Soybean Epoxide Hydrolase

IDENTIFICATION OF THE CATALYTIC RESIDUES AND PROBING OF THE REACTION MECHANISM WITH SECONDARY KINETIC ISOTOPE EFFECTS*

Received for publication, October 5, 2004, and in revised form, November 19, 2004 Published, JBC Papers in Press, December 13, 2004, DOI 10.1074/jbc.M411366200

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Soybean epoxide hydrolase catalyzes the oxirane ring opening of 9,10-epoxystearate via a two-step mechanism involving the formation of an alkylenzyme intermediate, which, in contrast to most epoxide hydrolases studied so far, was found to be the rate-limiting step. We have probed residues potentially involved in catalysis by site-directed mutagenesis. Mutation of His220, a residue predicted from sequence analysis to belong to the catalytic triad of the enzyme, considerably slowed down the catalytic reaction, an Asp126 taking place. By inference, it was postulated that epoxide hydrolases belong to the α/β-hydrolase fold superfamily of enzymes, many structural features including conserved residues known to be essential for catalytic activity. The haloalkane dehalogenases possess in their active site a nucleophile-His-acid catalytic triad of residues. During the catalyzed reaction, an Asp is acting in the first step (dehalogenation) as a nucleophile leading to the formation of a covalent alkylenzyme intermediate. A His is essential for the second step of the process, i.e. in the hydrolysis of the ester bond of the transient alkylenzyme. By inference, it was postulated that epoxide hydrolases belong to the α/β-hydrolase family (6, 7), and this assumption was recently validated by three-dimensional structure determinations (8–10). It was shown that murine and bacterial (Agrobacterium radiobacter) EHs also possess a catalytic triad and that the catalyzed reactions proceed via a two-step pathway involving the transient formation of an alkylenzyme intermediate. In analogy to the dehalogenases, a covalently bound ester intermediate is formed in a first step by an attack of the oxirane ring of a nucleophilic Asp residue. In a second step, the Asp-His pair of the active site might activate a water molecule that hydrolyzes the intermediate, and the reaction product is released. Because this latter half-reaction is rate-limiting in most EHs studied, the alkylenzyme intermediate, which accumulates under steady-state conditions, could be trapped (12, 14–17). Up to now, the driving force leading to the oxirane ring opening and formation of the alkylenzyme remains a matter of debate. Two limiting molecular mechanisms have been suggested: (i) A S_n2-type reaction (“push-pull” mechanism); according to this mechanism, which is prevalent in literature (8, 18–22), the nucleophilicity of the Asp residue is instrumental in the oxirane ring opening step, which is also assisted by tyrosine residues (19, 21) that protonate the developing oxynion. (ii) A pull mechanism, in which the oxirane ring is first destabilized by strong interactions with e.g. hydrogen bonding.
residues that polarize the scissile bond. Accordingly, the formation of the alkenylenzyme would thus be a consequence of the epoxide destabilization rather than the cause, i.e. the reaction is more $S_21$-like (23).

In contrast to the wealth of studies on the mechanism of mammalian and bacterial epoxide hydrolases, little work has been devoted to plant epoxide hydrolases. Based on sequence analysis of EHs isolated from different plants (24–28), it was hypothesized that these enzymes have a catalytic mechanism akin to that of the other α/β-hydrolases and involves, for example, the formation of a covalent alkenylenzyme intermediate (29).

During recent years, we have investigated the properties of purified and recombinant soybean epoxide hydrolases. Interestingly, these enzymes present a strict enantioselectivity, e.g. toward 9,10-epoxyeicaric acid, a likely endogenous substrate involved in the biosynthesis of cutin monomers (30, 31). Thus, 9R,10S-epoxyeicaric acid is the preferred substrate, but importantly, both enantiomers of 9,10-epoxyeicaric acid yield the same enantiopure product, 9R,10R-dihydroxyeicaric acid. In sharp contrast, most soluble mammalian epoxide hydrolases do not exhibit such stereoselective reactions (31). To better comprehend the origin of this enantioselectivity, which will be discussed in a separate report, it was of great importance to progress in our understanding of the molecular mechanism of plant EHs. It should be noted that such issues, which are of interest from a mechanistic standpoint, are also highly relevant in the bioengineering of enzymes that can be used in biotransformations and in chemoenzymatic synthesis of chiral building blocks used for the production of bioactive compounds (32).

To elucidate the molecular mechanism of recombinant soybean epoxide hydrolase, we have studied the incidence on catalysis of site-directed mutagenesis of residues Asp126, His320, and Asp285, which from sequence alignments with other EHs, are predicted to constitute the catalytic triad of this enzyme (33). We could demonstrate the formation of an alkenylenzyme intermediate (31). During recent years, we have investigated the properties of purified and recombinant soybean epoxide hydrolases. Interestingly, these enzymes present a strict enantioselectivity, e.g. toward 9,10-epoxyeicaric acid, a likely endogenous substrate involved in the biosynthesis of cutin monomers (30, 31). Thus, 9R,10S-epoxyeicaric acid is the preferred substrate, but importantly, both enantiomers of 9,10-epoxyeicaric acid yield the same enantiopure product, 9R,10R-dihydroxyeicaric acid. In sharp contrast, most soluble mammalian epoxide hydrolases do not exhibit such stereoselective reactions (31). To better comprehend the origin of this enantioselectivity, which will be discussed in a separate report, it was of great importance to progress in our understanding of the molecular mechanism of plant EHs. It should be noted that such issues, which are of interest from a mechanistic standpoint, are also highly relevant in the bioengineering of enzymes that can be used in biotransformations and in chemoenzymatic synthesis of chiral building blocks used for the production of bioactive compounds (32).

