Formation of Epoxyalcohols by a Purified Allene Oxide Synthase

IMPLICATIONS FOR THE MECHANISM OF ALLENE OXIDE SYNTHESIS*

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The allene oxide synthase (hydroperoxide dehydrogenase) of flaxseed is a cytochrome P450 that exhibits an exceptionally high catalytic turnover (≥1000/s) for hydroperoxy substrates. In a previous study, using a crude extract of flaxseed, we detected a secondary activity that could offer an insight into the mechanism of the enzymatic transformation of hydroperoxides. We observed that the substrate 8R-hydroxy-15S-hydroperoxyeicos-5,9,11,13,17-pentaenoic acid is converted not only to allene oxide, but also to epoxyalcohol derivatives (Brash, A. R., Baertschi, S. W., and Harris, T. M. (1990) J. Biol. Chem. 265, 6705-6712). The transformation of hydroperoxides to epoxyalcohols has been investigated extensively in other systems, and heterolytic or homolytic cleavage of the hydroperoxide is associated with characteristic rearrangements and stereochemistry of the epoxyalcohol products. Using the purified enzyme, we established that the epoxyalcohols are products of the allene oxide synthase. Their structures were determined by UV, gas chromatography-mass spectrometry, and NMR. The major epoxyalcohol is 8R,13R-dihydroxy-14R,15S-epoxyeicos-5Z,9E,11Z,17Z-tetraenoic acid, a trans-epoxide with an α-hydroxyl in the relative threo configuration. Two minor products are the corresponding 11E isomer and a cis-epoxide identified as 8R,13-dihydroxy-14S,15S-epoxyeicos-5Z,9E,11E,17Z-tetraenoic acid. Gas chromatography-mass spectrometry analysis of the reaction with [13C18]hydroperoxide substrate indicated complete retention of the hydroperoxy oxygens in the epoxyalcohol products. Mechanistic precedents support a homolytic hydroperoxide cleavage as the initial step in the synthesis of these epoxyalcohols. We suggest that the same process initiates allene oxide synthesis, a conclusion that is also most compatible with the known chemistry of cytochromes P450.

Allene oxide synthase activity has been detected in many plants, in certain soft corals, and in the oocytes of starfish (Vick and Zimmerman, 1979a; Hamberg, 1989; Corey et al., 1987, 1988; Brash et al., 1987, 1991). Recently, the allene oxide synthase of flaxseed was purified; and based on its spectral properties, it was identified as a member of the cytochrome P450 family of hemoproteins (Song and Brash, 1991a). This enzyme will convert the 13S-hydroperoxy derivatives of linoleic and linolenic acids to the respective allene oxides at turnover rates of over 103/s (Song and Brash, 1991a). The corresponding 15S-hydroperoxides of C20 fatty acids are also good substrates, whereas the enantiomeric “R” hydroperoxides and other positional hydroperoxides are metabolized slowly or not at all (Feng and Zimmerman, 1979; Vick and Zimmerman, 1979b; Baertschi et al., 1988). Certain other plants contain an allene oxide synthase that will metabolize efficiently both the 9S- and 13S-hydroperoxides of linoleic and linolenic acids (Gardner, 1970, 1991; Hamberg, 1987a), whereas in the animal systems studied so far, the enzymes are specific for the 8R-hydroperoxide of arachidonic acid (Corey et al., 1987, 1988; Brash et al., 1987, 1991). In both animals and plants, the different allene oxide synthases convert the natural substrates to a single product, the allene oxide derivative of the respective hydroperoxide. The work described here concerns the metabolism of a more complex hydroperoxide substrate. This 8R-hydroxy-15S-hydroperoxy eicosanoid is converted by the allene oxide synthase of flaxseed to a mixture of products comprised of allene oxides and epoxyalcohols.

