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Mechanism of Soluble Epoxide Hydrolase

FORMATION OF AN α-HYDROXY ESTER-ENZYME INTERMEDIATE THROUGH Asp-333*

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^{18}O-Labeled epoxides of *trans*-1,3-diphenylpropene oxide (tDPPO) and *cis*-9,10-epoxystearic acid were synthesized and used to determine the regioselectivity of sEH. The nucleophilic nature of sEH catalysis was demonstrated by comparing the enzymatic and nonenzymatic hydrolysis products of tDPPO. The results from single turnover experiments with greater or equal molar equivalents of sEH:substrate were consistent with the existence of a stable intermediate formed by a nucleophilic amino acid attacking the epoxide group. Tryptic digestion of sEH previously subjected to multiple turnovers with tDPPO in $H_2{}^{18}O$ resulted in the isolation and purification of a tryptic fragment containing Asp-333. Electrospray mass spectrometry of this fragment conclusively illustrated the incorporation of ^{18}O. After complete digestion of the latter peptide it was shown that Asp-333 of sEH exhibited an increased mass. The attack by Asp-333 initiates enzymatic activity, leading to the formation of an α-hydroxyacyl-enzyme intermediate. Hydrolysis of the acyl-enzyme occurs by the addition of an activated water to the carbonyl carbon of the ester bond, after which the resultant tetrahedral intermediate collapses, yielding the active enzyme and the diol product.

Mammalian epoxide hydrolases (E.C. 3.3.2.3) have been implicated in the metabolism of epoxide containing xenobiotics, many of which are believed to be mutagenic and/or carcinogenic (1–3). During the past 20 years the mammalian microsomal epoxide hydrolase (mEH)[1] has received a great deal of attention partly due to its higher selectivity for cyclic and arene oxides (4–6). A great deal of work has provided a clear picture of the regio-, stereo-, and enantiospecificity of mEH (7–10). Most researchers agree that mEH hydrolyzes the epoxide via an *anti* (commonly referred to as *trans*) opening of the oxirane, where attack of the nucleophile usually takes place at the least sterically hindered carbon. These results are substantiated by substituent effects on the rate of hydrolysis as investigated by Dansette *et al.* (11) and kinetic solvent isotope studies as reported by Armstrong *et al.* (12). By the use of single turnover experiments in $H_2{}^{18}O$ (13), Lacourciere and Armstrong have also postulated that the hydrolysis of epoxides by mEH proceeds via the intermediary of a nucleophilic amino acid, yielding an acyl-enzyme intermediate, which is hydrolyzed further to release the diol product and the active enzyme. This is in contrast with the previously more accepted hypothesis in which an activated water molecule was thought to be responsible for direct attack on the epoxide.

Much less work has been completed on sEH, but the preliminary data closely parallel those for mEH. Because of the critical role of sEHs in the metabolism of xenobiotics and their possible involvement in the biosynthesis of metabolites (14–16, 18),[2] a detailed understanding of the catalytic mechanism of sEH is imperative. The *anti* opening of epoxides has been demonstrated on a select few substrates along with $H_2{}^{18}O$ studies, which have shown that nucleophilic attack occurs on the less hindered carbon (7, 19, 20). Recently, Hammock *et al.* (21) were able to trap a postulated acyl-enzyme intermediate, suggesting the possible involvement of a nucleophilic amino acid in the hydration of epoxides by sEH. Pinot *et al.* (22) were able to isolate a tryptic fragment of sEH containing the postulated acylated amino acid. The latter discovery is very significant in light of studies that have compared the sequence homology of several EHs. These studies have shown significant sequence similarity among EHs and a bacterial haloalkane dehalogenase (DhlA, from the bacterium *Xanthobacter autotrophicus*), which, among other regions of homology, possesses a conserved histidine and aspartic acid tandem (23–25). DhlA belongs to a group of enzymes known as the α/β hydrolase fold family, all of which contain a catalytic triad consisting of a nucleophile, a histidine, and an acid (26). If EHs, and particularly sEH in our case, belong to the α/β hydrolase fold family of enzymes then it can be inferred that the conserved aspartic acid is responsible for the nucleophilic attack on the epoxide, resulting in the acyl-enzyme intermediate observed by Hammock *et al.* (21).

Herein we detail experiments associated with the mechanism of sEH. Results of regioselectivity of hydration, direction of oxirane opening, mechanism of hydration, and identification of the putative catalytic amino acid are reported. Also, possible mechanisms of inhibitory action by chalcone oxides and glycidols are discussed.

MATERIALS AND METHODS

General Procedures—^{1}H-NMR spectra were obtained on a QE-300 MHz spectrometer (General Electric Co.) with *deutero*-chloroform

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[1] The abbreviations used are: mEH, microsomal epoxide hydrolase; sEH, soluble epoxide hydrolase; GC, gas chromatography; MS, mass spectrometry; HPLC, high pressure liquid chromatography; BSTFA, bis(trimethylsilyl)trifluoroacetamide; MTBSTFA, *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide; BSA, bovine serum albumin.

[2] K. Motoba, M. F. Moghaddam, B. Borhan, F. Pinot, and B. D. Hammock, submitted for publication.

($CDCl_3$) as solvent and tetramethylsilane as the internal standard. GC/MS analyses were performed using a VG Trio-2 spectrometer equipped with a VG11–250 data system. Electron ionization (70 eV) was employed. GC separation was performed on a Hewlett Packard 5890A gas chromatograph fitted with a DB-5 (30-m) capillary column directly attached to the mass spectrometer. High performance liquid chromatography (HPLC) analysis employed a Perkin Elmer system consisting of a series 410 BIO pump and a Rheodyne injector. UV absorbance was monitored at 215 nm using a Perkin Elmer LC-235 diode array detector. HPLC separation was accomplished by the use of a Vydac C4 (4.6 mm × 25 cm) column. A microbore HPLC model UMA 600 (Michrom BioResources, Inc., Pleasanton, CA) equipped with a C18 preinjection peptide trap and a 5-μ, 300-Å pore size, 1 mm × 5 cm column packed with Vydac C4 particles (Michrom BioResources, Inc.) was used to concentrate tryptic digest peaks obtained from the analytical HPLC. UV absorbance was monitored at 210 nm. Electrospray mass spectra of peptides were generated in positive ion mode using a VG/Fisons Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK). Peptide sequencing was performed at the Protein Structure Laboratory (University of California, Davis) by automated Edman degradation, utilizing an Applied Biosystems gas phase sequenator (Applied Biosystems, Foster City, CA) coupled to an Applied Biosystems 120A PTH analyzer and M900 data system.