Experimental Procedures

Materials—Commonly used chemicals and reagents were of the highest purity available. Purified oligonucleotides used for mutagenesis and DNA sequencing were provided by Invitrogen, Custom Primers, or Eurogentec. Nickel-nitrilotriacetic acid Superflow affinity gel was used for purification of enzymes. Purified oligonucleotides used for mutagenesis and DNA sequencing were provided either by Invitrogen, Custom Primers, or Eurogentec. Nickel-nitrilotriacetic acid Superflow affinity gel was used for purification of enzymes.

Preparation of Epoxides—Racemic-terminated or 14C-labeled epoxides were prepared chemically from [9,10-3H]- and [1-14C]oleic acid, respectively (34). Preparation of pure 9S,10R-epoxyeicaric acid was based on the high enantioselectivity exhibited by soybean epoxide hydrolase, which preferentially hydrolyzes the 9R,10S-epoxyeicaric enantiomer (35). For this purpose, 1H/14C-labeled racemic 9,10-epoxyeicaric acid (2 μl) was incubated with soybean epoxide hydrolase (1 μg) in 0.1 M potassium phosphate buffer, pH 7.4 (final volume 400 μl) for 4 min at 26 °C. After the addition of acetonitrile, the residual epoxide was purified by TLC using n-hexanediethyl ether:formic acid (50:50:1) as a solvent system. After methylation with ethereal diazomethane, this epoxide was found to consist of 9S,10R-epoxyeicaric acid with >99% enantiomeric excess when analyzed by pressure liquid chromatography on column.

Hydrolysis of Epoxides—Enzymatic hydrolysis of epoxides was performed as described previously (31), and the kinetic constants, i.e. $k_m$ and $V_{max}$, were determined using purified wild-type and mutant EHs. Initial rates were determined under conditions where rates were proportional to enzyme concentrations (up to 1 μg for purified enzymes) and the reaction progress was linearly dependent upon time (<10% of substrate transformation). The specific activities determined on soluble fractions of crude yeast extracts were obtained with the same quantities of protein as assessed by SDS-PAGE and density scanning.

Determination of the Formation of a Covalent Intermediate by Mass Spectrometry—9,10-Epoxyeicaric acid (2 μl) was incubated in the presence of purified soybean epoxide hydrolase H320Y mutant (6 μg) in ammonium acetate buffer (25 mM, pH 5.0, final volume 100 μl) for 10 min at room temperature. The sample was then diluted to 15 μl (15 μg of enzyme) in a 1:1 water:acetonitrile mixture (√/√), acidified with 1% formic acid, which allowed measurement of molecular weights with good precision (<0.01%). Electrospray ionization mass spectrometric studies were performed on a BioQ triple quadrupole mass spectrometer (Waters-Micromass) upgraded to Quattro II performance standards. Ions were detected below m/z 4000 at the exit of the first analyzer. Mass spectra were recorded in the positive ion mode on the mass range m/z 500–1500 at an accelerating rate of 60 V. Calibration of the instrument was performed using the multiply charged ions produced by horse heart myoglobin (Sigma) diluted to 2 μM in 1:1 water:acetonitrile (acidified with 1% formic acid) mixture.

Secondary Kinetic Isotope Effects—Kinetic isotope effects were measured by adapting the protocol described by Cohen et al. (36). Typically, a mixture (2 μl) of [9,10-3H]- and [1-14C]epoxyeicaric acid (0.6 μCi) and (1-14C)-epoxyeicaric acid (0.06 μCi) in 0.1 M potassium phosphate buffer, pH 7.4 (300 μl), was pre-equilibrated at 26 °C, and three zero time points ($t_0$ for purified enzymes) were measured. The reaction was initiated by the addition of soybean epoxide hydrolase (0.5 μg), and aliquots of the reaction mixture were removed at 10 time points ($t_0$ and quenched with acetonitrile (50 μl). The quenched samples were analyzed by 14C radio detection TLC (35) to determine the extent of conversion of the epoxide into diol. Bands corresponding to diols were then scraped off the plate, eluted with dichloromethane, and the tritium 14C ratio in these reaction products was measured by liquid scintillation counting using a standard predefined dual label program. The isotope ratios [14C/3H] and [3H/14C] were determined, respectively, at the given time points and after complete transformation of the substrate, were determined. Because of the high enantioselectivity of the recombinant soybean EH, the hydrolysis of racemic 9,10-epoxyeicaric acid could be treated conveniently as a succession of two reactions involving first the transformation of the preferentially hydrolyzed 9R,10S enantiomer (up to 50% reaction progress) followed by the hydrolysis of the opposite 9S,10R enantiomer. The secondary kinetic isotope effect (KIEs) for formation of 9,10-epoxyeicaric acid from the two epoxide enantiomers were calculated according to Equation 1.

$$\frac{V({14C})}{V({3H})} = 1 - f \ln(1 - f) \left( \frac{V({14C})}{V({3H})} \right)$$

