**Mechanism of Reaction of Fatty Acid Hydroperoxides with Soybean Peroxygenase***

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Soybean peroxygenase, a membrane-bound hemoprotein, catalyzes sulfoxidation of alkylaryl sulfides (Blée and Durst, 1987; Blée and Schuber, 1989) and efficient epoxidation of unsaturated fatty acids such as oleic and linoleic acids (Blée and Schuber, 1990a). Epoxide fatty acids are of physiological importance because they are intermediates in the biosynthesis of plant defense compounds and of cutin monomers (Blée and Schuber, 1990a, 1990b). Peroxygenase is a unique oxidase, distinct from cytochrome P-450-dependent enzymes and peroxidases, which accepts only hydroperoxides as the source of oxidant. In both sulfoxidation (Blée and Durst, 1987) and epoxidation reactions (Blée and Schuber, 1990a), the enzyme catalyzes oxygen transfer from the hydroperoxide to the substrate. We have now investigated the molecular mechanism of reaction of peroxygenase with hydroperoxides derived from unsaturated fatty acids, i.e. the physiological cosubstrates of this enzyme. In addition to mechanistic considerations on the catalytic properties of the peroxygenase, this study also addresses the contribution of the enzyme to the formation of naturally occurring metabolites of fatty acid hydroperoxides (for a review, see Gardner, 1991).

The reactions of ferric hemoproteins or model systems with hydroperoxides have been well studied. Cleavage of the hydroperoxide O–O bond and oxidation of the iron to a ferryl-oxo complex is generally observed. Both heterolytic and homolytic cleavages are known to occur with metalloporphyrins, depending on the structure of the peroxy compounds, the coordination sphere of the metal (White et al., 1980; Lee and Bruce, 1985; Mansuy et al., 1989) and on the protein component (Labèque and Marnett, 1987). For example, it has been demonstrated that both homolytic (White et al., 1980; Blake and Coon, 1981) and heterolytic cleavages take place during the reaction of cytochrome P-450 enzymes with hydroperoxides (Thompson and Wand, 1985). The exact nature of the iron-oxo species formed when soybean peroxygenase reacts with hydroperoxides remains to be determined. However, the results obtained in our studies on the mechanism of sulfoxidation, e.g. 18O labeling experiments, and the highly stereospecific oxidation we have observed, led to the conclusion that soybean peroxygenase most probably mediates heterolytic cleavage of the peroxy O–O bond (Blée and Schuter, 1989). Similarly, in the epoxidation reactions catalyzed by this enzyme we could rule out a cooxidative process involving free radical species (Blée and Schuber, 1990a). Consistent with a heterolytic cleavage of the peroxy catalyzed by the peroxygenase, we have shown that the cosubstrate 13-hydro-
peroxyoctadeca-9(2),11(E)-dienoic acid (13-HPOD) was converted predominantly into its corresponding alcohol. However, side products were also formed among which we have tentatively identified as epoxidized 13-HOD (Blié and Schuber, 1990a). Some epoxy-hydroxy fatty acids are also formed by homolytic cleavage (Dix and Marnett, 1985).

Recently, Wilcox and Marnett (1991) have developed 13-hydroperoxyoctadeca-9(2),11(E),15(Z)-trienoic acid (13-HPOT) as a novel probe for the study of mechanisms of metal-catalyzed reactions with hydroperoxides. Using model porphyrin complexes, they demonstrated that heterolysis of the hydroperoxide O–O bond leads exclusively to the corresponding alcohol whereas homolysis generated multiple products such as aldehydes and ketones *inter alia* (Fig. 1). We have now used this hydroperoxide as a probe for the mechanism of hydroperoxide reduction by soybean peroxidase. These studies were performed with a detergent-solubilized and partially purified enzyme preparation (Blié and Schuber, 1989, 1990a) devoid of any other oxidative activity, such as cytochrome P-450 or lipoygenases, which might also react with hydroperoxides. Elucidation of the structures of the products formed during oxidation reactions catalyzed by soybean peroxygenase in the presence of 13-HPOT are consistent with an exclusive heterolytic cleavage of the hydroperoxide O–O bond. Moreover, results obtained in experiments with 18O-labeled hydroperoxides, demonstrate both intra- and intermolecular oxygen transfer reactions, indicating that peroxidase can epoxidize hydroperoxides in the absence of oxidizable substrates. A unifying mechanism of oxygenation by this hemoprotein is proposed which is consistent with all the structural and mechanistic data reported so far.

