d** and CPO used were 100 and 3 mg, respectively. The amount of purified **2d** obtained was 45.7 mg.

RESULTS

Kinetic Studies for S-Oxygenation. Equation 3 shows an electrophilic reaction of compound I to thioanisoles. Reaction rates would increase with the electron-donating property of substituents, and the logarithm of k_2 will correlate well with the σ_p parameter in a Hammett plot. An electron-transfer reaction from thioanisoles to compound I is shown in eq 3. In this mechanism, reaction rates would similarly increase with the electron-donating property of substituents. However, a good correlation between the logarithm of k_3 and the σ^+ parameter in a Hammett plot is expected, as was obtained for the oxidation by compound II.

Table I: Rate Constants for HRP- and CPO-Dependent S-Oxygenation of *p*-X-$PhSCH_3$[a]

substituents (X)	$k_{cat} \times 10^{-5}$ (M^{-1} s^{-1}) HRP	CPO	substituents (X)	$k_{cat} \times 10^{-5}$ (M^{-1} s^{-1}) HRP	CPO
$(CH_3)_2CHO$	35.5	3.97	Cl	17.7	3.72
CH_3O	25.4	5.01	CH_3CO_2	4.87	1.01
CH_3CONH	10.1	0.99	NC	4.36	0.32
CH_3	13.6	3.37	O_2N	4.49	0.19
H	3.78	9.14			

[a] Experimental conditions were as described under Materials and Methods.

On the assumption of steady-state kinetics, initial velocities v for one- and two-step oxygen-transfer reactions are given as eq 6 and 8, respectively, where [E], [S], and [H_2O_2] indicate initial concentrations of enzyme, substrate, and hydrogen peroxide. As can be seen from eq 7 and 9, both k_2 and k_3 will be obtained as 1/slope in [E]/v vs. 1/[S] plots. Since both rate constants are the same value, k_{cat} is used instead of k_2 and k_3.

one-step oxygen-transfer mechanism

$$v = \frac{k_1 k_2 [S][H_2O_2]}{k_1[H_2O_2] + k_2[S]}[E] \quad (6)$$

$$\frac{[E]}{v} = \frac{1}{k_1[H_2O_2]} + \frac{1}{k_2[S]} \quad (7)$$

two-step oxygen-transfer mechanism

$$v = \frac{k_1 k_3 k_4 [S][H_2O_2]}{k_1 k_3 [H_2O_2][S] + k_4(k_1[H_2O_2] + k_3[S])}[E] \quad (8)$$

$$\frac{[E]}{v} = \frac{1}{k_4} + \frac{1}{k_1[H_2O_2]} + \frac{1}{k_3[S]} \quad (9)$$

The calculated k_{cat} are listed in Table I. Reactivity of para-substituted thioanisoles was found to increase with the order of the electron-donating property of substituents. The kinetic data for **1a** and **1b** were excluded from further mechanistic analyses for both HRP and CPO systems for the following reasons. In the HRP system, zero-order kinetics was observed only when the substrates were **1a** and **1b**. In addition, the initial rate for these derivatives was not affected by the concentration of H_2O_2. These data suggested that the rate-determining steps for **1a** and **1b** are different from those for others. On the other hand, although zero-order kinetics was not observed for all derivatives in the CPO-dependent S-oxygenation, CPO competitively catalyzed both S-oxygenation and O-dealkylation on **1a** and **1b** (Kobayashi et al., 1986).

Hammett plots of the logarithm of k_{cat} vs. σ^+ and σ_p were examined. Correlations for σ_p ($\rho = -0.39$, $r = 0.513$) and σ^+ ($\rho = -0.31$, $r = 0.571$) were very poor for the HRP-dependent S-oxygenation. A poor correlation vs. σ^+ ($\rho = -0.75$, $r = 0.610$) and a relatively good correlation vs. ρ_p ($\rho = -1.40$, $r = 0.824$) (Figure 1A) were obtained for the CPO-dependent S-oxygenation.

Oxidation of Thioanisoles by HRP Compound II. According to electrochemical studies of sulfide oxidation (Uneyama & Torii, 1971), one-electron oxidation of a sulfide gives a cation radical intermediate that requires a water molecule as an oxygen source to yield the corresponding sulfoxide. Since compound II is a one-electron acceptor, one reasonable