(1)

where $f$ is the fractional conversion of each light isotope containing the 9,10-epoxyeicaric enantiomer as derived from the TLC 14C counting.
Because the substrate isotopic $^{3}H/^{14}C$ ratio at $t_{0}$ was equal to the $^{3}H/^{14}C$ ratio in the products at $t_{i}$, an average of the $t_{0}$ was used as ($^{3}H/^{14}C$) in Equation 1. The validity of this approach was established by showing that the calculated KIEs ($^{3}H/^{14}C$) in Equation 1 were independent of the reaction progress; e.g. the S.D. were <3% for 7-9 time points spanning an important fractional conversion of the substrate. KIEs on the chemical hydrolysis of racemic 9,10-epoxy-stearic acid were obtained similarly by following the formation of 9,10-dihydroxystearic acid from a mixture of 9,10-$^{3}H$- and 1-$^{14}C$-labeled epoxides incubated at 25 °C in a mixture of tetrahydrofuran:water (50:50, v/v) containing 0.6% perchloric acid. In this case, however, as for the enzymatic hydrolysis of enantio pure 9S,10R-epoxystearate, a single exponential progress curve was observed and $f$ (Equation 1) covered the total reaction (100% of reaction progress).

Analytical Procedures—Radioactivity was measured on TLC plates with a Berthold TLC linear detector LB 2821, and peak integration was obtained by using the program CHROMA 1D (Packard Instrument Company). Radioactivity was also determined in a liquid scintillation spectrometer (LS 9000, Beckman).

RESULTS AND DISCUSSION

In this work, we addressed three main issues pertaining to the mechanism of recombinant soybean epoxide hydrolase: (i) the experimental confirmation of the key catalytic residues, (ii) the occurrence of a two-step kinetic mechanism involving the formation of a transient covalent substrate–enzyme intermediate and identification of the rate-determining step of the catalytic process, and (iii) the molecular mechanism of the epoxide ring opening step.

The Occurrence of a Stable Covalent Intermediate Cannot Be Demonstrated Experimentally in Reactions Catalyzed by Wild-type Soybean Epoxide Hydrolase—To confirm that soybean EH-catalyzed reactions proceed via a two-step mechanism, as demonstrated previously with mammalian and bacterial EHs, we have attempted to trap the postulated $\alpha$-hydroxyalkylenzyme intermediate resulting from the opening of the epoxide by the putative nucleophilic Asp$^{126}$ residue. For that purpose, we have incubated the enzyme in the presence of [1-$^{14}C$]-9,10-epoxystearic acid and precipitated the mixture with an acidified solvent. This technique, which was successfully used for labeling, e.g. murine epoxide hydrolase with juvenile hormone III as substrate (15), did not permit the isolation of a labeled reaction intermediate in our case. Further attempts to covalently bind 9,10-epoxystearic acid to soybean epoxide hydrolase by varying the reaction conditions, including several enzyme or substrate concentrations with increasing incubation times, have all failed (not shown). Thus, we switched to a procedure that permitted a direct visualization of an enzyme–substrate ester bond in the soluble and membrane-bound mammalian rat EH-catalyzed transformation of [1-$^{14}C$]-9,10-epoxystearic acid (37). Accordingly, soybean epoxide hydrolase was incubated in the presence of labeled substrate, and after precipitation with acetone, the protein was subjected to SDS-PAGE and bound radioactivity detected by fluorography. This approach also proved unsuccessful (results not shown). Finally, we used hydroxylamine as an exogenous nucleophile, which could, in principle, compete with the water molecule that hydrolyzes the alkylenzyme ester bond and produce a hydroxamate derivative of Asp$^{126}$, which results in an inactivation of the enzyme. Such a strategy was used successfully to demonstrate the occurrence of a covalent intermediate in reactions catalyzed by L-2 haloacid dehalogenase, i.e. an enzyme mechanistically related to epoxide hydrolases (38). However, unlike the dehalogenase, soybean EH was not inhibited by hydroxylamine (up to 0.5 M), suggesting that the ester bond of the putative alkylenzyme intermediate was not accessible to nucleophiles other than water.

Our inability to detect a covalent intermediate during the transformation of 9,10-epoxy-stearic acid by soybean EH might be explained by considering the relative rates of the two steps of the catalytic process (Equation 2, where $E-I$ is the alkylenzyme).

\[
\begin{align*}
E + S & \rightleftharpoons ES \\
& \rightarrow E-I \\
& \rightarrow E + P \\
& \text{(Eq. 2)}
\end{align*}
\]

In the mammalian and bacterial epoxide hydrolases studied so far, the hydrolytic cleavage of the alkylenzyme intermediate, i.e. the dealkylation step $k_{3}$ in Equation 2, is rate-limiting; this kinetic barrier allows a significant accumulation of the intermediate, which can thus be trapped (14, 17). We speculated that if conversely, in soybean EH, the breakdown of the intermediate of the enzyme is more rapid than the alkylation step ($k_{3}$ in Equation 2), this change in the rate-limiting step might hinder the accumulation of E-I and make it more difficult to evidence. This would not be without precedent because a fungal EH was also suggested to have a slow alkylation step (39).

To test this hypothesis, we have attempted to turn the second half-reaction (reaction $k_{4}$) into the rate-determining step of the catalyzed reaction by slowing down the hydrolysis of the putative alkylenzyme. This was achieved by mutating His$^{320}$, the conserved residue suggested to activate the water molecule involved in the hydrolytic cleavage of the ester bond of the alkylenzyme intermediate.