Chemicals—^{16}O and ^{18}O *cis*-9,10-epoxystearic acid and *trans*-1,3-diphenylpropene oxide (tDPPO) were synthesized as described (27). The ^{16}O and ^{18}O methyl *cis*-9,10-epoxystearates were prepared by methylation of the ^{16}O and ^{18}O *cis*-9,10-epoxystearic acid with diazomethane (28). Bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-methyl-*N*-(*t*-butyldimethylsilyl)triflouroacetamide (MTBSTFA), immobilized protease Sg, and BCA reagent for protein concentration determination were obtained from Pierce. $H_2{}^{18}O$ (95% ^{18}O) and all other chemicals were purchased from Aldrich. Solvents used were reagent grade and were freshly distilled before use. Synthesis of *threo*-1,3-diphenyl-1,2-propanediol was achieved by *cis*-hydroxylation of *trans*-1,3-diphenylpropene with catalytic osmium tetroxide and *N*-methyl morpholine oxide (29).

Enzyme Preparation and Specific Activity Measurement—Recombinant murine sEH was produced in the baculovirus expression system as previously reported (22). The enzyme was purified from cell lysate by affinity chromatography as described by Wixtrom *et al.* (30). Specific activity of the purified enzyme was measured using ^{3}H-*trans*-stilbene oxide as described previously (31). Protein concentrations were determined with BCA reagent from Pierce using bovine serum albumin (BSA) as standard.

HPLC Analysis of tDPPO Hydrolysis Products—A 100 mM stock solution of tDPPO was prepared in dimethyl formamide. One μl of the stock tDPPO solution was added to 100 μl of sodium phosphate buffer (100 mM, pH 7.4) containing either 100 μg/ml BSA or 100 μg/ml BSA and 3.9 μg/ml sEH. Also, one μl of the stock tDPPO solution was added to a 5% solution of perchloric acid (100 μl) in order to generate *erythro*- and *threo*-diols of tDPPO. The latter two products are generated because acidic hydrolysis of the epoxide leads to a stabilized benzylic carbocation that can be attacked by water from either face of its trigonal planar geometry. The latter solutions were incubated in a 37 °C water bath, and 20 μl fractions were withdrawn at specific times for direct HPLC injection. The *erythro*- and *threo*-diols and the epoxide were separated on a reverse phase C18 column and monitored spectrophotometrically at 215 nm with a diode array detector. The solvent system used was as follows: 70:30::H_2O:acetonitrile from 0 to 12 min to resolve the two diols, followed by a step gradient at 12 min to 45:55::H_2O:acetonitrile to elute the epoxide. An aqueous solution of *threo*-1,3-diphenyl-1,2-propanediol was also prepared (100 μM final concentration) and analyzed under the same HPLC conditions in order to identify the *threo*-diol peak from the mixture of *threo*- and *erythro*-diols generated by acidic hydrolysis of tDPPO. The specific activity of the sEH used in this experiment was measured with ^{3}H-*trans*-stilbene oxide and found to be 895 nmol/min/mg protein.

GC/MS Analysis of Hydrolysis Products of tDPPO and cis-9,10-Epoxystearic Acid—Stock solutions of $^{16}O/^{18}O$-labeled tDPPO, $^{16}O/^{18}O$-labeled *cis*-9,10-epoxystearic acid, and $^{16}O/^{18}O$-labeled methyl *cis*-9,10-epoxystearate (100 mM) were prepared in dimethyl formamide. Enzymatic hydrolysis of the latter substrates was achieved by the addition of 1 μl of substrate (stock) to 100 μl of sodium phosphate buffer (100 mM, pH 7.4) containing 100 μg/ml BSA and 3.9 μg/ml sEH. Controls were also run with buffer containing only 100 μg/ml BSA to ensure that no hydrolysis had taken place due to the interaction of substrates with buffer and BSA. All enzymatic and control experiments were incubated in a 37 °C water bath for 30 min, after which saturated NaCl (100 μl) was added to quench the hydrolysis. The specific activity of the sEH used in this study was measured by ^{3}H-*trans*-stilbene oxide assay to be 916 nmol/min/mg protein.

Acidic hydrolyses of the same epoxides were performed by introducing 2 μl of the substrates (stock) into 5% perchloric acid solution (100 μl). After 30 min of incubation at 37 °C in a water bath, saturated NaCl (100 μl) and enough 10% KOH was added to neutralize the solution. Basic hydrolysis of $^{16}O/^{18}O$-labeled tDPPO was effected by the addition of 2 μl of each epoxide to a 10% KOH solution (100 μl) followed by incubation for 20 h at room temperature, at which time saturated NaCl (100 μL) was added and the reaction mixture was neutralized with 12 M HCl.

The enzymatic and chemical hydrolysis products were extracted with similar efficiency by the addition of 450 μl of ethyl acetate. Each test tube was vortexed vigorously and centrifuged in order to clearly separate the organic and aqueous phases. The top layer (ethyl acetate, 400 μl) was withdrawn and placed in a conical reaction vessel, isooctane (5 μl) was added as a trap solvent, and the solvent was evaporated under nitrogen flow. Each sample was derivatized 30 min prior to GC/MS analysis with BSTFA (50 μl). The latter experiments were performed in quadruplicate. The enzymatic hydrolyses were performed with three separate enzyme preparations. It should be noted that [^{3}H]tDPPO was synthesized for substrate activity studies[3] and was utilized here to measure the efficiency of ethyl acetate extractions of the epoxide and its corresponding diol. It was found that the ethyl acetate extracts contained >99.9% of the [^{3}H]diol and 100% of the [^{3}H]epoxide that were originally incubated in the assay buffer.

Single Turnover Experiments—The following ratios of sEH:^{16}O-substrate (tDPPO and *cis*-9,10-epoxystearic acid) were used: 1:2000, 1:20, 1:1, 2:1, 4:1, 10:1, 20:1, and 100:1. The 1:20, 1:1, 2:1, and 4:1 samples contained 156.25 μg of sEH (2.5 nmol), while the 10:1, 20:1, and 100:1 samples contained 312.5 μg of sEH (5.0 nmol). The 1:2000 sample contained 3.13 μg of sEH. The stock sEH solution (25 mg/ml) was diluted with sodium phosphate buffer (100 mM, pH 7.4) to achieve the indicated amounts of sEH in 50 μl of buffer in Eppendorf tubes. This would ensure that after lyophilization and addition of 50 μl of water, the ionic strength of the new solution would be identical to the standard assay conditions. The samples were frozen just prior to the experiment in an ethanol/dry ice bath and lyophilized until all $H_2{}^{16}O$ was removed. The Eppendorf tubes containing the dry sEH were kept on ice, and $H_2{}^{18}O$ (25 μl) was added to rehydrate the protein. For each sEH sample, substrate diluted in $H_2{}^{18}O$ (25 μl) was prepared in a separate Eppendorf tube and kept on ice. The following is a list of substrate volumes and concentrations used to achieve each molar ratio: 1) 1:1, 1.0 μl of 2.5 mM; 2) 2:1, 1.0 μl of 1.25 mM; 3) 4:1, 1.25 μl of 0.5 mM; 4) 10:1, 1.0 μl of 0.5 mM; 5) 20:1, 1.0 μl of 0.25 mM; 6) 100:1, 1.0 μl of 0.05 mM; 7) 1:20, 1.0 μl of 50 mM; 8) 1:2000, 1.0 μl of 100 mM. To initiate enzymatic hydrolysis the cold substrate solutions were added and thoroughly mixed with the enzymatic solution at 0 °C with a pipetter and incubated for 10 min in a 37 °C water bath. This was sufficient for total conversion of epoxide to diol. The hydrolysis was quenched with the addition of saturated NaCl (100 μl), and the buffer solution was extracted with ethyl acetate (450 μl). The biphasic solution was vortexed vigorously and centrifuged. The top layer (ethyl acetate, 400 μl) was withdrawn and placed in a conical reaction vessel, isooctane (5 μl) was added, and the solvent was evaporated under nitrogen flow. The *cis*-9,10-epoxystearic acid hydrolysis products were dissolved in 50 μl of methanol and were methylated with (trimethylsilyl)diazomethane (50 μl, 2 M in hexanes). After 10 min the esterified products were again dried under nitrogen flow. Each sample was derivatized 30 min prior to GC/MS analysis with BSTFA (10 μl). The diol was the sole metabolite detected chromatographically, and assignment of its structure was supported by co-chromatography and by full spectrum GC/MS. To achieve the highest degree of sensitivity and precision in the single turnover experiments the mass spectrometer was programmed to monitor only diagnostic fragments for each substrate (single ion monitoring). For the derivatized diol of tDPPO the masses monitored were m/z 179, 181, 193, and 195, and for the derivatized diol of *cis*-9,10-epoxystearic acid they were m/z 215, 217, 259, and 261. These correspond to fragments generated by cleavage of the C–C bond between the derivatized diol oxygens and can be used to establish the extent of labeling at each position.