The discovery of the epoxyalcohol synthesis was made in an earlier study in which we used model hydroperoxy substrates to examine the possibility of converting fatty acids to the A series prostaglandins via allene oxide intermediates (Brash et al., 1990). Crude extracts of flaxseed metabolized 8R-hydroxy-15S-hydroperoxy or 8S-hydroxy-15S-hydroperoxy derivatives of eicosapentaenoic acid to the corresponding allene oxides (14,15-epoxy). The unstable allene epoxides cyclized spontaneously to analogues of prostaglandin A2 or were hydrolyzed to α-ketols. Unexpectedly, an additional group of products was formed exclusively from the 8R-hydroxy-15S-hydroperoxide diastereomer. Preliminary analyses indicated that this extra series of products were isomeric epoxyalcohols (8,13-dihydroxy-14,15-epoxyeicos-5,9,11,17-tetraenoic acids) (Brash et al., 1990) as shown in Scheme 1.

These initial observations raised several important questions. Is the epoxyalcohol synthesis in this crude flaxseed extract catalyzed by the allene oxide synthase? This would constitute the first demonstration of an additional catalytic activity of this novel type of cytochrome P450. Furthermore, the mechanisms of transformation of hydroperoxides to epoxyalcohols have been investigated extensively in other systems, and the characteristic features of epoxyalcohol synthesis via
either homolytic or heterolytic cleavage of the hydroperoxide are well established (see Gardner, 1989, for references). Therefore, a more thorough study of the enzymatic epoxyalcohol synthesis in the flaxseed system offers a potential insight into the mechanism of hydroperoxide cleavage by the enzyme. With these ideas in mind, the objectives of this study were to determine the precise structures of the novel epoxyalcohol derivatives, to investigate their mechanism of formation, and to determine if the allene oxide synthase is the enzyme responsible for their biosynthesis. The results have a bearing on the catalytic activity on the allene oxide synthase and on the mechanism of the enzyme-catalyzed hydroperoxide cleavage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Eicosapentaenoic acid was obtained from Cayman Chemical Co. or was purified from the ~50% Special Prep from Nuchek Prep Inc. (Elysian, MN). 8R-HEPE was prepared from eicosapentaenoic acid using the 8R-lipoxygenase activity of aceton powders of the coral *Plexaura homomalla*, and 8R-HEPE was prepared by autoxidation (Brash et al., 1987, 1990; Peers and Coxon, 1983). The 8R-hydroxy-15S-hydroperoxide and 8S-hydroxy-15S-hydroperoxycis-5Z,9Z,11Z,13Z-pentaenoic acids were prepared by reaction of 8RS-HEPE with the soybean lipoxygenase (Brash et al., 1990). The 8R-hydroxy-15S-[18O]hydroperoxide was prepared by reaction of 8R-HEPE with the soybean lipoxygenase under an atmosphere of 18O gas. Purification of Flaxseed Allene Oxide Synthase—Acetone powder extracts of flaxseed were prepared as described (Bacro et al., 1988). Two isozymes were isolated from flaxseed acetone powder (Sog and Brash, 1991a). Peak fractions from the chromatofocusing column were used in the experiments described here.

**Incubation and Extraction**—Incubations with the crude extract of flaxseed acetone powder were conducted as described, before use, 2 ml of extraction buffer (200 mM potassium phosphate buffer (pH 7) for 15 min at room temperature (Brash et al., 1990). Incubations with the purified allene oxide synthase were carried out in a 0.5 ml volume containing 3 pmol of enzyme, 25 (150 mM) of substrate for 15 min at room temperature. Reactions were initiated by addition of substrate in a small volume of ethanol. The products were recovered by acidification to pH 3.5 and extraction with ethyl acetate. The organic phases were washed with ethereal diazomethane, followed by evaporation of the solvent to dryness, and then taken to dryness using a stream of nitrogen. Extracts were stored in methanol at -70 °C prior to HPLC.