Formation of a Stable Covalent Intermediate Evidenced by Mass Spectrometry in a His$^{320}$$\rightarrow$Tyr Mutant—Replacement of His$^{320}$ by a tyrosine gave a mutant that could be expressed in yeast in quantities similar to the wild-type enzyme. Compared with the wild-type enzyme, dramatic changes were observed in the transformation of [1-$^{14}C$]-9,10-epoxystearic acid catalyzed by the purified H320Y mutant (Fig. 1). Because of its low residual activity (<1/1500 of wild-type soybean EH activity), this mutant was used in a concentration range (about 7 μM) that was close to that of the labeled substrate (peak 3). As shown in Fig. 1, when aliquots of the reaction medium were directly spotted on a TLC plate, a new radioactive peak (peak 1) appeared within a few seconds, whereas essentially no diol (peak 2) could be detected during the first hour of incubation. The peak, which did not migrate under our conditions, accounted (after a few minutes) for most of the radioactivity used in the assay. Peak 1 then slowly evolved with time yielding the expected product, 9,10-dihydroxystearate (peak 2). This behavior strongly supported the idea that peak 1 on the base line corresponded to a stable substrate-enzyme adduct, which is turned over only very slowly and which could thus be amenable to further characterization. Importantly, no similar changes of substrate could be observed in control incubations using heat-inactivated mutant or wild-type soybean EH.

To verify whether a covalent alkylenzyme did indeed accumulate during the incubation of H320Y mutant with 9,10-epoxystearate, the enzyme was analyzed by electrospray mass spectrometry. The mutant, when incubated in the absence of substrate, showed the presence of multiply charged ions, allowing the determination of the molecular mass of the protein. Surprisingly, two different molecular masses could be calculated. The first one was 37,011 Da (Fig. 2A), which corresponds to the molecular mass predicted from the amino acid sequence of H320Y soybean EH possessing a hexahistidine tag. A second apparent molecular mass was 36,599 Da (Fig. 2A), which putatively corresponded to H320Y with a tag restricted to four histidine residues. Indeed, C-terminal sequencing of the enzyme revealed that ~40% of the H320Y epoxide hydrolase mutant had lost two terminal residues of its histidine tag. The mass of H320Y that had reacted with 9,10-epoxystearic acid was also determined by the same procedure (Fig. 2B). Values of 36,898 and 37,310 Da were found, corresponding to an increase of 299 mass units over the unreacted mutant proteins. This is in excellent agreement with the mass increment expected for the formation of an alkylenzyme (+298.5 Da) intermediate by
reaction of the mutant with 9,10-epoxystearic acid.

These results demonstrate that mutation of His 320 profoundly affected the kinetic mechanism of soybean EH. The H320Y mutant is an enzyme in which overall catalytic efficiency was much impaired and that, on incubation with the substrate 9,10-epoxystearic acid, readily yields a covalent reaction intermediate stable enough to be characterized by mass spectrometry and that evolves only very sluggishly to complete the catalytic process, giving the product 9,10-dihydroxystearate. Our results are also in favor of a two-step mechanism (Equation 2) and confirm the paramount role of His320 most probably as a general base in the hydrolytic $k_3$ step. They also strongly suggest that the rate-determining step in the soybean EH catalytic cycle is the formation of the intermediate, which does not accumulate in the wild-type enzyme. This difference in the nature of the rate-limiting step between the soybean enzyme and most other EHs studied so far, including with 9,10-epoxystearic acid as substrate (37), is worthy of comment. It was recently hypothesized that, for EHs involved in the disposition of toxic metabolites, a fast step leading to the formation of an alkylenzyme intermediate might correspond to a biological advantage; i.e. provided they are expressed in high quantities, these EHs could rapidly sequester such epoxides (40). Soybean EH, in which the formation of the covalent enzyme-substrate intermediate is the slow step of the catalytic process, is clearly different. However, as already pointed out by Fersht (41), enzymes that are crucial in physiological processes must avoid saturation at low substrate concentrations and consequently the accumulation of reaction intermediates; this implies, in a multistep reaction, a fast conversion of the intermediate relative to its formation, as found here with 9,10-epoxystearate, a compound involved in cutin biosynthesis (30).

**Kinetic Parameters of Asp285 Mutant**—In almost all epoxide hydrolases studied to date, the ionized acid residue of the catalytic triad that is hydrogen bonded to His (herein His320) in a charge relay system is an aspartate (presumably Asp285 for soybean enzyme), except in membrane-bound mammalian EHs, where a glutamic acid is invariably found at this position (42). This residue seems to make an important contribution to the catalytic activity of some EHs. For example, replacement of the glutamate residue by an aspartate in rat mEH strongly increased the turnover rate of this enzyme (42). On the other hand, the exchange of the triad aspartate by a glutamate in Aspergillus niger EH, i.e. a soluble enzyme related to mammalian microsomal EHs, resulted in a switch of the rate-limiting
Table 1: Specific activities of wild-type and mutant soybean epoxide hydrolases

| Enzyme          | Specific activity a (pmoles/min/mg protein) |
|-----------------|-------------------------------------------|
| Wild type       | 877 ± 25                                  |
| D285C           | 298 ± 10                                  |
| D285L           | 1.5 ± 0.2                                 |
| E195A           | 888 ± 30                                  |
| D285C/E195A     | 135 ± 9                                   |
| D285L/E195A     | <0.02                                     |

a All assays were performed on the soluble fraction of yeast extracts and with 9,10-epoxystearic acid as substrate. The reported results are means ± S.D. (n = 3).