**Experimental Procedures**

Materials—[1,14C]Linolenic acid (54 Ci/mol) and [1,14C]linoleic acid (56 Ci/mol) were purchased from Du Pont-New England Nu.

**Preparation of Products—** [1,14C]13-HPOT was obtained by treatment, at 0 °C, of [1,14C]linolenic acid (780 μM final concentration) in 700 ml of O2-saturated 47 mM sodium borate buffer, pH 9.0, with soybean lipoygenase (2.1 × 104 units/ml). After stirring for 10 min, the reaction medium was acidified to pH 3.5 and the products extracted with dichloromethane. Purification was achieved by HPLC on a Whatman 10 μm Magnum 9 (10 × 500 mm) column eluted isocratically, at room temperature, with n-hexane/isopropanol/acidic acetic acid (98.7:1.2:0.1, v/v) as solvent, at a flow rate of 3.0 ml/min. Under these conditions 13-HPOT had a retention time of 46 min. The corresponding 13-HOT was obtained by treating the hydroperoxide with an excess of triphenylphosphine in diethyl ether for 1 h at 0 °C. It was purified by chromatography on thin layer silica gel plates (F254, Merck) using diethyl ether/n-hexane/formic acid (70:30:1, v/v) as solvent (solvent A). The RF was 0.58 and 0.49, respectively, for 13-HPOT and its corresponding alcohol. A similar procedure was applied to obtain [1,14C]13-HPOD and [1,14C]13-HOD (Blié and Schuber, 1990a).

[18O]13-HPOT was prepared by soybean lipoygenase-catalyzed oxidation of linolenic acid under O2 atmosphere following a procedure already described for the preparation of [18O]13-HPOT (Blié and Schuber, 1990a). The products were separated by TLC using solvent A. 18O content of the hydroperoxide was determined by GC/MS analysis of the product obtained after reduction by triphenylphosphine, methylation with ethereal diazomethane, and trimethylsilylation with Bis(trimethylsilyl)trifluoroacetamide. The atom % excess 18O in the labeled hydroperoxide was found to be 97% as determined from the ratio of ion intensities at m/z 313 (M[18O]-C9H14) and 311 (M[18O]-C9H12) or 382 (M[18O]) and 380 (M[18O]) relative to a standard sample of 18O13-HPOT similarly derivatized. Concentrations of the hydroperoxides were determined spectrophotometrically, using ε230 = 22.5 × 103 M-1cm-1 (Gibian and Vandenberg, 1997).

**Analytical Procedures—** Products obtained after reaction of [1,14C]13-HPOT with peroxygenase were separated by analytical HPLC on a Altech Partisil Silica 10 μm (4.5 × 250 mm) column using a Varian 9010 pump. Isocratic elution was performed, at room temperature, with n-hexane/tetrhydrofuran/acetic acid (88.5:11:0.5, v/v) at a flow rate of 1.5 ml/min. The compounds were detected by their absorbance at 230 and 275 nm, using a Varian 2550 variable wavelength detector and by their radioactivity, using a Radiomatic Flow-One beta detector. GC/MS was performed on a LKB 9000S apparatus with ionizing energy of 16 eV. The separations were carried out on a DB5 fused silica capillary column (0.25 mm, J&W Scientific) with temperature programming between 35 and 300 °C at the rate of 3 °C/min. 1H NMR spectra were recorded, using
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C_{18}a solutions, on a Brucker AM-400 spectrometer. Radioactivity was read on TLC plates by a Berthold TLC linear analyzer LB 283 and the peaks integrated by a data acquisition system LB 512. Spectrophotometric measurements were obtained with a Shimadzu model MPS-2000 spectrophotometer.

Enzyme Preparation—Peroxygenase was solubilized with emulphogene BC-720 from carefully washed microsomes, obtained from soybean (Glycine max) seedlings, and partially purified by ion-exchange chromatography as described before (Blee and Schuber, 1989, 1990a). Proteins concentrations were determined (Bradford, 1976) using bovine serum albumin as standard.

Enzymatic Assays—In a typical procedure to study the transformation of 13-HPOT catalyzed by peroxygenase, ~6 µg of enzyme was incubated at 26 °C, in 10 mM sodium acetate buffer, pH 5.5, containing 20% glycerol and 0.1% emulphogene (final volume, 200 µL), in the presence of [1-14C]13-HPOT (300 µM) with or without oleic acid (300 µM) or thiobenzamide (300 µM) as substrates. After the incubation time, the reaction products were extracted with dichloromethane (more than 95% of the initial radioactive compounds were routinely recovered) and analyzed: (i) by TLC on silica gel plates using solvent A or (ii) by HPLC as described above. Epoxidation of [1-14C]oleic acid was determined in the presence of cumene hydroperoxide as already described (Blee and Schuber, 1990a).