Tryptic Digestion of $^{16}O/^{18}O$-Labeled sEH—In order to label the catalytic amino acid with ^{18}O, 1.0 mg of sEH (200 μl) was lyophilized and subsequently rehydrated in $H_2{}^{18}O$ (200 μl). The hydrated sEH was

[3] B. Borhan, T. Mebrahtu, S. Nazarian, M. J. Kurth, and B. D. Hammock (1995) *Anal. Biochem.*, in press.

incubated with 100 mM tDPPO (4 μl) in a 37 °C water bath for 1 h, at which time the enzymatic solution was charged again with 100 mM tDPPO (4 μl) and incubated for another hour (50 theoretical turnovers). The reaction was quenched by precipitating the protein with the addition of 100% trichloroacetic acid (22 μl). The precipitated protein was pelleted by centrifuging the Eppendorf tube for 4 min at 16,000 × *g* at 4 °C. The pellet was washed twice with cold acetone (200 μl) and resuspended in double distilled water (200 μl) containing 1% acetic acid and then reprecipitated and washed as described above. The 1.0 mg of precipitated enzyme was dissolved in 250 μl of 8 M urea, 500 mM sodium phosphate buffer (pH 7.4). The urea solution was divided into 10 separate Eppendorf tubes for digestion on a smaller scale. To each Eppendorf tube 45 mM dithiothreitol (5 μl) was added, the tube was vortexed, and the mixture was incubated at 50 °C for 15 min. After a 15-min incubation at room temperature, distilled water (60 μl) and 5 μg of trypsin (1 mg/ml) were added and vortexed. The tryptic digestion was allowed to proceed for 2 h at 37 °C and was stopped either by freezing or by directly injecting into a reverse phase HPLC. The above procedure was duplicated with 1.0 mg of lyophilized sEH, which was rehydrated in $H_2{}^{16}O$ and incubated with excess tDPPO to achieve multiple turnovers.

Purification and Concentration of Tryptic Fragments Obtained from $H_2{}^{16}O$- and $H_2{}^{18}O$-treated sEH—The tryptic digests from the $H_2{}^{16}O$- and $H_2{}^{18}O$-treated sEH were subjected to reverse phase HPLC analysis. Fragments were eluted with the following acetonitrile/H_2O gradient: 0–2 min at 100% A, changed to 75% B from 2 to 112 min, up to 100% B in 3 min, followed by 15 min at 100% B (A and B were 0.06% trifluoroacetic acid, H_2O and 0.052% trifluoroacetic acid, 80% acetonitrile, H_2O, respectively). The flow rate was set at 0.5 ml/min, and the peptide elution was monitored by UV absorbance at 215 and 280 nm. The elution profiles of the $H_2{}^{16}O$- and $H_2{}^{18}O$-treated sEH fragments were virtually identical. Nine peaks eluting from 84 up to 121 min were collected. The region containing these nine peaks was chosen for collection of the tryptic fragment containing the postulated catalytic Asp-333 by calculating the relative hydrophobicity of each theoretical tryptic fragment. It was found that T26, the tryptic fragment that contains Asp-333 (T is the designation for tryptic, and 26 refers to the 26th theoretical fragment obtained from the digestion of sEH by trypsin, counting from the N terminus) had the second highest hydrophobic score. Therefore, it was expected that T26 would elute toward the end of the elution profile. The program utilized for the hydrophobicity calculations was the Fisons Biopolymer analysis routine in the VG MassLynx software system. The pooled individual peaks from multiple HPLC injections (2–4 ml) were diluted with an equal volume of doubly distilled water and were subjected to microbore HPLC. The large volume of eluant for each peak was slowly loaded onto a peptide trap (C18 cartridge), which was installed inline on the microbore HPLC's injection port. The microbore HPLC was programmed to produce the following gradient: 0–30 min at 50% A, changed to 100% B (A and B were 0.06% trifluoroacetic acid, H_2O and 0.052% trifluoroacetic acid, 80% acetonitrile, H_2O, respectively). The flow rate was set at 100 μl/min. The eluting fragments were monitored by UV absorbance at 215 nm, and the peaks were collected in Eppendorf tubes (~50 μl total volume, >100-fold reduction in volume).

Electrospray Mass Spectrometry of Tryptic Fragments—Electrospray mass spectra of the concentrated peptides were generated using a H_2O:acetonitrile:formic acid::49:50:1 mobile phase. An Isco μLC-500 syringe pump delivered the mobile phase at 5 μl/min. Solutions of purified peptides were analyzed by direct flow injection using an injection volume of 10 μl. Spectra were obtained in positive ion mode using a capillary voltage of +3.5 kV, a cone voltage of 50 V, and a source temperature of 65 °C. Spectra were scanned over the range of 200–2,000 *m/z* at a rate of 20 s/scan. Twenty scans were combined using the VG MCA acquisition mode. Molecular weights were determined by using the maximum entropy deconvolution algorithm (MaxEnt™) to transform the range of 500-1500 *m/z* to give a true mass scale spectrum. Mixed polyethylene glycols were used for mass calibration.