The step of the enzymatic reaction (39). We found that in soybean EH, substitution of Asp285 by a glutamate affected only moderately its $V_{\text{max}}$ which decreased from 18.0 (wild-type) to 3.75 μmol/min/mg, indicating that this location of the active site can accommodate, without too great a consequence, another acidic residue. Importantly, the $K_m$ of the mutant remained essentially unchanged, as expected in the absence of a change in the nature of the rate-limiting step.

Two other mutants of Asp285 gave contrasting results. First, D285C remained fairly active (Table I), suggesting that Asp285 could be replaced by a cysteine residue. Second, replacement of Asp285 by a leucine caused a large decrease of activity, which represented about 0.17% of the wild type (Table I). A similar observation was reported for an EH mutant from A. radiobacter when the Asp residue was replaced by an alanine (14). The crystal structure of this enzyme suggested that the residual activity of the mutant might be due to the presence of a second aspartate close to the active site (9). By modeling the active site of soybean EH (Fig. 3), it appears that Glu195 could be a good candidate to play such a role in our enzyme. To test this hypothesis, we have mutated this residue and also constructed double mutants altered at positions 285 and 195. The single mutant E195A hydrolyzed 9,10-epoxystearic acid at the same rate as the wild-type enzyme (Table I), suggesting that this glutamate residue does not play a decisive role when Asp285 is unaffected. Double mutations gave contrasting results. Although D285L/E195A had no measurable activity, the specific activity of D285C/E195A decreased only by half compared with that of the single mutant D285C (Table I). These results indicate that: (i) Glu195 might be an alternative residue of the catalytic triad, and (ii) if Asp285 does indeed play a role in the catalytic triad, we must assume that an (ionized) cysteine residue is also effective in the assistance of the proton abstraction step.

In analogy to studies where attempts were made to convert serine proteases into cysteine proteases, we have investigated the catalytic activity of the D126C mutant. No diol formation could be readily detected after incubation of [14C]9,10-epoxystearic acid with this mutant. However, when the reaction mixture was analyzed by TLC at different time points, a new radioactive peak (X), which did not migrate in our solvent conditions, appeared within the first minutes of reaction (Fig. 4). This compound was stable. When using the same substrate concentration, we found that the amount of peak X was proportional to the quantity of the mutant enzyme used (not shown).

In analogy to the results obtained with the H320Y mutant (see above and Fig. 1), we have tentatively assigned X as the product of the hydrolysis of the ester bond of the alkylenzyme intermediate. Interestingly, the initially inactive mutant D126N slowly recovered hydrolytic activity, i.e., about 80% of the wild type after an overnight incubation at 26 °C, confirming that this asparagine residue, which was prone to a spontaneous deamination process (44), was indeed part of the active site of the enzyme. One could suggest that, in analogy to the mechanism proposed for the hydrolytic cleavage of the ester bond of the alkyl enzyme intermediate, the carboxamide moiety of Asn285 might also be hydrolyzed by an activated water molecule generated, in the absence of substrate, by the general base His195. A similar observation was made previously for an analogous mutant of a mammalian EH (18).

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In analogy to the results obtained with the H320Y mutant (see above and Fig. 1), we have tentatively assigned X as the covalent thioether intermediate that could be formed via a nucleophilic attack of the substrate epoxide by the Cys126 residue of the mutated enzyme. This observation implies that Cys126 could replace Asp285 within the active site, presumably generating a stable thioether bond on opening of the substrate epoxide ring. Because such a bond is not prone to hydrolysis, the enzyme becomes irreversibly blocked, and one could consider for this particular mutant of soybean EH, that the normal substrate 9,10-epoxystearate becomes the equivalent of a mechanism-based enzyme inhibitor. Such cysteine-histidine ion pairs were already shown to play a central role in the catalytic activity of chalcone synthase and cysteine proteases, such as papain (45, 46).
results contrast with those obtained by Janssen and co-workers (19) with a bacterial EH where mutations of the catalytic tyrosine residues resulted in only a 2–4-fold decrease of \( k_{cat} \) values, whereas the \( K_m \) increased by three orders of magnitude. This can, however, be explained by the difference of the rate-determining step of the two EHs and by a reversal of this step in the Tyr mutants of the bacterial enzyme. Thus, in the bacterial EH, the second half-reaction, which was limiting in the wild-type enzyme and in which the rate is unaffected by Tyr mutations, becomes faster in the mutants relative to the alkyl-enzyme intermediate formation step.