Sulfoxidation of thiobenzamide, catalyzed by peroxygenase, was assayed by an adaptation of a method described earlier by Cashman and Hanzlik (1981) for flavin-dependent monooxygenases. Thiobenzamide sulfoxide formation was monitored spectrophotometrically at 370 nm.

RESULTS

In our previous studies we have used 13-HPOD or cumene hydroperoxide as cosubstrates in the oxidation reactions catalyzed by soybean peroxygenase. For the present study, we have first established that 13-HPOT was also able to act as an oxygen donor. When incubated with this hydroperoxide, peroxygenase epoxidized oleic acid less efficiently than with cumene hydroperoxide. In a typical experiment, the yield of 2,10-epoxyoleic was 5-fold higher with cumene hydroperoxide than with 13-HPOT.

Identification of the Reaction Products of 13-HPOT Formed during Sulfoxidation or Epoxidation Catalyzed by Soybean Peroxygenase—When [1-14C]13-HPOT was incubated with soybean peroxygenase, in the presence of oleic acid or thiobenzamide, three major peaks were detected by radio-HPLC. Fig. 2A depicts a representative experiment with oleic acid; on integration these peaks account, respectively, for 20, 47, and 25% of the total recovered counts. The first peak (elution time, 37 min) is unreacted hydroperoxide: it coeluted with an authentic standard and was reduced by treatment with triphenylphosphine to its corresponding alcohol. The second peak (retention time, 41 min) was identified as 13-HOT; it coeluted with an authentic sample (see “Experimental Procedures”), and its structure was confirmed by mass spectrometry. The methyl ester-trimethylsilyl ether derivative gave the expected molecular ion at m/z 380 and prominent ions at 311, base peak (M-69, loss of 'CH2,CH=CHCH2,CH3) and 365 (M-15, loss of 'CH3).

The third peak (X in Fig. 2) eluted with a retention time of about 150 min. Analysis by GC of the methyl ester-trimethylsilyl ether derivative of compound X gave a single peak, which was over 95% pure. Mass spectra taken across this peak were identical (Fig. 3); they featured a molecular ion at m/z 396, indicative of the presence of an additional oxygen atom, compared to 13-HOT. Representative fragments were found at m/z 381 (M-15, loss of 'CH3), 378 (M-18, loss of H2O), 337 (M-59, loss of 'CO2,CH3), 311 (M-85, loss of O, 306 (M-90, loss of (CH3)2SOH'), 239 CH3CH=CHCH2,CH3) (M-157, loss of 'CH2,CO2,CH3), 131 (306-157-18) and 75

FIG. 2. Radiochromatogram of the products formed by reaction of soybean peroxygenase with 13-HPOT. [1-14C]13-HPOT (300 µM) was incubated for 7 min at 26 °C in the presence (A) or absence (B) of oleic acid (300 µM). The reaction products were extracted and analyzed by HPLC as described under “Experimental Procedures.”

The 'H NMR (400 MHz, CD3OD) spectrum of methylated X provided further proof for the structure of this compound. It gave characteristic signals at δ 6.75 (dd, J = 15 and 11 Hz, 1H, H-11), 6.12 (t, J = 11 Hz, 1H, H-10), 5.66 (dd, J = 15 and 6 Hz, 1H, H-12), 5.44 (dt, J = 11 Hz, 1H, H-9), 4.33 (q, J = 6 Hz, 1H, H-13), 3.35 (s, 3H, OCH3), 2.86 (dt, J = 6.2 and 4.2 Hz, 1H, H-15), 2.56 (dt, J = 6.8 and 4.2 Hz, 1H, H-16), 2.53 (dt, J = 6.2 and 4.2 Hz, 1H, H-17), 0.77 (t, J = 7.5 Hz, 3H, H-18). The spectrum confirmed the presence of two double bonds of opposite configuration: Z (J = 11 Hz) and E (J = 15 Hz), one hydroxyl group (H-13 at 4.33 ppm), and one epoxide group (having a cis configuration, J15,16 = 4.2 Hz) in the molecule. Thus, X was identified as 15,16-cis-epoxy-13(S)-hydroxyocta-9(Z),11(E)-dienoic acid (15,16-EHOD). It is important to note that peroxygenase does not epoxidize the conjugated diene of 13-HOT, but rather its 15,16-cis double bond. No other product was detected by HPLC analysis. Importantly, no aldehyde or ketone derivatives, resulting from the homolysis of the hydroperoxide O=O bond (see the Introduction and Fig. 1), could be found. Finally, no transformation
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**Table I**