Enzymatic Hydrolysis of T26 and GC/MS Analysis—Immobilized protease Sg (250-μl suspension) was pelleted by centrifuging at 16,000 × *g* for 10 min. The aqueous solution was withdrawn, and the pellet was resuspended in 250 μl of NH_4CO_3 buffer (50 mM, pH 8.0) containing 5 mM $CaCl_2$, centrifuged, and resuspended again. The latter wash was repeated three times, after which the protease, suspended in the latter buffer, was added to lyophilized T26 isolated from the tryptic digestion of $H_2{}^{16}O$- and $H_2{}^{18}O$-treated sEH. The latter reaction was incubated at 37 °C for 18 h, at which time the immobilized protease Sg was pelleted by a 30-min centrifugation at 16,000 × *g*. The $NH_4CO_3/CaCl_2$ buffer containing the hydrolyzed amino acids was removed from the pellet, and the solution was frozen and lyophilized. The residue was derivatized with MTBSTFA (20 μl) 1 h prior to GC/MS analysis. Controls for the detection of derivatized aspartic acid and glutamic acid were also prepared by derivatization of each amino acid with MTBSTFA. Samples were analyzed using a DB-5 (15-m) capillary column (0.25-mm inner diameter, 0.25-μm film) using splitless injection and 70 eV electron ionization. Helium was used as carrier gas at a flow rate of 35 cm/s at 150 °C, and the injector was held at 285 °C. The column temperature was programmed from 150 °C (1-min hold) to 275 °C at 10 °C/min. These chromatographic conditions have been found to give complete separation of *tert*-butyldimethylsilyl (TBDMS) derivatives of all 20 amino acids. Single ion monitoring of each GC/MS run was performed to detect the abundant $[M - C_4H_9]^+$($[M - 57]^+$) fragments characteristic of the TBDMS derivatives for unlabeled Asp (*m/z* 418), $[^{18}O_1]$Asp (*m/z* 420), $[^{18}O_2]$Asp (*m/z* 422), unlabeled Glu (*m/z* 432), $[^{18}O_1]$Glu (*m/z* 434), and $[^{18}O_2]$Glu (*m/z* 436) using sample times of 80 ms/channel and a delay of 20 ms between channels.

RESULTS

General Base Catalysis of sEH Demonstrated with tDPPO—Early in our investigation of sEH we set out to determine the mechanism by which the epoxide is opened, *i.e.* A1 (S_N1 type) *versus* A2 (S_N2 type) (32). As can be seen in Fig. 1*a*, acid hydrolysis of tDPPO led to the expected equal amounts of *erythro*- and *threo*-diols (t_R = 6.92 and t_R = 8.08 min) due to the trigonal planar geometry of the resultant benzylic carbocation transition state, which allows attack of water to occur from either face of the carbocation. Osmium tetroxide *cis*-dihydroxylation of *trans*-1,3-diphenylpropene results in formation of the *threo*-diol, exclusively (Fig. 1*b*, t_R = 8.15 min). Therefore, the *erythro*-diol was identified as the peak at 6.92 min. Fig. 1*c* illustrates the incubation of sEH with tDPPO after 5 min. As the epoxide (t_R = 18.47 min) is turned over by the enzyme only the *erythro*-peak (t_R= 6.57 min) is formed, and after 40 min (Fig. 1*d*) all the epoxide has been converted to one single diastereoisomer. The *anti* (A2 type) opening of *trans*-epoxides exclusively produces *erythro*-diols, which was the only diastereoisomer observed in the hydrolysis of tDPPO. The *anti* opening of *cis*-9,10-epoxystearic acid with sEH has been previously established by Gill and Hammock (33) by demonstrating that *threo*-9,10-dihydroxystearic acid was the sole enzymatic product.

Regioselectivity of sEH with tDPPO and cis-9,10-Epoxystearic Acid—Next, we turned our attention to the regioselectivity of epoxide opening with sEH. The oxygen-labeled $[^{18}O]$tDPPO and $[^{18}O]$*cis*-9,10-epoxystearic acid were synthesized as described previously (27). $[^{18}O]$Methyl *cis*-9,10-epoxystearate was synthesized from the reaction of excess diazomethane and $[^{18}O]$*cis*-9,10-epoxystearic acid. Each ^{18}O-labeled epoxide was incubated with sEH for 10 min at 37 °C, after which the enzymatic hydrolysis products were extracted with ethyl acetate, concentrated, and analyzed by GC/MS. Fig. 2 illustrates the result of the latter experiment. The enzymatic epoxide opening of $[^{18}O]$tDPPO is accomplished largely by the attack of enzyme at the benzylic position (97.1% ± 0.8%). This leads to the labeled oxygen on the homobenzylic carbon; thus, after derivatization of the hydroxyls with BSTFA, the mass spectra showed abundant peaks at *m/z* 179 and 195 and much weaker signals at *m/z* 181 and 193. The result of KOH opening of $[^{18}O]$tDPPO was practically identical to the sEH opening of $[^{18}O]$tDPPO with regard to the position of the ^{18}O oxygen. The attack of hydroxyl anion occurred at the benzylic position (97.0% ± 1.0%). Acidic hydrolysis of $[^{18}O]$tDPPO was even more selective, exhibiting 100% attack of the benzylic position. This result is not surprising due to the stability of the benzylic carbocation.

$[^{18}O]$Epoxystearic acid also shows some regioselectivity of epoxide opening with sEH. Acidic hydrolysis of $[^{18}O]$*cis*-9,10-epoxystearic acid results in a 1:1 distribution of carbon 9- and carbon 10-labeled diol, a testament to the chemical equivalence

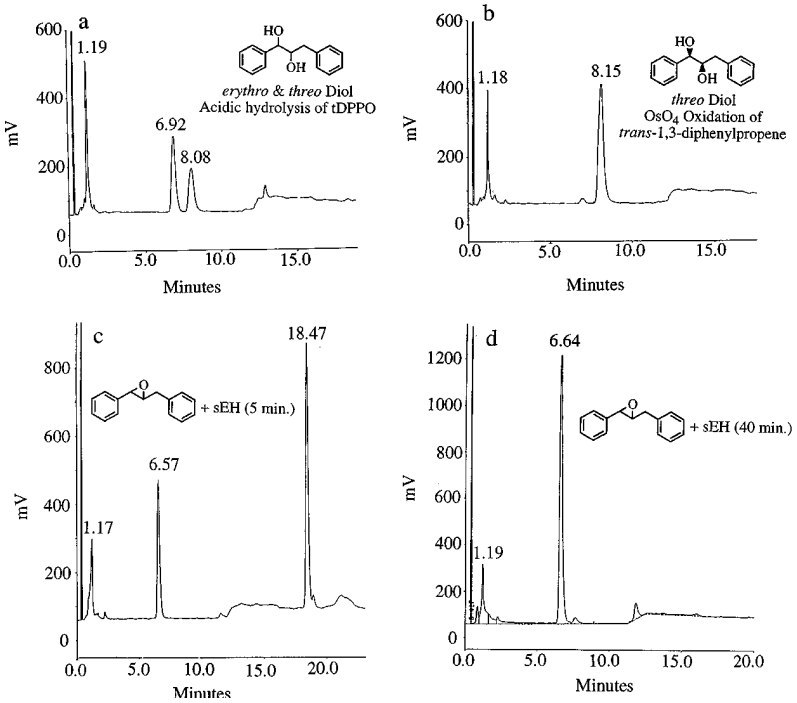

FIG. 1. **Reverse phase HPLC chromatograms of tDPPO and its corresponding diols.** *a*, the *threo*- and *erythro*-diols of tDPPO generated by acidic hydrolysis of tDPPO. *b*, *threo*-diol of tDPPO (*threo*-1,3-diphenyl-1,2-propanediol) generated by *cis*-hydroxylation of *trans*-1,3-diphenylpropene. *c*, 100 mM tDPPO incubated at 37 °C with 3.9 μg/ml sEH in sodium phosphate buffer (100 mM, pH 7.4) containing 100 μg/ml BSA after 5 min. *d*, same as *panel c* after 40 min.