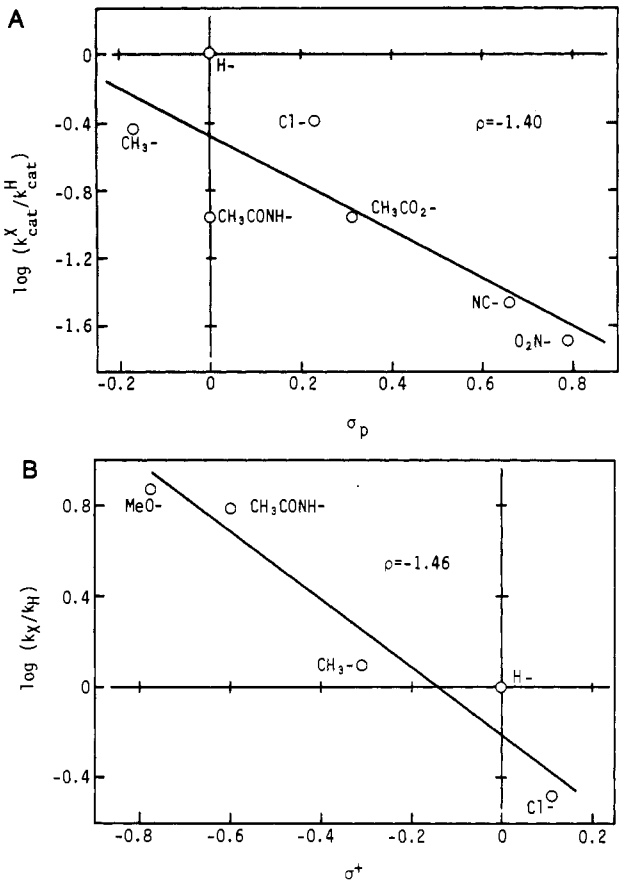

FIGURE 1: (A) Hammett plot of the logarithm of relative k_{cat} for CPO-dependent S-oxygenation of para-substituted thioanisoles vs. σ_p parameters and (B) Hammett plot of the logarithm of relative second-order rate constant for reduction of compound II by para-substituted thioanisoles vs. σ^+ parmeters. Substituent constants taken from Murov (1973).

mechanism for the reaction of compound II with the substituted sulfides similarly involves formation of the sulfide cation radical in the rate-determining step (eq 10). Subsequent disproportionation of the cation radical (eq 11) generates the dication which is converted to the corresponding sulfoxide (eq 12).

$$(Fe{=}O)^{2+} + ArSCH_3 + 2H^+ \xrightarrow{k} Fe^{3+} + ArS^{\cdot+}CH_3 + H_2O \quad (10)$$

$$2ArS^{\cdot+}CH_3 \rightarrow ArS^{2+}CH_3 + ArSCH_3 \quad (11)$$

$$ArS^{2+}CH_3 + H_2O \rightarrow ArSOCH_3 + 2H^+ \quad (12)$$

Six thioanisoles (**1a–1f**) reduced compound II, whereas the other three (**1g**, **1h**, and **1i**) were unable to reduce compound II. Reaction rates were calculated on the assumption of the stoichiometrical conversion of ferric form to compound II. The reduction of compound II obeyed pseudo-first-order kinetics. The rate constant for the spontaneous decay of compound II (2.10×10^{-4} s^{-1}) was subtracted from observed pseudo-first-order rate constants. The values obtained were proportional to the concentration of the thioanisoles. Calculated second-order rate constants (k) were 291 M^{-1} s^{-1} for **1b**, 243 M^{-1} s^{-1} for **1c**, 48.6 M^{-1} s^{-1} for **1d**, 39.0 M^{-1} s^{-1} for **1e**, and 13.0 M^{-1} s^{-1} for **1f**. The rate constant is decreased with increasing electron-withdrawing ability of the substituents.

A Hammett plot of the logarithm of the relative second-order rate constant gave a good linear correlation ($r = 0.968$) for σ^+ with a ρ value of −1.46 (Figure 1B) and a much poorer correlation for σ_p ($\rho = -2.17$, $r = 0.722$).

Stereochemical Studies. Stereochemical studies for the S-oxygenation by peroxidases were carried out, and results were compared with those of P-450. P-450 is known to produce chiral sulfoxides to a degree dependent on the structure of sulfides, for example, 14.1 ee % in **2d** (Takata et al., 1980). In this study, we chose the same thioanisole (**1d**) as a substrate for peroxidases. HRP induced no chirality on **2d** within experimental error (c 0.528, acetone). CPO produced chiral **2d**, $[\alpha]^{25}_D$ +16.2° (c 0.672, acetone). Pure (*R*)-**2d** showed $[\alpha]^{25}_D$ +128.5° (c 0.394, acetone). From a comparison of these results, the absolute configuration induced on **2d** by CPO is confirmed as *R* and the optical yield became 12.7 ee%.

Discussion

The one-electron oxidations by compound II showed the excellent correlation vs. σ^+ but not ρ_p in Hammett plots. The results are consistent with the mechanism that the oxidation of thioanisoles with compound II involves the cation radical intermediate.

