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An Evidence of the Peroxidase-dependent Oxygen Transfer from Hydrogen Peroxide to Sulfides

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Summary: Horseradish- and chloro-peroxidase catalyzed oxidation of sulfides have been investigated. Thioanisoles were oxygenated to the corresponding sulfoxides by such peroxidases at the expense of H_2O_2. Dealkylation was observed only in the chloroperoxidase-dependent oxidations of p-methoxy- and p-iso-propoxy-thioanisoles. The experiments with ^{18}O-labeled H_2O_2 indicated that an oxygen atom of H_2O_2 is incroporated into the sulfoxides. These research lead to the conclusion that compound I or II is capable of acting as an oxygen donor as well as an electron acceptor.

Peroxidases catalyze the oxidation of a wide variety of organic molecules at the expense of peroxides. The catalytic cycle, the so-called peroxidase-oxidase reaction, involves two active species, compounds I and II, which are able to abstruct one electron from substrate to produce a free radical followed by coupling, disproportionation and/or reaction with molecular oxygen (1). Under certain conditions, compound I has been considered to be directly reduced to the ferric form (2). It is unknown whether the two electron transfer process accompanies an oxygen transfer.

While electronic structures of compounds I and II have been well characterized by various physical methods, little is known of the active form of P-450. A compound I-like species is believed to be an active form of P-450 (3). A com-

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Abbreviations: HRP, horseradish peroxidase; CLP, chloroperoxidase; THA, thioanisole; P-450, cytochrome P-450.

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pound II-like species has been also proposed as an active form of P-450 in the peroxide-supported reactions (4). Thus, although the active species of P-450 is still a mystery, the oxygen transfer is a characteristic of P-450 even when activated by different systems.

Peroxidases are known to catalyze the N-and O-dealkylations as does P-450, the different rate determining steps being indicated from studies of deuterium isotope effects (5,6,7). These results give rise to the important question as to whether either compound I or II acts as an oxygen donor to a substrate. To solve this question, thioanisole derivatives were chosen as substrates of the peroxidases for the reason that the sulfoxide oxygen atom is unexchangeable with that of water molecule. Although P-450 is well known to oxygenate sulfides to corresponding sulfoxides (8), there has been no report with respect to S-oxygenation by perodiases. The present paper includes the characterization of S-oxygenation by HRP and CLP, and evidence of the peroxidase-catalyzed oxygen transfer from H_2O_2 to thioanisoles.

Materials and Methods: HRP and CLP were obtained from Sigma Chemical Co. p-Substituted thioanisoles were prepared by reaction of the corresponding thiophenols with CH_3I in basic methanol solution (9). P-Substituted phenylmethyl sulfoxides were synthesized by photosensitized oxidation of the thioanisoles. Oxygen-18-water (98% ^{18}O excess) and oxygen gas (97% ^{18}O excess) were purchased from Cambridge Isotope Lab. and Amersham, respectively. Labeled-hydrogen peroxide was prepared by the methods of Sawaki and Foote (10) using HNO_3 substituted for HCl. Product analysis; The concentrations of HRP and CLP were determined spectrophtometrically at 403 nm using milimolar extinction coefficients of 102 (11) and 75.3 (12), respectively. Hydrogen peroxide was diluted to 50 mM with 100 mM acetate (pH 5.0) and the concentration was determined by titration with permanganate (13).

Incubation conditions were varied with the substituents based on the kinetic results. Incubation mixtures contained 390 nmol of HRP or 1 pmol of CLP, 200 nmol of thioanisoles, and 350 nmol of H_2O_2 in 1.5 ml of 100 mM acetate (pH 5.0). The incubations were carried out for 1-6h at 25° C. The products were extracted with $CHCl_3$, concentrated, and subjected to analysis. The sulfoxides were identified by a comparison of the retention time with those of the authentic samples and quantified relative to an internal standard by HPLC.