From a mechanistic standpoint (see below), a general acid-catalyzed activation of the epoxide ring of 9,10-epoxystearate by Tyr\(^{175}\) and Tyr\(^{255}\) can be interpreted in light of recent theoretical studies on the activation of oxiranes by 1,8-biphenylenediol, a molecule that nicely mimics the situation occurring in the active site of EHs. Compared with a monodentate acid, this molecule, which was taken as a model for bidentate Bronsted acids, is much more efficient in removing the electronic charge from the oxirane ring through paired interaction orbitals, thus facilitating the nucleophilic attack of the C–O bond and also reducing (by serving as a temporary reservoir of electronic charges) the overlap repulsion between the opening epoxide and the incoming nucleophile (47). Thus, from a catalytic standpoint, the activation of an oxirane ring by the enzyme is rather enhanced by the presence, in an optimal geometry (48), of two tyrosine residues that serve as an H-bond donor and one of them as a proton donor; this might be particularly crucial for a poorly reactive epoxide as the one used here as the substrate. An important issue, nevertheless, remains, which is related to the stabilization/reprotonation of the tyrosine residue that gave its proton to the epoxide oxygen to generate the alkyl-enzyme intermediate. Hammock and co-workers (20) have suggested that the tyrosinate could be H-bonded to the other Tyr residue of the active site, whereas Schiott and Bruice (22) have predicted the occurrence of a proton shuttle between the His residue of the catalytic triad and this tyrosinate. Fig. 5 summarizes the residues that were identified in this work by site-directed mutagenesis and their putative role in the mechanism of hydrolysis of 9,10-epoxystearate into 9\(R\),10\(R\)-dihydroxy-stearate catalyzed by soybean EH.

**Secondary Tritium Kinetic Isotope Effects on the Hydrolysis of 9,10-Epoxystearate**—The main question we wanted to answer in the present work was: is the nucleophilic attack at the S-carbon of 9,10-epoxystearic acid by the soybean EH-active site Asp\(^{126}\) a concerted process with the epoxide ring opening or is it this reaction more stepwise, involving in the rate-limiting step a polarization of the oxirane by Tyr\(^{175}\) and Tyr\(^{255}\) acting via H-bonding/general acid catalysis and resulting in the formation at the S-carbon of a (partial) positive charge, which is subsequently trapped by Asp\(^{126}\) leading to the alkyl-enzyme intermediate? To answer this question, we used secondary KIEs that have been widely exploited to elucidate the mechanism of reactions and determine the structure of their transition states (49–51).

In enzyme-catalyzed processes, techniques have been devel-

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**Characterization of Tyr\(^{175}\) and Tyr\(^{255}\) Mutants**—The discovery, by crystallography, of the presence within the active site of two tyrosines was of importance with regard to the molecular mechanism of the epoxide ring opening step catalyzed by epoxide hydrolases (19, 21). These residues have been identified as essential and assigned as general acid catalysts, which via H-bonding could polarize the epoxide moiety of the substrate and be involved as proton donors in the formation of the alkyl-enzyme intermediate. Sequence alignment of soybean EH with eukaryotic and microbial EHs predicts that the conserved Tyr\(^{175}\) and Tyr\(^{255}\) could play such a role in the soybean enzyme. To investigate the functions of these residues, Tyr\(^{175}\) was mutated to Ser and Tyr\(^{255}\) to Phe and His and the kinetic parameters determined for 9,10-epoxystearic acid with the purified mutants. Y255F and Y255H mutations resulted in enzymes in which the specificity constant (Table II) and in which the rate is unaffected by Tyr mutations, becomes faster in the mutants relative to the alkyl-enzyme intermediate formation step.

**TABLE II**

 Kinetic parameters of purified recombinant wild-type and Tyr\(^{255}\), Tyr\(^{175}\) soybean epoxide hydrolase mutants

|          | \( V_{max} \) | \( K_m \) | \( V/K_m \) |
|----------|---------------|----------|-----------|
| Wild-type | 18            | 25        | 0.72      |
| Y255F    | 0.005         | 15        | 0.00033   |
| Y255H    | 0.0016        | 60        | 0.000026  |
| Y175S    | 0.0112        | 200       | 0.000056  |

* 9,10-epoxystearic acid was used as substrate.

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**FIG. 4.** Radiochromatogram of the hydrolysis of [1-\(^{14}\)C]9,10-epoxystearic acid catalyzed by the D126C mutant of soybean epoxide hydrolase. The mutant (20 \( \mu \)g) was incubated in the presence of substrate (20 \( \mu \)M, 2 \times 10\(^{4}\) disintegrations/min) for 3 h at 27 °C in 0.1 M KH\(_2\)PO\(_4\) buffer (pH 7.4) (final volume 100 \( \mu \)l). The reaction mixture was analyzed on a TLC plate developed in hexane:diethyl ether:formic acid (50:50:1, v/v) as solvent. X was tentatively assigned as a covalent thioether intermediate formed by the reaction of the mutant with the substrate. S, start; F, solvent front.