The reaction mechanism for HRP-dependent S-oxygenation could not be judged from Hammett treatment because of the small correlation coefficients against both σ^+ and σ_p parameters. This failure is probably due to competitive formation of sulfoxides via at least two mechanisms, as was previously

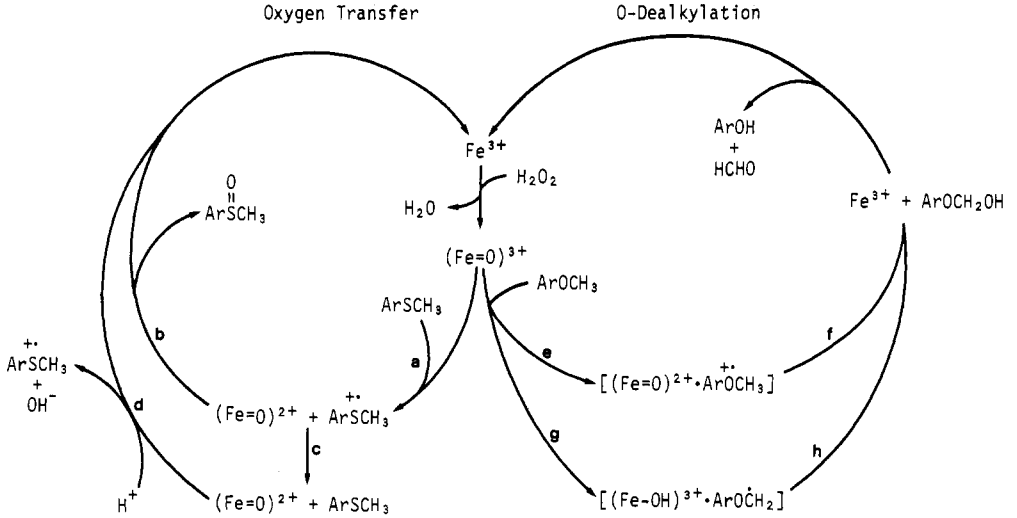

FIGURE 2: Proposed mechanism for S-oxygenation by peroxidases. $[(Fe{=}O)^{2+}{\cdot}ArO^{\cdot+}CH_3]$ and $[(Fe{-}OH)^{3+}{\cdot}ArO^{\cdot}CH_2]$ express the complex of compound II and a cation radical and the complex of a protonated form of compound II and a radical, respectively.

pointed out from the ^{18}O-labeling study (Kobayashi et al., 1986). The labeling study showed that the oxygen atom of sulfoxides was incorporated from both hydrogen peroxide and water. The sulfoxide with the oxygen atom obtained from water must be produced via cation radical intermediates, which could be formed by one-electron oxidations of thioanisoles with compound I (pathway a in Figure 2). The resultant compound II is also able to oxidize another thioanisole to produce the cation radical (pathway d). However, pathway d does not function on the oxidation of **1g–1i**, suggesting that those oxidation potentials are higher than that of compound II. Thus the formation of the cation radical is highly possible in the HRP system. It would be reasonable to consider that the oxygen atom transfers by the reaction of the cation radical and the compound II (pathway b), the two-step oxygen-transfer mechanism. Compound II competitively reacts with both thioanisoles and the corresponding cation radicals. The competition of two reactions would be dependent on the oxidation potentials of the cation radical and compound II.

Meanwhile, HRP did not catalyze O-dealkylation. It might not be able to abstract either the α-hydrogen atom or one electron from the ether oxygen atom, whereas it did abstract the α-hydrogen atom from *N*-methylaniline (Miwa et al., 1983b).

CPO utilized mostly hydrogen peroxide as an oxygen source. The one-step oxygen-transfer mechanism by which compound I stoichiometrically transfers its oxygen atom to thioanisoles was considered for the CPO system. Although the kinetic results of a relatively good correlation for σ_p parameters were obtained for CPO-dependent S-oxygenation, lack of the parameters for **1a** and **1b** in the Hammett treatment did not allow us to consider the reaction mechanism. CPO catalyzed O-dealkylation under the present reaction conditions. A possible mechansim involves the formation of cation radical followed by the deprotonation from α-carbon (pathways e and f). The high incorporation of the oxygen-atom of hydrogen peroxide and the chiral induction might be anticipated, only when pathway b proceeds faster than pathway c. However, the involvement of the cation radical intermediate is not confirmed in the CPO system.

The rebound mechanism is proposed for S-oxygenation by P-450 because of a ρ value of –0.16 vs. σ^+ and because of 1.3% of ^{18}O incorporation from water with respect to **2d** (Watanabe et al., 1980). The different behavior of the HRP and P-450 systems obviously suggests the involvement of a different transition state between them. The low incorporation of the water oxygen atom and the induction of chirality might be anticipated, assuming that a cation radical intermediate reacts with a compound II like species of P-450 faster than with the compound II of HRP.

Oxidation of halogen ion (X^-) by peroxidases leads to halogenation of biological substances. Hypohalous ion (XO^-) and halonium ion (X^+) have been long proposed as possible active species in the oxidation of X^-. Although ^{18}O labeling is one method to identify a primary oxidized species, its application is quite difficult because the oxygen atom of XO^- is exchangeable to that of water. The results presented in this paper coupled with recent studies demonstrate that oxygen transfer would be an intrinsic function of peroxidases. Thus, peroxidases could catalyze the oxygenation of X^- to produce XO^- rather than two-electron oxidation of it to produce a much more labile species, X^+.

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