**HPLC Analyses**—Samples were analyzed initially by RP-HPLC with UV monitoring using a Hewlett-Packard 1040 diode array detector. An Altex 5-μm ODS UltraSphere column was employed with a solvent system of water/acetoniitrile/acetic acid (65:35:0.01, by volume). Usually, the ODS UltraSphere column was 25 × 0.46 cm in dimensions, and the flow rate used was 1 ml/min; but extractions of incubations with the pure allene oxide synthase were analyzed using a narrow bore column (25 × 0.2 cm) at a flow rate of 0.2 ml/min. The main products from RP-HPLC were extracted out of the reversed-phase solvent with ethyl acetate and then run on straight-phase HPLC using an Alttech 5-μm silica column (25 × 0.46 cm) and a solvent system of hexanes/propyl alcohol/acetic acid (100:5:0.1, by volume) at a flow rate of 1 ml/min with UV monitoring using the diode array detector or with monitoring at 220 nm using an LDC/Milton Roy variable wavelength UV detector.

**Derivatization**—Samples were derivatized to the methyl ester by reaction in a small volume of methanol and reacting for a few seconds with ethereal diazomethane, followed by evaporation of the reagent under a stream of nitrogen. Pentafluorobenzyl derivatives were formed by addition to the dried sample of 35 μl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 10 μl of 10% propyl alcohol/acetic acid as reaction medium. After 15 min at room temperature under argon, samples were evaporated to dryness, and trimethylsilyl (TMS) ester and TMS ethyl ester derivatives were prepared by reaction with bis(trimethylsilyl)trifluoroacetamide:pyridine for a least 30 min at room temperature. Samples were dissolved in dodecane for GC-MS analysis.

**GC-MS Analyses**—Positive-ion electron impact spectra were acquired using a Finnigan Inco 50 mass spectrometer operated at 70 eV and coupled to a Hewlett-Packard 5890 gas chromatography unit fitted with an SPB-1 fused silica capillary column (15 m, 0.25 mm, 0.25 μm). The oven temperature was programmed from 190 to 320 °C at 20 °C/min. Negative-ion analyses of pentafluorobenzyl ester derivatives was carried out using a Nermag R10-10C instrument (Blair, 1990).

**Measurement of O2 Content**—The oxygen-18 content of the 8R-hydroxy-15S-[18O]hydroperoxide derivative of eicosapentaenoic acid was measured after reduction to the saturated 8,15-di-hydroxyeicosanoic acid. The latter step was necessary because of instability of the eicosapentaenoate derivative on the GC. The saturated 8,15-diol was converted to the pentafluorobenzyl ester/TMS ether derivative and analyzed by GC-MS in the negative-ion/chemical ionization mode. Repetitive spectra were acquired on unlabeled compound and on the 18O-labeled analogue by scanning over the mass range m/z 485-495. This range encompasses the ion clusters corresponding to the M-PFB ions of the unlabeled 8,15-diol (m/z 487) and of the corresponding 18O-labeled molecules. About 30 spectra were acquired during elution of the peak from the GC; these were averaged to give the raw data used to calculate the 18O content. The oxygen-18 content of the epoxyalcohols was determined by the same type of analysis using the PFB ester/TMS ether derivative; this derivative gave satisfactory GC characteristics, and catalytic hydrogenation was not necessary. The instrument was set to scan over the mass range m/z 491-501, encompassing the M-PFB ions of the unlabeled epoxyalcohol derivatives (M-PFB at m/z 493) and of the 18O analogues.

**NMR—** Spectra were recorded using a Bruker AM-400 or an IBM/ Bruker NR-300 instrument at 400 and 300 MHz, respectively. Some of the initial scans were acquired using acetone-δ6 as solvent; but subsequently, this was substituted with CDCl3 to measure the signals from the epoxide protons. Chemical shifts are reported in relation to trimethylsilane (δ = 0.0). Typical parameters for one-dimensional spectra were 10K of sampling and data points, 60° pulse, and 2.5-s relaxation delay. Sufficilent scans were acquired to yield a good signal-to-noise ratio after transformation. For measurement of coupling constants, gaussian multiplication was employed, and the data were zero-filled to 32K before transformation. COSY spectra were obtained using the standard Bruker microprogram. 256 1K spectra were recorded using a 90°-45° pulse sequence. Sine-bell apodization, magnitude calculation, and symmetrization were employed.