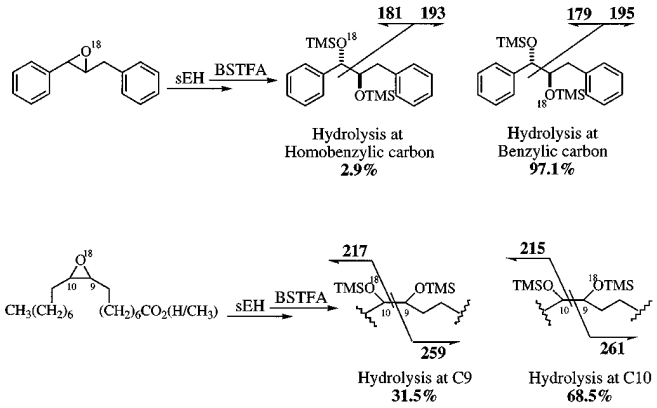

FIG. 2. **sEH exhibits a high regioselectivity of epoxide opening for tDPPO with over 97% of the attack occurring at the benzylic position.** The regioselectivity of sEH was less discriminating in the hydrolysis of *cis*-9,10-epoxystearic acid and methyl *cis*-9,10-epoxystearate.

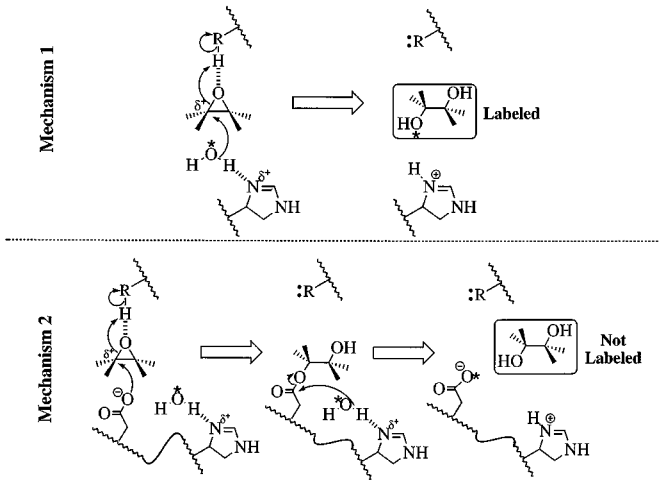

FIG. 3. **Two proposed schemes for the hydrolysis of epoxides by sEH.** *Mechanism 1* presupposes the activation of water by a histidine residue, which leads to the direct opening of the epoxide. If performed in $H_2{}^{18}O$, the resulting diol *would* incorporate ^{18}O oxygen after one turnover. *Mechanism 2* presupposes that a nucleophilic amino acid such as a carboxylate anion opens the epoxide, which would lead to the formation of an α-hydroxyester-enzyme intermediate. Subsequently, an activated water hydrolyzes the ester, which releases the diol product, and the enzyme is regenerated. If performed in $H_2{}^{18}O$, the resulting diol *would not* incorporate ^{18}O oxygen after one turnover. Acidic activation of the epoxide oxygen, which could enhance catalytic activity, is consistent with both mechanisms.

of the two epoxide carbons. However, as depicted in Fig. 2, 68.5% ± 1.3% of the enzymatic attack occurs at carbon 10. The ^{18}O label distribution following enzymatic hydrolysis of [^{18}O] methyl *cis*-9,10-epoxystearate is identical with the distribution following enzymatic opening of its corresponding free acid, suggesting that the anchoring of the carboxylic acid or ester on the enzyme is not the driving force for the observed regioselectivity.

Single Turnover Experiments with sEH—Fig. 3 illustrates the difference in products obtained in a single turnover experiment based on the two differing mechanisms proposed. Various concentrations of sEH:substrate, ranging from 100 to 0.01: 1 molar equivalents were incubated utilizing tDPPO and *cis*-9,10-epoxystearic acid as substrates. The sEH was lyophilized just prior to analysis, and it was rehydrated in $H_2{}^{18}O$. The products of enzymatic hydration generated at 37 °C for 10 min were extracted and derivatized with BSTFA as described above. During initial single turnover experiments it was noticed that tDPPO's high rate of hydrolysis led to complete consumption of substrate by a fraction of the enzyme population and yielded inconclusive data. However, after insuring rapid mixing of diluted substrate (in $H_2{}^{18}O$) at O °C with hydrated enzyme (in $H_2{}^{18}O$) and subsequent incubation at 37 °C, the results obtained conclusively supported a nucleophilic attack of enzyme upon tDPPO, which was followed by the hydrolysis of the resultant acyl-enzyme intermediate.

The latter result was substantiated further by single turnover hydrolysis and analysis of *cis*-9,10-epoxystearic acid in $H_2{}^{18}O$. The K_m and V_{max} for epoxy stearic acid and tDPPO were found to be 11.0 μM and 3460 nmol/min/mg protein, and 2.80

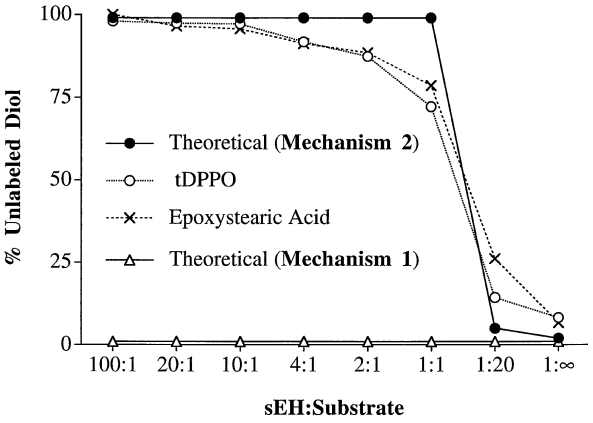

FIG. 4. **The *graph* illustrates single turnover experiments performed with various ratios of sEH:substrate.** The *theoretical line* (●—●) represents the theoretical data based on *mechanism 2*. The data representing single turnover experiments performed with tDPPO (○···○) and *cis*-9,10-epoxystearic acid (×···×) clearly follows the pattern dictated by *mechanism 2*. The *theoretical line* (△—△) represents the theoretical data obtained based on *mechanism 1*.