Influence of substrate concentrations of the conversion of 13-HPOT into 13-HOT and 15,16-EHOD catalyzed by soybean peroxygenase

| Substrate | Reaction product ratio | Thiobenzamide (mM) | Oleic acid (mM) |
|-----------|------------------------|---------------------|-----------------|
| 0         | 0.1                    | 0.3                 | 0.7             |
| 0.7       | 0.1                    | 0.3                 | 0.7             |

| 15,16-EHOD/13-HOT | 1.84 | 1.05 | 0.82 | 0.53 | 2.0  | 1.23 | 1.16 | 0.89 |

**TABLE II**

Transformation of 

of 13-HPOT was observed in the presence of boiled soybean peroxygenase.

Effect of Substrate Concentration on the Formation of 15,16-EHOD—The results obtained above suggest that 13-HOT, formed after the heterolytic cleavage of the peroxy O-O bond of 13-HPOT, can also serve as a final acceptor of the oxygen atom originating from the hydroperoxide group. This implies that the epoxidation of this reaction product should be in competition with the oxidation of other substrates. In order to test this point, varying amounts of thiobenzamide or oleic acid were incubated with the enzyme in the presence of fixed concentration of [14C]13-HPOT. As expected, increasing concentrations of the competing substrates, which were, respectively, S-oxidized and epoxidized (not shown), resulted in a decreased formation of 15,16-EHOD (Table I). However, even at high concentrations, thiobenzamide and oleic acid could not totally abolish the formation of the epoxy alcohol. Conversely, the highest yield of epoxy-alcohol (about 65% of the reaction products) was observed when peroxygenase acted on 13-HPOT in the absence of competing substrates (Table I and Fig. 2B).

Analysis of the kinetics of transformation of [14C]13-HPOT by the peroxygenase (Fig. 4) revealed that the disappearance of the hydroperoxide was paralleled by the formation of the epoxy alcohol. In contrast, after an initial increase, the formation of 13-HOT plateaued, consistent with its role as a precursor in the formation of 15,16-EHOD. This was borne out by the fact that [14C]13-HOT (300 μM) was efficiently transformed by soybean peroxygenase, in the presence of unlabeled 13-HPOT (300 μM), into [14C]15,16-EHOD (not shown). Moreover, 13-HOT was found to be a better substrate for epoxidation than oleic acid, e.g., under comparable conditions the yield of 15,16-EHOD was three times higher than the yield of 9,10-epoxysearic acid.

Transformation of [18O]13-HPOT by Peroxygenase-Intramolecular Versus Intermolecular Oxygen Transfer Mechanism—Peroxygenase is able to epoxidize 13-HOT formed by reduction of the corresponding hydroperoxide. As noted above, however (Table I), if oxidation of substrates such as thiobenzamide or oleic acid can compete with epoxidation reaction, it cannot suppress it. This suggests that the oxygen atom originating from the hydroperoxide could be transferred to 13-HOT according to two distinct pathways, i.e., intramolecular and intermolecular. In order to test this point, we determined the fate of the 18O-label during the reaction of [18O]13-HPOT with soybean peroxygenase. As indicated in Table II (experiment A), transformation of [18O]labeled hydroperoxide into 13-HOT was accompanied by an incorporation of about 86% of the label into the epoxy alcohol. This figure is very similar to the one found previously with 18O transfer catalyzed by peroxygenase to substrates, e.g., in sulfoxidation
products similarly derivatized.

expected, which contain, respectively, two $^{18}$O, one $^{17}$O and

two $^{16}$O atoms at the C(13)-hydroxyl and 15,16-epoxide groups. The fragments obtained in the mass spectra of these epoxy alcohols (Fig. 3) allow an easy determination of the relative atom % excess $^{18}$O in these two locations. The results are summarized in Tables II and III. They cannot be explained by exclusive intramolecular mechanisms.

A good agreement is reached, however, assuming a 5:1 ratio of an intramolecular to intermolecular transfer.