**RESULTS**

**Formation of Epoxylcohols by Purified Allene Oxide Synthase**—We found before that a crude solution of flaxseed enzymes converts two diasteromeric 8-hydroxy-15S-hydroperoxy derivatives of eicosapentaenoic acid to distinctly different patterns of products. The product pattern is determined by the hydroxyl configuration at C-8 (although the enzymatic transformations involve only reactions of the 15S-hydroperoxide). Whereas the "8S" diastereomer is converted almost exclusively to allene oxide-derived products (α-ketols and prostaglandin A analogues), metabolism of the "8R" substrate is associated with the formation of three additional products with an earlier elution on RP-HPLC (Brash et al., 1990). As shown in the reversed-phase chromatograms in Fig. 1, the same contrast in the metabolism of the 8R and 8S diastereomers was found using purified allene oxide synthase. The typical Altex UltraSphere column resolved these three extra products from the 8R-substrate hydroperoxide (8R-substrate, see inset). These were slight variations in the properties of individual Altex UltraSphere columns; and in Fig. 1 (main panels), using the narrow bore UltraSphere column (0.2 cm, inner diameter), only two earlier eluting peaks are resolved. Good substrates of the flaxseed allene oxide synthase are transformed with initial rates of over 1000 turnovers/s (Song and Brash, 1991a). The 8S-hydroxy-15S-hydroperoxycis-epoxycisapentaenoic acid reacts at about one-third of this rate, whereas the 8R,15S diastereomers react at about one-tenth the rate (Brash et al.,

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1 The abbreviations used are: HEPE, hydroxyeicosapentaenoic acid; RP-HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrometry; PFB, pentafluorobenzyl; TMS, trimethylsilyl.
Mechanism of Allene Oxide Biosynthesis

89-OH reaction

UV at 205 nm

FIG. 1. RP-HPLC analysis of products from reaction of 8-hydroxy-15S-hydroperoxyeicosapentaenoic acid with purified flaxseed allene oxide synthase. The major and minor isozymes purified from flaxseed were each reacted with 8R- and 8S-hydroxy diastereomers. Each isozyme gave the same pattern of products (chromatograms from the main isozyme are shown). Upper, products from the 8R-hydroxy diastereomer; lower, products from the 8S-hydroxy diastereomer. The following conditions were used: column, narrow bore Altex/Beckman 5-μm Ultrasphere ODS (25 × 0.2 cm); solvent, water:acetonitrile:acetic acid (65:35:0.01, by volume); flow rate, 0.2 ml/min; and UV detection at 205 nm. Panels, chromatogram from a separate experiment in which two purified isozymes were reacted with the 8R diastereomer and analyzed on a 25 × 0.46-cm Ultrasphere column. Each isozyme gave indistinguishable chromatograms, one of which is shown. This particular Ultrasphere column resolved P1, P2, and P3 and also partially resolved the two α-ketols (the earlier eluting 11Z isomer, followed by the less prominent 11E isomer). PAG, prostaglandin A.

1990). Incubations with boiled enzyme gave no detectable allene oxide products and ≤2% of the amount of epoxyalcohols (attributed to nonenzymatic rearrangements) (data not shown).

Aliquots of crude flaxseed extracts incubated and analyzed in parallel with incubations of the pure isozymes gave exactly the same pattern of products on RP-HPLC. Further analyses were carried out on the larger-scale incubations that were possible with the crude enzyme. The RP-HPLC peaks were further resolved by straight-phase HPLC. The straight-phase chromatograms were examined for additional products that might be masked by the main components on RP-HPLC (especially the large peak of α-ketols), but we could find only the three products in the early group. These were designated as P1, P2, and P3, in order of elution from the reversed-phase column. The relative abundance of the products appeared to vary slightly between individual experiments (Fig. 1, cf. main panels and inset). The relative amounts purified from large incubations and quantified by UV were in the relative proportions of ~1:6:1.5 for P1, P2, and P3, respectively.