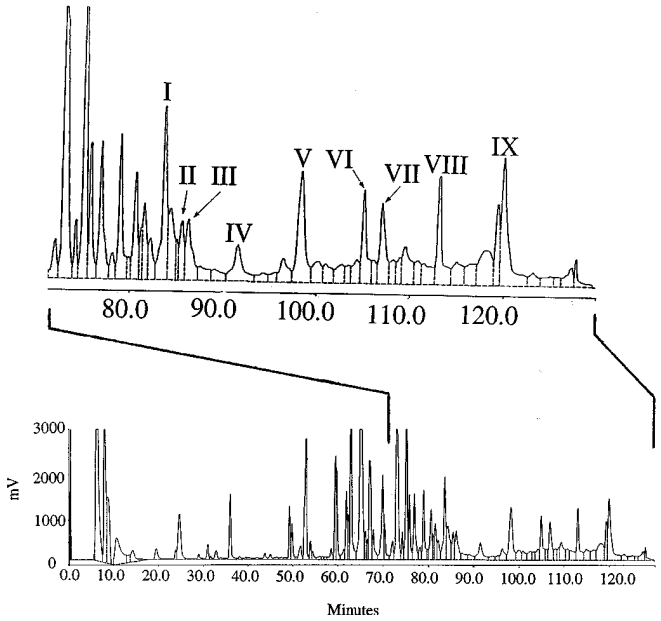

FIG. 5. **Reverse phase HPLC spectra for the tryptic digestion of sEH treated with excess tDPPO in $H_2{}^{18}O$.** *Peaks I–IX* were isolated and concentrated by microbore HPLC (>100 fold) as described under "Materials and Methods."

μM and 26200 nmol/min/mg protein, respectively.[3] Since the oxidized fatty acid turned over more slowly than tDPPO, a smaller fraction of the enzyme population experienced multiple turnover. This hypothesis is supported by the better fit of the *cis*-9,10-epoxystearic acid data to what is expected theoretically as compared with the data obtained for tDPPO (Fig. 4).

From our previous observations the expected site of ^{18}O insertion within tDPPO is at the benzylic carbon. The amount of ^{18}O label incorporated in the product was monitored by GC/MS for each experiment. As can be seen in Fig. 4, equal or greater molar equivalence of sEH (*i.e.* ≤1 theoretical turnover) led to minimal ^{18}O incorporation from the $H_2{}^{18}O$ solvent. The same pattern was observed with *cis*-9,10-epoxystearic acid (Fig. 4). These data suggest ^{16}O incorporation from enzyme onto the epoxide leading to the formation of an acyl-enzyme intermediate.

^{18}O Labeling of Catalytic Amino Acid and Isolation of Labeled Tryptic Fragment—The nucleophilic amino acid responsible for the initial attack of sEH onto epoxides was labeled with ^{16}O and ^{18}O by multiple turnovers of sEH incubated with excess tDPPO in $H_2{}^{16}O$ and $H_2{}^{18}O$. Both enzyme preparations were precipitated, isolated, and digested with trypsin as described under "Materials and Methods." HPLC purification of the tryptic digests (Fig. 5) yielded identical chromatograms for both the $H_2{}^{16}O$ and $H_2{}^{18}O$ incubated sEH with base line-resolved separation of most fragments. Theoretical computer calculation of possible tryptic fragments assigned a relatively high hydrophobic score for the tryptic fragment T26, which contains Asp-333 (T26 refers to the 26th theoretical fragment obtained from the tryptic digestion of sEH by counting from the N terminus). Therefore, the last nine major peaks in the reverse phase separation of the tryptic fragments were collected (Fig. 5). Table I lists the molecular weights obtained by electrospray mass spectrometry for the nine isolated fragments for the $H_2{}^{16}O$ and $H_2{}^{18}O$ incubated enzymes, along with the theoretical masses expected for each fragment. Peak III could not be assigned because it was a combination of many coeluting substances. The mass accuracy for peak I was also poor due to the low signal-to-noise ratio, which impedes very accurate mass assignments. However, the remaining peaks were assigned very easily with a very high degree of confidence due to excellent signal-to-noise ratio. Peaks II, VI, VII, and VIII do not exhibit any increase in mass between sEH turned over in $H_2{}^{16}O$ *versus* sEH turned over in $H_2{}^{18}O$. Conversely, peaks IV, V_a, V_b, IX_a, and IX_b exhibit an average of 3.6 Da increase due to the incorporation of two ^{18}O oxygen atoms for the multiply turned over sEH in $H_2{}^{18}O$. Peak IV was identified as the tryptic fragment T26 and is the common peptide stretch among the peptides in the peaks isolated that exhibit increased mass due to ^{18}O incorporation.

Isolation of ^{18}O-Labeled Asp-333—The labeled and unlabeled tryptic fragments ([$H_2{}^{16}O$]T26 and [$H_2{}^{18}O$]T26) obtained as described above by multiple turnover of sEH with tDPPO in $H_2{}^{16}O$ and $H_2{}^{18}O$ and subsequent HPLC purification after tryptic digestion were hydrolyzed completely with a nonspecific protease (immobilized protease Sg). After removal of the protease and lyophilization, the residue of both samples was derivatized with MTBSTFA and analyzed by GC/MS in the single ion monitoring mode. The fragments monitored were generated by the loss of a *t*-butyl group ($[M - 57]^+ = A^+$). Table II lists the percentages of unlabeled Asp, [$^{18}O_1$]Asp, and [$^{18}O_2$]Asp obtained for Asp-333 from the complete hydrolysis of [$H_2{}^{16}O$]T26 and [$H_2{}^{18}O$]T26, along with free aspartic acid that was derivatized as control. The control Asp exhibits 14.6% $[A + 2]^+$ (equivalent to [$^{18}O_1$]Asp) and 0.5% $[A + 4]^+$ (equivalent to [$^{18}O_2$]Asp). It should be noted that the natural abundance of heavy isotopes of silicon and other elements in each mass fragment contributes to the latter percentages, and therefore, the $[A + 2]^+$ and $[A + 4]^+$ values for control Asp and control Glu can be assumed as normal background (control Asp and Glu do not have any ^{18}O incorporation). As can be seen from Table II, Asp-333 obtained from [$H_2{}^{16}O$]T26 exhibits percentages closely matching the values obtained for control Asp. However, Asp-333 obtained from [$H_2{}^{18}O$]T26 clearly has incorporated ^{18}O as indicated by the large increase in the $[A + 2]^+$ and $[A + 4]^+$ percentages. The ratios of A^+, $[A + 2]^+$, and $[A + 4]^+$ observed for control Glu and Glu-348 obtained from [$H_2{}^{16}O$]T26 and [$H_2{}^{18}O$]T26 are very similar (all background due to isotopic impurity), demonstrating that the mass increase within T26 is solely due to the involvement of Asp-333 in catalytic action, and that Glu-348 does not participate in the catalytic mechanism. The latter result clearly implicates Asp-

TABLE I

Molecular weights of peaks I–IX determined by electrospray mass spectrometry

R. T. refers to retention time of peaks isolated from reverse phase HPLC of sEH tryptic digestion. Subscripts a and b for peaks V and IX refer to two unseparable peptides within each peak that were identified by electrospray mass spectrometry.