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**Molecular Mechanism of Soybean Epoxide Hydrolase**
opened that measure KIEs on $V/K_m$, i.e. the kinetic parameter, which in a multistep reaction, reflects the events leading to the first irreversible step of the pathway (52). In soybean epoxide hydrolase-catalyzed reactions, which involve the intermediary formation of an alkylenzyme, such KIE determinations would be particularly powerful tools in analyzing the molecular mechanism of the epoxide ring opening step, which in the wild-type enzyme, is rate-determining. We have measured secondary tritium kinetic isotope effects by the competitive method (53) with the labeled pair [9,10-3H]9,10-epoxystearic acid and [1,14C]9,10-epoxystearic acid (remote label) as substrates (see “Experimental Procedures”). Because of the high enantioselectivity of the enzyme (31), the first part of the soybean EH-catalyzed reaction will overwhelmingly consist, in the attack at C-10, of [9,10-3H]R9,10S-epoxystearic acid by Asp126, leading to the formation of the alkylenzyme and ultimately to [9,10-3H]R9,10R-dihydroxystearic acid. The rate of epoxide opening is expected to be affected by two secondary tritium KIEs (Fig. 6). First, an $\alpha$-secondary KIE resulting from the presence of a $^3$H substituent on C-10; this isotope effect is the most informative as a mechanistic probe, because it is sensitive to changes of hybridization at C-10 (e.g. $sp^3 \rightarrow sp^2$), and one can predict that it will be normal ($\nu(V/K_m) > 1$) and large (>1.2) if a (partial) positive charge develops at the transition state (dissociative pathway); conversely it will be small (or slightly inverse) when the epoxide opening reaction is a $S_N2$-type process (associative pathway) (54, 55). Second, a $\beta$-secondary KIE due to the presence of a $^3$H substituent on C-9; this KIE is observed principally when the $^3$H–C-9 bond can hyperconjugate to the empty $p$-orbital of an adjacent (i.e. at C-10) cationic species. $\beta$-Secondary KIEs, which are, in general, of lesser magnitude than $\alpha$-secondary KIEs (54), are very sensitive to the conformation of the transition state, i.e. in our case, on the dihedral angle between the $^3$H–C-9 bond and the putative C-10 cationic $p$-orbital; they are minimal at $\theta = 90^\circ$ (perpendicular) and maximal at $0^\circ$ (ellipsed or anti) (56). Because [9,$^3$H]- and [10,$^3$H]10-epoxystearic acid were not available, we could not determine separately and precisely both the $\alpha$- and $\beta$-secondary KIEs. We believe, however, that despite the fact that precise numbers cannot be reached by using [9,10-3H]9,10-epoxystearic acid as substrate (i.e. the measured KIEs will consist of a contribution of both $\alpha$- and $\beta$-secondary KIEs), the magnitude of the observed KIE will nevertheless be quite informative, from a phenomenological standpoint, on the nature of the transition state of the oxirane ring opening step. Because in our particular case the dihedral angle between the $^3$H–C-9 bond and a putative $p$-orbital at C-10 is not particularly favorable to hyperconjugation, the contribution of the $\beta$-secondary KIEs to the total value should not be very important. Altogether, the information reached with the KIEs should be particularly illuminating, allowing us to differentiate between limiting mechanisms, i.e. stepwise ($S_N1$-type) or a concerted process ($S_N2$-type), and providing information on the catalytic driving force of the oxirane ring opening by soybean EH.

The results given in Table III indicate that substitution of hydrogens at positions 9 and 10 by tritium results in large secondary KIEs for soybean EH-catalyzed transformation of 9,10-epoxystearate. These data were obtained with the racemic substrate, but because of the large enantioselectivity of the recombinant enzyme (31) (specificity ratio $E \approx 200$), the same progress curve could be used to measure KIEs on both 9R,10S- and 9S,10R-epoxystearic acids. Within experimental errors, both substrates gave the same secondary KIE values of $\approx 1.30$. The validity of this approach was borne out by the study of the hydrolysis of enantiopure 9S,10R-epoxystearate, i.e. the slow reacting enantiomer, which gave a single exponential progress curve and which yielded a similar KIE. Interestingly, acid-catalyzed hydrolysis of racemic 9,10-epoxystearic acid is also accompanied by a high secondary KIE (Table III). Altogether, our results show that the epoxide ring opening of 9,10-epoxystearate, catalyzed either by soybean EH or acid, is accompanied by very large secondary tritium kinetic isotope effects.

To reveal the specific contribution of EH catalytic residues, it is of interest to compare the chemical and enzymatic mechanisms of epoxide hydrolysis. The acid-catalyzed epoxide ring opening has been the subject of much scrutiny in the chemical literature (57). Although, in general, rate-determining C–O bond fission occurs, after a rapid $O$-protonation pre-equilibrium, at the carbon that stabilizes best the development of a positive charge, many examples are known where the epoxide ring opening seems to follow borderline reaction mechanisms. These latter reactions between $S_N1$ and $S_N2$ are characterized by late transition states, i.e. a highly dissociative reaction with a nearly complete C–O bond breaking accompanied by the occurrence of a pronounced positive charge at the reaction center, with little participation of the solvent (nucleophile) molecule (57). The KIE values found here, $\nu(V/K_m) \approx 1.30$, can be compared with a compendium of secondary $\alpha$-KIEs measured in glycosidase/nucleosidase-catalyzed reactions, which involve in their transition states the formation of oxocarbenium ions (54, 58). From a phenomenological standpoint, the magnitude of the secondary kinetic isotope effects that we have measured are clear-cut; they indicate that the oxirane ring opening process occurs via a highly dissociative mechanism that generates a (partial) positive charge on the carbon atom of the scissile C–O bond. Of importance only in this case, a $\beta$-KIE

![Putative reaction mechanism of soybean epoxide hydrolase-catalyzed hydrolysis of 9,10-epoxystearate and residues involved in the catalytic process.](Image)
the presence of recombinant soybean epoxide hydrolase (EH) or acid epoxystearic acid from the first half (the nearby anionic nucleophile Asp126 to generate, via a back-
residual bond order to the leaving oxygen, can then react with obtained as described under “Experimental Procedures.”

Values of the secondary kinetic isotope effects on the transformation of 9-3H,10-3H-labeled 9,10-epoxy-
stearic acid (conditions) T(4), 1.31 (EH) 1.30 (acid).