Structural Analysis of Epoxyalcohols—P1, P2, and P3 each exhibited a UV spectrum typical of a conjugated diene chromophore. The spectrum of the most abundant product, P2, had a λmax at 233–234 nm, consistent with a cis,trans-conjugated diene chromophore. The other two epoxyalcohols had a UV λmax at 231 nm, indicative of a trans,trans-conjugated diene.

Analysis by negative-ion/chemical ionization GC-MS of the PFB ester/TMS ether derivative was useful for establishing the molecular weight of the products. Each derivatized product gave a prominent ion at m/z 493, corresponding to the carbohydrate anion formed by loss of the PFB group. The deduced molecular weight for the parent compounds corresponds to a C36 fatty acid having two hydroxyl groups, an epoxide, and four double bonds. The electron impact spectra of the TMS ether/TMS ester and TMS ester/methyl ester derivatives were recorded, but these spectra were not particularly informative (data not shown).

The complete structures and key stereochemical features of P1, P2, and P3 were deduced from the proton NMR spectra, presented in Tables I—III, respectively. The NMR data establish that the three products are stereoisomers of 8,13-dihydroxy-14,15-epoxyeicos-5,9,11,17-tetraenoic acid. The structures (and the stereochemistry as far as it is established) are illustrated in Fig. 2. (The geometry of the 5,6- and 17,18-double bonds and the hydroxyl configuration at C-8 were not determined and are assumed to retain the original 5Z, 8R, and 17Z stereochemistry.)

The values for coupling constants for cis and trans double bonds (~10 and ~15 Hz, respectively) and for cis- and trans-epoxides (~4 and 2.5 Hz, respectively) are well known. Assignment of the relative configuration of the C-13 hydroxyl to the C-14 epoxide oxygen is based on the original observations of Mercier and Agoh (1974) and on later reports by

**Table I**

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<th>Chemical shift</th>
<th>Multiplicity</th>
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<td>6.28-6.35 (6.315)</td>
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<td>H10 (or H11); J_{10,11} = 10.6 Hz, J_{11,12} = 14.9 Hz</td>
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<td>6.19-6.26 (6.225)</td>
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<td>H11 (or H10); J_{11,12} = 10.6 Hz, J_{12,13} = 14.7 Hz</td>
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<td>5.75-5.79 (5.775)</td>
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<td>H8, J_{8,9} = 14.7 Hz, J_{8,10} = 6.6 Hz</td>
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<td>5.73-5.75 (5.74)</td>
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<td>H12; J_{12,13} = 14.9 Hz, J_{12,13} = 5.5 Hz</td>
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<tr>
<td>3.5-3.6</td>
<td>m</td>
<td>H5, H6, H17, H18</td>
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<tr>
<td>4.21</td>
<td>t</td>
<td>H8; J = 6.3 Hz</td>
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<tr>
<td>4.965</td>
<td>t</td>
<td>H13; J = 4.7 Hz</td>
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<tr>
<td>3.025</td>
<td>dd</td>
<td>H15; J_{14,15} = 2.3 Hz, J_{14,16} = 5.4 Hz</td>
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<td>2.28</td>
<td>dd</td>
<td>H14; J_{14,15} = 4.5 Hz, J_{14,16} = 2.3 Hz</td>
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<td>2.25-2.45</td>
<td>m</td>
<td>H2a, b, H7a, b, H16a, b</td>
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<tr>
<td>2.15-2.00</td>
<td>m</td>
<td>H4a, b, H19a, b (H3a, b were obscured by H2O)</td>
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<tr>
<td>0.97</td>
<td>t</td>
<td>H20a, b, c</td>
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**Table II**