**Identification of the Reaction Products of 13-HPOD with Soybean Peroxygenase**—In our previous study of the epoxidation of oleic acid by peroxygenase, we have shown that about 1.7 hydroperoxides were consumed per epoxide formed, in the presence of 13-HPOD as cosubstrate (Blé and Schuber, 1990a). This was a good indication that 13-HOD might also serve, after its formation, as substrate for an oxygen transfer reaction. The reaction of 13-HPOD with peroxygenase produced 13-HOD and an additional compound which on TLC had a mobility expected for the corresponding epoxycalcohol, i.e. in solvent A (see "Experimental Procedures") the following $R_f$ values were found: 0.36 for the epoxycalcohol, 0.46 for 13-HOD, and 0.58 for 13-HPOD. The mass spectrum of the methyl ester-trimethylsilyl ether derivative of this compound was identical to that obtained for authentic 9,10-EHOE. It showed a molecular ion at $m/z$ 398 and prominent ions at $m/z$ 383 (M-$^{18}$O, loss of $^{18}$O); 327 (base peak); 298 (M-$^{16}$O); 241 (M-$^{34}$O, loss of $^{16}$O); 237 (M-$^{15}$O, loss of (CH$_2$)$_2$COOCH$_3$); 237 (M-$^{16}$O, loss of (CH$_2$)$_2$COOCH$_3$); 237 (M-$^{18}$O, loss of (CH$_2$)$_2$COOCH$_3$); 237 (M-$^{15}$O, loss of (CH$_2$)$_2$COOCH$_3$); 237 (M-$^{18}$O, loss of (CH$_2$)$_2$COOCH$_3$).

Under the same experimental conditions used in the reaction of 13-HPOD with peroxygenase, the yield of epoxy alcohol obtained from 13-HOD (initial concentration, 300 $\mu$M), in the absence of any other substrate, was only 28% of the reaction products, i.e. about half of that obtained in the 15,16-double bond epoxidation of 13-HPOD (Table IV). Addition of oleic acid to the reaction mixture slightly decreased the formation of this epoxy alcohol (Table IV). These results indicate that an epoxidation of the conjugated double bond system of 13-HOD can occur. The regioselectivity of the oxidation was again very high, i.e. only the 9,10-unsaturation, which has a cis-configuration, was epoxidized.

**TABLE II**

Isotopic composition of 13-HOT and 15,16-EHOD after incubation of

$^{18}$O-labeled 13-HPOD with soybean peroxygenase

Mixtures of $^{18}$O-labeled and unlabeled 13-HPOD (100 $\mu$M) were incubated for 15 min, at 26 $^\circ$C, in 10 mM sodium acetate buffer, pH 5.5, containing 0.1% emulphogene. Reactions were initiated by addition of purified peroxygenase (0.1 mg). The reaction products (trimethylsilylated derivatives of methyl esters) were analyzed by GC/MS as described under "Experimental Procedures.

| Experiment* | Compound analyzed | Isotopic composition$^a$ |
|-------------|------------------|-------------------------|
| A | 13-HOT | 3% $^{18}$O, 97% $^{16}$O |
| 15,16-EHOD | 0% $^{18}$O, 14% $^{17}$O, 86% $^{16}$O |
| B | 13-HOT | 30% $^{18}$O, 70% $^{16}$O |
| 15,16-EHOD | 21% $^{18}$O, 14% $^{17}$O, 65% $^{16}$O |
| C | 13-HOT | 64% $^{18}$O, 36% $^{16}$O |
| 15,16-EHOD | 56% $^{18}$O, 12% $^{17}$O, 32% $^{16}$O |

$^a$ The initial $^{18}$O/$^{16}$O isotope ratios for 13-HPOD were, respectively, 97:3 (A), 70:30 (B), and 36:64 (C).

$^b$ $^{18}$O, $^{17}$O, or $^{16}$O contained, respectively 0, 1, or 2 $^{18}$O atom(s).

The atom % excess $^{18}$O in the products were determined from the ion intensities for: (i) 13-HOT at $m/z$ 313 (M+$^{18}$O)-C$_3$H$_7$) and 311 (M+$^{17}$O)-C$_3$H$_7$) or 382 (M+$^{18}$O) and 380 (M+$^{17}$O) or M($^{16}$O) and (ii) 15,16-EHOD at $m/z$ 400 (M+$^{18}$O$\_2$), 398 (M($^{18}$O$\_1$)), and 396 (M($^{18}$O) or M($^{16}$O)) relative to a standard sample of $^{18}$O-containing products similarly derivatized.

or epoxidation reactions (Blé and Durst, 1987, Blé and Schuber, 1990a).