Peak	Tryptic fragment[a]	R. T.	Theoretical M_r	$H_2{}^{16}O$ M_r	$H_2{}^{18}O$ M_r	Asp[b]	Glu[b]
		min					
I	(T2)	84	2002.1	2006.0	2003.4	2	0
II	(T21)	85	2992.3	2992.5	2992.2	0	2
III	Not assigned	87		Mixture	Mixture		
IV	(T26)	93	3218.8	3218.3	3221.8	1	1
V_a	(T25-T26)	100	4183.0	4182.5	4185.7	2	2
V_b	(T25-T26-T27)	100	4438.3	4438.2	4441.4	2	2
VI	(T16)	106	4777.6	4775.3	4775.2	3	3
VII	(T11-T12-T13-T14-T15-T16)	108	8579.2	8578.8	8578.2	6	3
VIII	(T16)	113	4777.6	4777.3	4777.3	3	3
IX_a	(T24-T25-T26)	120	6483.6	6483.4	6487.2	3	6
IX_b	(T24-T25-T26-T27)	120	6738.9	6738.4	6742.6	3	6

[a] The notation T followed by a number refers to the tryptic fragment obtained from the theoretical tryptic digestion of sEH by counting from the N terminus. Peaks containing multiple tryptic fragments (as indicated by hyphens) are the result of incomplete tryptic digestion and are covalently bound by peptide bonds. *These are not separate peptides.* The contents of each set of parentheses is one single peptide fragment consisting of one or more theoretical tryptic fragment(s).

[b] Number of Asp and Glu residues in each fragment.

TABLE II

Abundance of A^+, $[A + 2]^+$ and $[A + 4]^+$ masses detected by selected ion monitoring

The percentages refer to integrated peak areas for the diagnostic fragment ($A^+ = [M - 57]^+$, loss of *t*-butyl group from parent isotopically nonenriched molecule) monitored by single ion monitoring.

Amino acid residue[a]	A^+ (unlabeled)	$[A + 2]^+$ ($[^{18}O_1]$)	$[A + 4]^+$ ($[^{18}O_2]$)
Asp-Control	84.8	14.6	0.5
Asp-333-$H_2{}^{16}O$-T26	84.9	14.0	1.1
Asp-333-$H_2{}^{18}O$-T26	40.2	34.8	25.0
Glu-Control	82.3	15.9	1.8
Glu-348-$H_2{}^{16}O$-T26	84.5	14.5	1.0
Glu-348-$H_2{}^{18}O$-T26	81.3	15.9	2.8

[a] Tryptic fragment T26 isolated from digestion of sEH that had been previously subjected to multiple turnovers with tDPPO in $H_2{}^{16}O$ or $H_2{}^{18}O$ was hydrolyzed with a nonspecific protease. The amino acid residues were derivatized with MTBSTFA and analyzed by GC/MS by single ion monitoring. The percentages of $[A + 2]^+$ and $[A + 4]^+$ obtained for Asp-control and Glu-control are due to natural abundance of heavy isotopes of the elements within each mass fragment and can be assumed to be background. The latter results directly implicate Asp-333 as the catalytic amino acid.

333 (the only aspartic acid in T26) as the nucleophilic amino acid responsible for the first step in the hydration of epoxides.

DISCUSSION

In this study we set out to examine the mechanism of epoxide hydration by sEH. Previous publications from this and other laboratories have reported some stereo- and regiochemical controls exerted by sEH. It is generally accepted that sEH opens epoxides by nucleophilic attack (as compared with a general acid catalysis) often at the least hindered carbon in an S_N2 fashion. This is normally accomplished by the back side attack of the epoxide carbon (*anti* opening) (20). Therefore, the initial stereochemistry of the epoxide dictates the stereochemical outcome of the diol product. *trans*-1,3-Diphenylpropene oxide was synthesized as a general substrate for sEH. The asymmetry about the epoxide allows regiochemical studies of nucleophilic attack. Also, the nature of the epoxide carbons vary greatly, one being benzylic, which greatly favors nucleophilic attack, and the other being homobenzylic, which is slightly less hindered. As can be seen from Fig. 1, sEH opens tDPPO via a back side displacement of the epoxide oxygen. This is evident from the exclusive production of *erythro*-diol from the *trans*-epoxide substrate. A completely acid-catalyzed mechanism would probably result in a mixture of *threo*- and *erythro*-diols since the carbocation involved in the mechanism would be greatly stabilized by the phenyl group and would allow water to attack the carbocation from either face due to its trigonal planar geometry. However, the latter data do not preclude the possibility of an acid assisted catalysis occurring in concert with a base catalyzed attack of the epoxide. Transient carbocations can retain their tetrahedral geometry, and therefore, block one face of nucleophilic water attack, which would result in the formation of either *erythro*- or *threo*-diol (depending on the stereochemistry of the parent epoxide) and not both.

Next, the regioselectivity of sEH with tDPPO, *cis*-9,10-epoxystearic acid and methyl *cis*-9,10-epoxystearate was investigated. The [^{18}O]epoxides of the latter substrates were synthesized as described previously. The chemically (base- and acid-catalyzed) and enzymatically generated diols were analyzed by GC/MS to determine label incorporation into each position. In the case of sEH-hydrolyzed tDPPO, 97.1% of the attack occurred at the benzylic position (Fig. 2). The same pattern was observed for the base-hydrolyzed [^{18}O]tDPPO (97.0%). Even though the homobenzylic carbon is slightly less sterically hindered, the nucleophilic attack occurs at the benzylic carbon, which can better stabilize the transition state. As expected, the acid-catalyzed hydrolysis of [^{18}O]tDPPO led to products that exclusively exhibited attack of the benzylic position due to the stability of the benzylic carbocation.

We were also able to show that sEH is able to discriminate two chemically similar epoxide carbons. The enzymatic hydrolysis of [^{18}O]*cis*-9,10-epoxystearic acid led to a 68.5% attack of C-10 in favor of C-9 (Fig. 2). The methyl ester of [^{18}O]*cis*-9,10-epoxystearic acid was also subjected to sEH hydrolysis with identical results, discounting the importance of the carboxylate anion of this fatty acid being anchored by the enzyme. The observed selectivity of sEH between the two nearly identical epoxide carbons can be due to several reasons. The accessibility of the nucleophilic amino acid to C-9 *versus* C-10 can account for the observed data. Another possibility is the different spatial orientations by which the fatty acid can be absorbed onto the catalytic site. This could lead to a different population of C-9 *versus* C-10 positioned closer to the catalytic amino acid for nucleophilic attack (if one spatial orientation is favored over others). Of course, the true explanation might be a combination of the latter two hypotheses. The regioselectivity data were instrumental in the calculations of single turnover data.