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<td>H10; J_{10,11} = 15.1 Hz, J_{11,12} = 11.2 Hz</td>
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<td>6.175</td>
<td>t</td>
<td>H11, J_{11,12} = 11.0 Hz</td>
</tr>
<tr>
<td>5.85</td>
<td>dd</td>
<td>H9; J_{8,9} = 5.9 Hz, J_{9,10} = 15.1 Hz</td>
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<td>5.57 to 5.42</td>
<td>m</td>
<td>H5, 6, 11, 12, 17, 18 (on a shoulder of CH2Cl2 peak)</td>
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<td>4.45</td>
<td>dd</td>
<td>H13; J_{12,13} = Hz, J_{13,14} = Hz</td>
</tr>
<tr>
<td>4.22</td>
<td>q</td>
<td>H8; J = 6.2 Hz</td>
</tr>
<tr>
<td>4.27</td>
<td>dd</td>
<td>H15; J_{14,15} = 2.2 Hz, J_{14,16} = 5.4 Hz</td>
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<tr>
<td>2.86</td>
<td>dd</td>
<td>H14; J_{14,15} = 5.1 Hz, J_{14,16} = 2.2 Hz</td>
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<td>2.42 to 2.25</td>
<td>m</td>
<td>H2a, b, H7a, b, H16a, b</td>
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<td>2.12</td>
<td>q</td>
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<tr>
<td>2.03</td>
<td>p</td>
<td>H19a, b</td>
</tr>
<tr>
<td>1.69</td>
<td>p</td>
<td>H3a, b</td>
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<tr>
<td>0.95</td>
<td>t</td>
<td>H20a, b, c</td>
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Metabolism of \(^{18}O\) Hydroperoxy Substrate—In the synthesis of epoxyalcohols from hydroperoxides, formation of the \(-\)OH group can occur by incorporation of an oxygen from \(O_2\) from water, or from the hydroperoxide itself (Gardner, 1989). We studied the oxygen-18 content of the products formed from an incubation conducted in \(H_2^{18}O\) and from a separate incubation in normal buffer using \(8R\)-hydroxy-15S-\(^{18}O\) hydroperoxyeicosapentaenoic acid substrate. The measurements were made by purification of the individual products and then analysis of the \(^{18}O\) content by negative-ion/chemical ionization GC-MS of the PFB ester/TMS ether derivative. There was no measurable incorporation of \(^{18}O\) in the epoxyalcohols formed in \(H_2^{18}O\) (data not shown). The results for the epoxyalcohols formed from the \(^{18}O\)-labeled hydroperoxide substrate are shown in Fig. 3. The hydroperoxide substrate contained 92% \(^{18}O\); and the measured values for P1, P2, and P3 were 94, 93, and 89% \(^{18}O\), respectively (Fig. 3, legend). Thus, within the precision of the method, we conclude there is complete retention of both of the hydroperoxy oxygen in the epoxyalcohol products.

**DISCUSSION**

The finding that the purified allene oxide synthase can form epoxyalcohols establishes a new catalytic activity for this type of enzyme. That the formation of epoxyalcohols is a bona fide enzymatic reaction and does not result from non-specific hemoprotein-catalyzed degradation of the hydroperoxide is supported by several lines of evidence. First, no allene oxide products and ±2% of the amount of epoxyalcohols are recovered from incubations with heat-denatured allene oxide synthase. Second, the \(8S\)-hydroxy-15S-hydroperoxy fatty acid is converted only to the allene oxide and not to epoxyalcohols. Finally, unlike the non-specific hemoprotein-catalyzed degradation of fatty acid hydroperoxide, which would produce both erythro and threo diastereomers of the C-13 alcohol (Dix and Marnett, 1985; Gardner, 1989), the allene oxide synthase formed a single C-13 diastereomer. (Although there were three

**FIG. 2. Structures of P1, P2, and P3.**

![Structures of P1, P2, and P3.](image)

**TABLE III**

Proton NMR spectrum of product P3 in CD2Cl2

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Multiplicity</th>
<th>Assignment: coupling constant</th>
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<td>6.38 to 6.31</td>
<td>dd</td>
<td>H10 (or H11); (J_{13,14} = 10.7) Hz, (J_{15,16} = 14.8) Hz</td>
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<tr>
<td>6.31 to 6.23</td>
<td>dd</td>
<td>H11 (or H10); (J_{13,14} = 10.6) Hz, (J_{11,12} = 14.8) Hz</td>
</tr>
<tr>
<td>5.85 to 5.80</td>
<td>dd</td>
<td>H9; (J_{13,14} = 14.8) Hz, (J_{9,10} = Hz)</td>
</tr>
<tr>
<td>5.815 to 5.76</td>
<td>dd</td>
<td>H12; (J_{13,14} = 14.8) Hz, (J_{12,13} = Hz)</td>
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</table>

\[\approx 5.5\]