In order to investigate the mode of oxygen transfer in the formation of the epoxycalcohol (intra- versus intermolecular mechanism), we incubated mixtures of different proportions of $^{18}$O- and $^{16}$O-labeled 13-HPOD and analyzed by GC/MS the percentage of $^{18}$O in the resulting epoxycalcohols. Under such conditions, three different species of 15,16-EHOD are expected, which contain, respectively, two $^{18}$O, one $^{17}$O and one $^{16}$O.
DISCUSSION

Reaction of 13-HPOT with solubilized and partially purified soybean peroxygenase produces 13-HOT and 15,16-EHOD. This epoxy product is formed even in the presence of competing substrates such as oleic acid or thiobenzamide. Experiments carried out with \(^{18}O\)-labeled 13-HPOT indicate that the oxygen atom incorporated into the epoxide group of 15,16-EHOD, originates primarily (\(\approx 86\%\)) from the hydroperoxide. Similarly, when 13-HPOT was incubated with peroxygenase, 13-HOD and 9,10-EHOE were formed. Very recently, the formation of 15,16-EHOD by soybean seed extracts incubated in presence of 13-HPOT was reported (Gardner et al., 1991).

Several mechanisms could account for the formation of such epoxides. For example, intramolecular oxidative mechanisms consisting of isomerization of unsaturated fatty acid hydroperoxides are well known to lead to epoxy alcohol derivatives. They are produced, along with other compounds, in nonenzymatic reactions with hemes (Graveland, 1970; Gardner et al., 1984; Claeyts et al., 1985), e.g. in the presence of hemoglobin (Garssen et al., 1976) or hematin (Dix and Marrett, 1985), or by lipoxygenases (Hamberg, 1975; Gardner, 1991). Several of these processes involve radical species which are generated by an homolytic cleavage of the hydroperoxide.

**Table IV**

Comparison of the proportions of epoxy alcohols and hydroxy derivatives formed from 13-HPOT and 13-HPOD in the absence or presence of oleic acid as substrate

| Reactants                  | Epoxide alcohol/hydroxy product ratio |
|----------------------------|---------------------------------------|
| 13-HPOT                    | 1.78                                  |
| 13-HPOT + oleic acid       | 1.33                                  |
| 13-HPOD                    | 0.38                                  |
| 13-HPOD + oleic acid       | 0.32                                  |

*Epoxycarboxyls are 15,16-EHOD and 9,10-EHOE; hydroxy derivatives are respectively 13-HOT and 15-HOD.*

O-O bond followed by allylic rearrangement reactions. None of these reactions, however, were found to convert 13-HPOD or 13-HPOT into products described here. 13-Oxotrideca-9(Z),11(E)-dioinoic acid, 13-oxyoctadeca-9(Z),11(E),15(Z)-trienoic acid, and other alkoxyl radical-derived products account for all of the metabolites of 13-HPOT under conditions of hydroperoxide homolysis, i.e. 13-HOT is not detected. Since none of these alkoxyl radical-derived products could be detected in the reaction of 13-HPOT with peroxygenase, the occurrence of a homolytic pathway can be ruled out in the formation of the epoxy alcohol. Thus, a different mechanism must be invoked to explain the formation of 15,16-EHOD by epoxidation of 13-HOT involving the transfer of the oxygen atom from the hydroperoxide group.