Values listed are the mean (±S.D.) of n independent determinations obtained as described under “Experimental Procedures.”

| Substrate a,b | 1/\(K_{iso}^f\) |
|--------------|-------------|
| 9R,10S-epoxy-
stearic acid a | 1.31 ± 0.06 (n = 4) |
| 9S,10R-epoxy-
stearic acid a | 1.29 ± 0.02 (n = 4) |
| Racemic 9,10-
epoxy-
stearic acid b (acid) | 1.31 ± 0.04 (n = 3) |
| 9S,10R-
epoxy-
stearic acid b (EH) | 1.31 (n = 2) |

Values of the secondary kinetic isotope effects on the transformation of 9^-3H^-1^-3H-labeled racemic substrates (a) or enantiopure 9S,10R-
epoxy-
stearic acid (b) into 9,10-dihydroxy-
stearic acid were obtained in the presence of recombinant soybean epoxide hydrolase (EH) or acid catalyzed (acid).

*Progress curves for the transformation of a mixture of 1^-14C^- and 9^-3H^-1^-3H-labeled racemic substrates (a) or enantiopure 9S,10R-
epoxy-
stearic acid (b) into 9,10-dihydroxy-
stearic acid were calculated respectively from the first half (f < 0.5) and second half (f > 0.5) part of the progress curves (7–9 time points each) and from the single exponential progress curves obtained in the acid catalyzed of the racemic substrate and EH catalyzed transformation of the single enantiopure enantiomer.

The secondary KIEs for the transformation of the racemic mixture of 9R,10S- and 9S,10R-epoxy-
stearic acid were calculated respectively from the first half (f < 0.5) and second half (f > 0.5) part of the progress curves (7–9 time points each) and from the single exponential progress curves obtained in the acid catalyzed of the racemic substrate and EH catalyzed transformation of the single enantiopure enantiomer.

could also contribute, via a hyperconjugation mechanism, to the large observed KIEs. It should be noted that relatively few studies exist in literature related to the present study. Thus, determinations of \(\alpha\)-secondary deuterium KIEs on acid-catalyzed solvolysis of epoxides involving the development of partial positive charges at benzylic positions have been reported (59, 60); they are also normal but of lesser magnitude than the one found here (Table III).

Altogether, the results obtained in the present work could be tentatively summarized as follows (Figs. 5 and 6). An optimal interaction by hydrogen bonding of the oxirane oxygen with the two Tyr a,b and Tyr a,b residues, within the active site environment, will result in a strong polarization of the epoxide ring leading to its opening (without substantial nucleophilic assistance) via a late transition state in which the scissile C–O bond is substantially elongated/broken (i.e. dissociative or limiting \(S_{\alpha\beta}\) mechanism), generating in the rate-determining step a protonated oxynion (one Tyr residue donating a proton) and a largely developed positive charge on the C atom, which adopts a near planar \(sp^2\)-like hybridization. This carbon, with little residual bond order to the leaving oxygen, can then react with the nearby anionic nucleophile Asp a,b to generate, via a backside attack, the alkylenzyme intermediate with inversion of configuration. The fact that one Tyr residue most probably plays the role of a proton donor, yielding (during the epoxide opening step) an alcohol rather than an oxynion, also helps to limit an internal return reaction, i.e. reformation of the epoxide by reaction of an oxynion with the C–Asp a,b bond of the alkylenzyme intermediate. Altogether, our results indicate that the oxirane ring opening step leading to the formation of the covalent enzyme-substrate intermediate occurs via a very loose transition state in which bond breaking is well in advance of bond making; i.e. the driving force of the reaction catalyzed by soybean EH is most probably because of the polarization/protonation of the epoxide by the catalytic Tyr residues (pull mechanism) rather to an associative mechanism involving a nucleophilic attack by Asp a,b (push mechanism). Our results are, however, somewhat at odds with the published mechanisms of EHs of mammalian (61) or bacterial origin, which in their vast majority, favor a coordinated push-pull mechanism where the reaction is initiated by the nucleophilic attack (\(S_{\alpha\beta}\)-type reaction mechanism) by the catalytic Asp residue on an activated epoxide ring H-bonded to the neighboring Tyr residues (8, 18–22). It should be stressed, however, that in contrast to most reactions catalyzed by these EHs, the rate-determining step of 9,10-epoxide transformation by soybean EH is the formation of the covalent enzyme-substrate intermediate and not its hydrolysis. This might explain why the epoxide activation step is so critical for the soybean enzyme. Moreover, other studies exist in literature that support the epoxide ring opening mechanism found for the soybean EH. Thus, Hammett relationships obtained in the hydrolysis of \(p\)-substituted aryl oxides by fungal EHs were in favor of a predominant electrophilic activation during the oxirane ring opening (23). Similarly, an anomalous course of the transformation of mesonorbornane epoxides by a rabbit liver microsomal EH could only be explained by the transient formation of a carbocation-like species, generated by an electrophilic activation of the oxirane, in which rearrangement is faster than its capture by the catalytic Asp residue of the active site (62).

**Concluding Remarks**—Our work represents the first comprehensive attempt to solve the molecular mechanism of a plant epoxide hydrolase. From these investigations, an original catalytic scheme has emerged; because it presents subtle but significant differences from the prevalent mechanism accepted for mammalian and bacterial enzymes, it indicates the occurrence of an unforeseen variety of catalytic options open to this family of enzymes.
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Acknowledgment—The homology-modeled structure of soybean EH was determined by the “Service de bioinformatique et biologie structurale” headed by Marc Bergdoll, IBMP (Strasbourg, France).

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