Kinetics; Kinetics were carried out at pH 5.0 and 25° C. The reaction mixtures contained 1.02 nmol of HRP or 6.6 nmol of CLP, 30-900 nmol of thioanisoles, and 1.24 μmol of H_2O_2 in 3 ml of 100 mM acetate buffer. Reaction was initiated by the addition of H_2O_2 and followed by the decrease of the absorption of thioanisoles with a Cary 219 spectrophotometer. The Vmax values were determined from the double reciprocal plots of more than 10 data points with a least squares fits.

^{18}O-Tracer Studies; Three thioanisoles were subjected to this experiment. Oxygen-18-labeled H_2O_2 and H_2O were diluted to 80 mM with 100 mM acetate buffer (pH 5.0) and one-tenth by volume with the same buffer, respectively. The initial mixtures contained 150 nmol of HRP or 200 pmol of CLP, 0.2 mg of thioanisoles, and 200 nmol of the labeled H_2O_2 in 3 ml of 100 mM acetate buffer (pH 5.0). To the reaction mixtures were added 15 nmol of HRP or 20 pmol of CLP, and 20 nmol of

the labeled H_2O_2 several times at 2-4 h interval. After the 12 h incubation, the products were extracted with ether, dried over Na_2SO_4 , and subjected to G.C. mass spectometry.

In the experiments using the labeled water, the reaction was carried out under the same conditions as described above, but substituting unlabeled H_2O_2 for the labeled one.

Results

Although thionaisoles are spontaneously oxidized by H_2O_2 alone, the reaction is accelerated markedly by HRP and CLP, with the initial rate increasing in proportion to the concentration of the enzyme. Boiled HRP lost 98% of the native activity under the same reaction conditions. Lactoperoxidase showed no activity against the thioanisoles used in this tudy. The values of Vmax/[E] indicated that CLP was much more active than HRP as shown in Table 1. In the HRP system, the reactivity increased with the electron donating substituents which reduce the oxidation potentials, while obviously different results were obtained in the CLP system. From these results, dealkylation was expected of p-CH_3O-and p-iso-propoxy-THA by CLP-dependent oxidation. p-Hydroxy-THA was actually produced from such derivatives in the CLP systems, but not the HRP systems. The absence of any other products in HPLC analyses indicated that the dealkylation occured only on the oxygen atom.

CLP and HRP showed different patterns of product accumulation (Figure 1). In the CLP system, the amounts of sulfoxide increased rapidly and soon leveled off.

Table 1. Comparison of S-oxygenation activity of HRP and CLP to P-substituted - thioanisoles

substituents	HRP	CLP	Ep (V)[a]
	Vmax/ E, min^{-1}		
ISO-propoxy-	24.0	1720	1.10
Methoxy-	19.4	3110	1.13
Methyl-	4.1	34600	1.27
H-	1.3	23100	1.41

a. The Ep values were measured by cyclic voltammetry in 0.1 M $(C_2H_5)_4NClO_4$ /CH_3CN

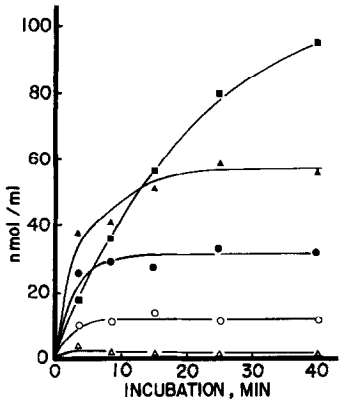

Figure 1. Accumulation patterns of oxygenated and dealkylated products in the HRP and CLP systems. Closed symbols express the sulfoxides; ■, p-iso-propoxy-phenylmethyl sulfoxide in the HRP system; ▲, the same compound and ●, p-CH_3O-phenylmethyl sulfoxide in the CLP systems. Open symbols express p-HO-thioanisole in the CLP systems; ○, from p-CH_3O-THA and △, from p-iso-propoxy-THA .