Our results support a mechanism in which a ferryl-oxo intermediary complex (compound 1-like), resulting from the heterolytic cleavage of the O-O bond of 13-HPOT, serves as oxygen donor for the substrates. An important observation is that oxidizable substrates (oleic acid, thiobenzamide) decrease the proportion of epoxy alcohol in the reaction products derived from the hydroperoxide (Table I). This is consistent with the notion that some 13-HOT is oxidized intermolecularly, i.e. after its formation it diffuses out of the active site sufficiently to be in competition with other substrates for the oxidative step. In fact, it is likely that epoxidation of 13-HOT generated from 13-HPOT proceeds through a similar mechanism of oxygenation by the peroxygenase as the other substrates (unsaturated fatty acids, sulfides). We have shown that this enzyme oxidizes with a high stereoselectivity, only cis-double bonds in unsaturated fatty acids, sulfides. We have shown that this enzyme oxidizes with a high stereoselectivity, only cis-double bonds in unsaturated fatty acids, yielding cis-epoxides (Blée and Schuber, 1990a, 1990b). Indeed, only cis-double bonds were epoxidized in 13-HPOT or 13-HPOD yielding cis-epoxides. The high regioselectivity in the oxidation of 13-HPOT is also remarkable. Only the 15,16-epoxide was produced and no epoxidation on the conjugated 9,10-cis double bond could be detected despite the fact that this position was epoxidized by the enzyme, albeit less efficiently, in 13-HPOD. It seems reasonable to assume that, besides topological considerations, the enzyme epoxidizes the more reactive nonconjugated cis-double bond. This regioselectivity can be compared with the monoeoxidation of linoleic acid by peroxygenase which formed the 9,10- and 12,13-epoxides in a 2:1 ratio (Blée and Schuber, 1990a, 1990b).

![Fig. 5. Biosynthesis of 15,16-EHOD from 13-HPOT.](image)
and Schuber, 1990b). In order to undergo epoxidation it is not, however, necessary for 13-HOT to diffuse out of the active site. The intermediary iron-oxo complex can transfer its oxygen atom to the same molecule from which it was generated (intramolecular oxygen transfer mechanism, Fig. 5). Experiments with mixtures of unlabeled and 18O-labeled 13-HPOT have revealed that 15,16-EHOD was formed predominantly (75%) via such a mechanism. Importantly, the same epoxide regioisomer was formed both via intramolecular and intermolecular mechanisms. Finally, the observation that reaction of 13-HPD with peroxygenase yields less epoxy alcohol than 13-HPOT, but is less sensitive to competing oleic acid (Table IV), might indicate that 9,10-EHOE is formed predominantly via an intramolecular pathway. It results that 13-HOD is a relatively poor substrate for an intermolecular oxygen transfer mechanism, suggesting that the unconjugated 15,16-cis double bond in 13-HOT may be important for the intermolecular pathway. In favor of this hypothesis, under comparable conditions, the yield of epoxidation of oleic acid was 2-fold higher with 13-HPOT than with 13-HPOD.

Taking into account the different results obtained in this study we propose the reaction scheme illustrated in Fig. 6. Accordingly, soybean peroxygenase catalyzes the reduction of 13-HPOT into 13-HOT with the concomitant 2-electron oxidation of the enzyme, resulting in the formation of a ferryl-oxo complex. This intermediate then epoxidizes the distal cis-double bond (at positions 15 and 16) of 13-HOT either before it diffuses out of the active site (caged reaction, pathway A) or after recomplexation with the active site of the molecules (pathway B). In the absence of any other substrates, the latter pathway accounts for about one-third of the epoxy alcohol formed. The simultaneous occurrence of intramolecular and intermolecular epoxidation indicates that the rate of reaction of the iron-oxo complex with the hydroxy fatty acids is comparable to the rates of their diffusion out/in the active site. This means that in the kinetic mechanism the rate-limiting step in the overall oxidative process is the transfer of the oxygen atom from the iron-oxo complex to the substrate.

Fig. 6 explains and predicts the following features of the reactions catalyzed by soybean peroxygenase.

(i) It explains why, in the presence of compounds such as oleic acid or thiocarbamide, 13-HOT must compete for the active site (pathway B). Since these molecules are also substrates, the enzyme catalyzes their co-oxidation in competition with the intermolecular oxygen transfer yielding the epoxy alcohol (pathway B).

(ii) A single enzyme catalyzes the different types of reactions such as alkylaryl sulfide sulfoxidation or unsaturated fatty acid epoxidation (Blé and Schuber, 1989, 1990a) depending on the substrate present. Peroxygenase is able to act on compounds of widely different structures. Accordingly, epoxidation of oleic acid by peroxygenase, in the presence of cumene hydroperoxide, could be inhibited by sulfides such as p-NH₂ and p-NO₂ thioanisoles (not shown). Interestingly, the inhibition potency was greater for p-NH₂ compared to p-NO₂ thioanisoles, i.e. parallel to the ease of oxidation of these molecules (Blé and Schuber, 1989).