(H5, 6, 17, 18 were obscured by \(CH_2Cl_2\))

4.22 q H8; \(J = 6.3\) Hz
4.07 t H15; \(J_{14,15} = 4.0\) Hz, \(J_{15,16} = 7.1\) Hz
3.05 dt H15; \(J_{14,15} = 4.0\) Hz, \(J_{15,16} = 6.6\) Hz
2.93 dd H14; \(J_{13,14} = 7.7\) Hz, \(J_{14,15} = 4.0\) Hz
2.55 to 2.45 m H7b
2.40 to 2.30 m H2a, b, H7a, H16a, b
2.15 to 2.05 m H4a, b, H19a, b
1.70 p H3a, b
0.98 t H20a, b, c

**FIG. 3. Mass spectrometric analysis of oxygen-18 retention in epoxyalcohols formed from 8R-hydroxy-15S-\(^{18}O\) hydroperoxyeicosapentaenoic acid.** From left to right are shown partial mass spectra of an unlabeled epoxyalcohol and the products from the \(^{18}O\) experiment (P1, P2, and P3). The samples were analyzed by negative-ion/chemical ionization GC-MS as the pentafluorobenzyl ester/TMS ether derivative. The intensities of ions corresponding to the prominent M-PFB ion were recorded by fast repetitive scanning from \(m/z\) 491 to 501. The partial mass spectra shown are the average of all scans collected during elution of the peak from the GC. The oxygen-18 content of the substrate in this experiment was measured on the corresponding 8,15-dihydroxy derivative: it comprised 3% \(^{18}O\), 1% \(^{16}O\), and 96% \(^{18}O\). From these values, the percent of hydroperoxide molecules containing \(^{2}O\) was estimated as 0.96 × 0.96 = 92%. The measured content of \(^{18}O\) in the epoxyalcohol products was P1 = 94%, P2 = 93%, and P3 = 89%.
epoxyalcohols, they were isomeric in the 11,12-double bond or the 14,15-epoxide; and no 13-hydroxy diastereomers of P1, P2, or P3 were detected.)

Although the reaction is enzymatic, three isomeric epoxyalcohols are formed from one substrate by the pure allene oxide synthase. P1 and P2 differ only in the configuration of the 11,12-double bond. This is very likely related to the isomerization that gives two allene oxides (11Z and 11E) from a single 8-hydroxy-15S-hydroperoxy substrate (Brash et al., 1990). It may be that the Z to E isomerization occurs before the reaction is committed to epoxyalcohol or allene oxide. Product P3 is a cis-epoxide, meaning that a fraction of the molecules are in the more hindered cis configuration when ring closure occurs. The appearance of both cis- and trans-epoxides apparently is indicative of an certain lack of stereochemical control by the enzyme.

Other instances of enzymatic epoxyalcohol synthesis have been described. The soybean lipoxygenase-1 converts the 13S-hydroperoxide of linoleic acid or 15S-hydroperoxyeicosatetraenoic acid to an epoxyalcohol in association with the transition from the “purple” to the “yellow” enzyme (de Groot et al., 1975). The product is a trans-epoxide with an α-hydroxyl in the relative three configuration, and there is a high retention (70%) of the two hydroperoxy oxygens in the biosynthesis (Garsen et al., 1976). These properties match those of the main epoxyalcohol (P2) in the experiments described here. Formation of a product of the same relative stereochemistry was described in an extract of potato, although the enzyme responsible was not identified (Galliard et al., 1975). Individual epoxyalcohols, not fully characterized in stereochemistry and that appear to be enzymatic products, have been reported in such diverse sources as marine algae and coral (Moghadam et al., 1990; Song and Brash, 1991b), products of mammalian lipoxygenases (Narumiya et al., 1981; Bryant et al., 1985), and products of 15S-hydroperoxyeicosatetraenoic acid metabolism in rat liver microsomes (Weiss et al., 1987).