CLP was found to be abraptly inactivated during the reaction. However HRP, slowly but smoothly, oxidized the thioanisoles. In the peroxidase-oxidase reaction, molecular oxygen is utilized as either an electron acceptor or an oxygen donor, or as both (1). The oxidation of p-CH_3O-THA under aerobic and anaerobic conditions had no difference in either the initial rates or the stoichiometries between the produced sulfoxides and added H_2O_2 (data not shown), suggesting no involvements of the molecular oxygen in the S-oxygenation by peroxidases.

The experiments with ^{18}O-H_2O_2 show that the oxygen atom originates predominantly from H_2O_2 (Table 2). In the HRP/p-CH_3O-THA/H_2O_2 systems, a relatively low incorporation of the oxygen atom from H_2O_2 indicated the utilization of other oxygen sources. The experiments with ^{18}O-H_2O showed that 10% of the

Table 2. ^{18}O-Incorporation from H_2O_2 to P-substituted-thioanisoles

Enzymes	Methoxy-	Methyl-	H-
	% incorporated in		
HRP	66(10)[a]	85	—
CLP	—	90	92

a. The value in parenthesis was the result obtained from the experiment with ^{18}O-H_2O

oxygen atom came from water. These results suggest that the oxygen source might vary depending on the substituents of the substrate.

Discussion

Like N-and O-dealkylations, S-oxygenation has now been characterized as one of the common reactions between peroxidases and P-450. Peroxidase-dependent oxygen transfer from H_2O_2 to thioanisoles was demonstrated by ^{18}O-labeled experiments. Two mechanisms, oxene and rebound, are to be considered in the light of these quite interest results. Scheme 1 depicts direct oxygen transfer from compound I to the sulfur atom by an oxene process. On the other hand, scheme 2 shows the electron transfer from the sulfur atom to compound I followed by the coupling of the resulting sulfur radical cation and compound II. The rebound mechanism has been already proposed to the S-oxygenation (14) as well as N- and O-dealkylations by P-450.

$$(Fe{=}O)^{3+} + \rangle S \longrightarrow Fe^{3+} + \rangle S{=}O \qquad \text{(Scheme 1)}$$

$$(Fe{=}O)^{3+} + \rangle S \longrightarrow (Fe{=}O)^{2+} + \rangle S^{+\bullet}$$

$$(Fe{=}O)^{2+} + \rangle S^{+\bullet} \longrightarrow Fe^{3+} + \rangle S{=}O \qquad \text{(Scheme 2)}$$

In the HRP/p-CH_3O -THA/H_2O_2 system, not only H_2O_2 but also H_2O was able to be an oxygen source. The results suggested that the reaction involves at least one more different mechanism other than the oxene or rebound mechanism. According to electrochemical studies of sulfide oxidation (15), one electron oxidation of a sulfide affords a cation radical as an intermediate, then two radical cations disproprotionate to give one molecule each of sulfide and dication. The dication, then, reacts with a water molecule to give a sulfoxide and two protons. If peroxidases catalyze the one electron oxidation of thioanisoles, the oxygen atom of sulfoxide should be supplied from water as represented in scheme 3.

$$(Fe{=}O)^{3+} \text{ or } (Fe{=}O)^{2+} + \rangle S \longrightarrow (Fe{=}O)^{2+} \text{ or } Fe^{3+} + \rangle S^{+\bullet}$$

$$2\,\rangle S^{+\bullet} \longrightarrow \rangle S + \rangle S^{++} \qquad \text{(Scheme 3)}$$

$$\rangle S^{++} + H_2O \longrightarrow \rangle S{=}O + 2\,H^+$$

The importance of the one electron transfer mechanism might be highly dependent on the stability of the radical cation. The lifetime of the radical cation of p-

CH_3O-THA is relatively long so that any radical cations which escape free from the proximity of the heme could undergo the reactions shown in scheme 3. If the radical cation is less stable, no incorporation of oxygen from H_2O would be expected, even though some of the radical cation may have escaped from the enzyme active site.

Even thought the present results did not clarify the mechanism of S-oxygenation by the peroxidases, they indicate that oxygen transfer may be a fundamental function of the mon-oxygenated heme complexes,i.e. compound I or II.

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