(iii) The proportion of intramolecular versus intermolecular oxygen transfer depends on the nature of the product formed by reduction of the hydroperoxide and on its relative affinity for the peroxygenase, i.e. the ease with which it engages in an intramolecular oxygen transfer reaction (Tabie IV). The important consequence is that a hydroperoxide generating a product which is a good substrate for the iron-oxo intermediate should support little co-oxidation by peroxygenase because of the incidence of high levels of intramolecular oxygen transfer. Conversely, one can postulate that a hydroperoxide which after reduction has no readily oxidizable functions (e.g. cumene hydroperoxide) should be an excellent co-oxidant because of the lack of an intramolecular pathway. This was confirmed by showing that oleic acid was less efficiently epoxidized (5-fold), in the presence of 13-HPOT than cumene hydroperoxide.

(iv) Although not developed in this work, an immediate consequence of the previous point is the extent of the mechanism-based inactivation that accompanies catalysis. The ferryl-oxo intermediate leads to the inactivation of the enzyme that is paralleled by a modification of the heme prosthetic group, i.e. disappearance of the Soret absorbance (Blé and Schuber, 1990a). One can predict that peroxygenase will be less susceptible to such a deleterious effect when reacting with hydroperoxides whose products are easily oxidizable. This is precisely the case, i.e. cumene hydroperoxide is much more destructive than 13-HPOD (Blé and Schuber, 1990a).

![Fig. 6. Reactions catalyzed by soybean peroxygenase.](image-url)
The demonstration that soybean peroxygenase catalyzes an exclusive heterolytic cleavage of the hydroperoxide O-O bond of 13-HPOT gives some information about the molecular mechanism of this enzyme. According to considerations developed by Lee and Bruice (1985) and Bruice et al. (1988), a homolytic scission of the hydroperoxide bond should be expected when alkyl hydroperoxides such as 13-HPOT react nonenzymatically with an iron(III) porphyrin (see also Labèque and Marnett (1988) and Traylor et al. (1989) for heterolytic processes in hydroxy solvents). Our data therefore suggest the occurrence of an additional catalytic contribution of the active site of the peroxygenase to the cleavage reaction. The heterolytic mechanism operating, e.g. for the peroxidases (Poulos and Kraut, 1980), is generally believed to be facilitated by an electron-donor axial ligand of the heme (i.e. imidazole) and a polar environment which by acid base-catalyzed assistance facilitates the protonation of the alkoxy leaving group (Dawson, 1988). Similar effects have been reported in biomimetic systems where reactions can be modified to favor 2-electron reduction of alkylhydroperoxides by use of electron donor axial ligands such as imidazole (Mansuy et al., 1989). This contrasts with cytochrome P-450 enzymes which also have the ability to react with hydroperoxides ("peroxide shunt"). In this case however, both homolytic and heterolytic cleavages of hydroperoxides are supported (reviewed in Ortiz de Montellano, 1986; Guenguerich, 1991; White, 1991), which can occur as competing processes (Thompson and Yumibe, 1985). In line with our results, the reaction of cumene hydroperoxide with a cytochrome P-450, according to the heterolytic mechanism, also resulted in an oxygenation of the intermediate alcohol in a caged reaction (Thompson and Wand, 1985).

It is interesting to compare soybean peroxygenase with some other enzymes in their reaction with hydroperoxides. An epoxide activity, associated with microsomes of Vicia faba, was recently described by Hamberg and Hamberg (1990). The scheme in Fig. 6 can readily explain the different reactions observed with this enzymatic fraction. In analogy to soybean peroxygenase (Bie and Schuber, 1990a), it epoxidizes unsaturated fatty acids such as linoleic acid in the presence of hydroperoxides. As found here, 13-HPOD was converted into 13-HOD and 9,10-EHOE. This epoxide alcohol was formed by 1:1 competition of intramolecular and intermolecular oxygen atom transfer from the hydroperoxide. From these results, it seems likely that this epoxidegenase is very similar, if not identical to the peroxygenase we are studying, and should therefore catalyze other types of oxidations. Recently, Song and Brash (1991) have described a new cytochrome P-450 from plants which also uses 13-HPOT as substrate. Like peroxygenase, it is a ferric, high spin heme protein but it dehydrates the hydroperoxide into an intermediary allene oxide, the precursor of "prostaglandin-like" compounds and ketones. In contrast to peroxygenase, it is likely that allene oxide synthase proceeds via a hemolytic scission of the hydroperoxide, illustrating again the importance of the apoprotein in determining the mechanism of hydroperoxide cleavage by hemoproteins. Nevertheless, both enzymes employ a direct cooperation of lipoxigenase with another oxygenase (peroxygenase or allene-oxide synthase) in different biosynthetic pathways.

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