Mechanistically unrelated to the transformations considered here is the epoxyalcohol biosynthesis via intra- or intermolecular epoxidation of an alcohol by “peroxygenase” enzymes (Hamberg et al., 1986; Blée et al., 1993).

There is a substantial body of literature on the mechanisms of fatty acid hydroperoxide rearrangement to epoxyalcohols (e.g. Hamberg (1974, 1987b), Pace-Asciak (1984), Dix and Marnett (1985), Labeque and Marnett (1989), Gardner et al. (1974, 1984), and Gardner (1989)). It is generally accepted that the heme- or iron-catalyzed reaction is initiated by homolytic cleavage of the hydroperoxide. This one-electron hydroperoxide reduction generates an iron-oxygen complex and an alkoxyl radical that cyclizes to a more stable carbon radical. Although cytochromes P450 can catalyze heterolytic as well as homolytic cleavage of hydroperoxides, the heterolytic reactions do not lead to the type of epoxyalcohols considered here. To form the epoxyalcohols via a heterolytic mechanism, the enzyme has to act as a Lewis acid, abstract OH−, and thus generate an epoxyallylic cation. Two lines of evidence suggest that this mechanism is unlikely to be executed by a cytochrome P450. Liebler and Guengerich (1983) have shown that metalloporphyrin complexes lack the Lewis acidity of the free metals. The strong electron-donating cysteinyl ligand of the P450 heme also dictates that the enzyme is unlikely to act as a Lewis acid and is more likely to behave as a two-electron donor and to reduce the hydroperoxide to an alcohol (Guengerich, 1990, 1991). Consequently, alcohols and the equivalent of Compound I enzyme-oxo complexes rather than epoxyallylic cations result from heterolytic cleavages of alklyhydroperoxides by cytochromes P450.

The precedent from nonenzymatic reactions and the known chemistry of cytochromes P450 both strongly suggest that homolytic cleavage of the hydroperoxide group is the initial step in the formation of the epoxyalcohols. More significantly, as the purified enzyme catalyzes the formation of both epoxyalcohols and allene oxides, it seems logical to infer that homolytic cleavage of the hydroperoxide may similarly represent the first step in allene oxide biosynthesis. Previously, heterolytic mechanisms have been proposed that involve an epoxyallylic cation as a possible transition state in allene oxide biosynthesis (Crombie and Morgan, 1988, 1991; Gardner, 1991). Our evidence favors homolytic cleavage as the initial event, with formation of an epoxyallylic cation implicated in a subsequent step (Scheme 2). Thus, starting from the fatty acid hydroperoxide substrate, heme-assisted homolytic scission of the oxygen-oxygen bond gives an alkoxyl radical and an iron-oxo complex. One-electron oxidation of the cyclized alkoxyl radical by the iron-oxo complex leads to an epoxyallylic cation. Loss of a proton from such a cation will give the allene oxide.

Apparently, addition of an 8R- but not an 8S-hydroxy group to the 15S-hydroperoxy fatty acid substrate interferes with the proper orientation on the enzyme, and the normal oxidation of the epoxyallylic radical by the iron-oxo complex is compromised by oxygen rebound to give the observed epoxyalcohols. Notably, the formation of epoxyalcohols is not related directly to the rates of reaction. The 8S,15S and 8R,15S diastereomers of eicosapentaenoic acid both react faster than the corresponding analogues of arachidonic acid, yet only the 8R-hydroxy substrates of each fatty acid form epoxyalcohols (Brash et al., 1990). There is complete retention of the original hydroperoxy oxygens in the epoxyalcohols, in keeping with the intermediate being positioned close to the heme in the active site of the enzyme. Presently, there is no clear understanding of the interactions between the substrate and the active site of the enzyme that result in allene oxide or epoxyalcohol synthesis; but mechanistically, it is evident that the two processes are closely related.

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