After firmly establishing the regioselectivity and mode of attack with both tDPPO and *cis*-9,10-epoxystearic acid, the mechanism of action could be probed. Two scenarios could be envisioned. The first hypothesis, which has been the generally

accepted theory, alleges an activated water (possibly by a histidine) delivery onto the epoxide carbon and subsequent protonation of the generated alkoxide to yield the product (Fig. 3, *mechanism 1*). The second theory supposes that the side chain of a nucleophilic amino acid such as a carboxylate anion within sEH attacks and opens the epoxide. The resultant acyl-enzyme intermediate would then be hydrolyzed by an activated water (again postulated via a histidine) that would generate the native enzyme and release the diol product (Fig. 3, *mechanism 2*). Even though there has not been direct evidence for the second postulated mechanism, recent discoveries from the mechanism of DhlA and single turnover experiments performed with mEH by Lacourciere and Armstrong (13) raised the possibility that sEH could also follow the same mechanistic path (25, 35). X-ray crystallographic study of DhlA with bound substrate has clearly shown the involvement of an aspartic acid as the nucleophile, which in the first step of enzymatic catalysis attacks the substrate leading to the formation of an acyl-enzyme intermediate (α-hydroxyester-enzyme) (36). Also, single turnover experiments with mEH incubated in $H_2{}^{18}O$ have shown that the oxygen introduced within the epoxide is not ^{18}O-labeled during the first turnover. The latter data suggest that mEH also hydrolyzes epoxides via an acyl-enzyme intermediate (13).

Fig. 3 illustrates the difference in products which would be obtained from a single turnover experiment performed in $H_2{}^{18}O$ based on the two postulated mechanisms. Since the first mechanism presupposes a direct attack of an activated water onto the epoxide, it follows that under any conditions of hydrolysis the ^{18}O would be incorporated within the product. Therefore, after a single turnover of sEH with tDPPO the diol product should be labeled with ^{18}O. However, if the hydrolysis follows the second mechanism suggested, then the oxygen incorporated during the first run of the enzyme is supplied by the enzyme. Since the amino acids of sEH contain only ^{16}O oxygens it is clear that the diol product obtained from the first turnover would contain only ^{16}O oxygen. The labeled oxygen from water would hydrolyze the acyl-enzyme intermediate and, therefore, incorporate itself within the enzyme. The enzyme would therefore contain a 1:1 ^{16}O:^{18}O labeled amino acid after one turnover. As multiple turnovers occur, the catalytic amino acid would be completely labeled with ^{18}O and would yield diol products identical to the first suggested mechanism, hence the importance of performing single turnover experiments.

As indicated from the data in Fig. 4, the lack of ^{18}O within the diol product for equimolar or greater sEH:substrate refutes the direct incorporation of ^{18}O from $H_2{}^{18}O$ into the epoxide. Care must be taken in such an experiment to insure each enzyme does not turn over more than one substrate by adding diluted substrate and mixing enzyme and substrate at cold temperatures. This is particularly important for substrates turned over with a high k_{cat} such as tDPPO. Initially our results were inconclusive since we added concentrated substrate to the enzyme buffer solution. Based on our initial results it is safe to assume most of the substrate was consumed as the mixing occurred within pockets of high substrate concentration. This would lead to a population of enzyme experiencing multiple turnovers. Proper mixing resulted in the data in Fig. 4, which are consistent with expected results of the second mechanism involving the intermediary of an acyl-enzyme.

Results of single turnover experiments conclusively showed the involvement of a nucleophilic amino acid within sEH responsible for the di-^{16}O oxygen observed in the product that had been obtained by hydrolysis of substrate in $H_2{}^{18}O$. With the recent investigation into the homology of epoxide hydrolase genetic sequences (23, 24) and their probable evolutionary connection with the α/β hydrolase fold family of enzymes (26), a conserved region containing an aspartic acid at position 333 of sEH has been identified. Consequently, the probable catalytic Asp-333 could be labeled with ^{18}O with excess substrate hydrolyzed by sEH in $H_2{}^{18}O$ (via multiple enzyme turnovers that would incorporate ^{18}O within the catalytic amino acid). Tryptic digestion of sEH labeled with $H_2{}^{16}O$ and $H_2{}^{18}O$ yielded identical HPLC elution profiles, and each peak could be separated for further analysis. Table I lists the molecular weights obtained for reverse phase HPLC-purified, microbore HPLC-concentrated tryptic fragments I-IX by electrospray mass spectrometry for sEH incubated with excess tDPPO in $H_2{}^{16}O$ and $H_2{}^{18}O$ buffer. Peaks I and III did not produce results that were interpretable in light of the protein sequence. Peaks II, VI, VII, and VIII in both the $H_2{}^{16}O$- and $H_2{}^{18}O$-treated groups exhibit molecular weights identical to those predicted theoretically. These observations indicate the absence of any labeled oxygen within those fragments. The difference between fragments VI and VIII is 2 Da, which could be attributed to disulfide bridge formation within T16. These data suggest that in the absence of specific catalysis there is no rapid exchange of water into carboxylic acid functionalities under the conditions used here.

As can be seen from Table I, there is an average increase of 3.6 Da per fragment for peaks IV, V_a, V_b, IX_a, and IX_b for the $H_2{}^{18}O$-treated sEH as compared with the $H_2{}^{16}O$-treated sEH, which corresponds to almost two isotopic oxygens substituted per fragment. The molecular mass of the fragment isolated as peak IV from the $H_2{}^{16}O$-treated group was 3,218.3 Da, which matches the expected molecular mass for the tryptic fragment T26 (3218.8 Da). The T26 fragment isolated from the $H_2{}^{18}O$-treated sEH increased 3.5 Da, therefore implicating T26 as the fragment containing the catalytic amino acid. Peaks IV, V_a, V_b, IX_a, and IX_b, which also contain the labeled oxygen are the result of incomplete tryptic digestion upstream and downstream from T26. The identity of T26 was further confirmed by amino acid sequencing of its N terminus (not shown), which clearly supported our assignment of the fragment. The reason an increase of 3.6 Da is observed as opposed to 4.0 Da (from the incorporation of two ^{18}O atoms) might be attributed to experimental error in molecular mass determination. However, close examination of the MaxEnt-transformed spectra reveals that the peak widths are broader for the ^{18}O-labeled peptides than for the unlabeled peptides. This suggests the presence of a small amount of peptide that contains only a single ^{18}O, and this would account for the molecular weights being slightly less than expected for incorporation of two ^{18}O units. It should be noted that the isotopic purity of the [^{18}O]water used for experiments was 95% at best.

The presence of two ^{18}O units within T26 clearly indicates the involvement of an amino acid in the delivery and opening of tDPPO. The failure to see any increase in mass in peaks II, VI, VII, and VIII and the increase in 4 Da rather that 8 Da or more in peaks IV, V_a, V_b, IX_a, and IX_b support the hypothesis that there is minimal exchange of $H_2{}^{18}O$ with the carboxylic acids of aspartic and glutamic acids unless there is a catalytic involvement. Single turnover experiments with sEH preincubated at 37 °C in $H_2{}^{18}O$ for 1 h yielded the exact same results as the normal single turnover experiment, discounting any catalytic exchange of ^{18}O into the catalytic amino acid without the presence of substrate. The cDNAs and predicted peptide sequence of T26 contains Asp-333 as the only aspartic acid within the fragment. As discussed previously, Asp-333 has been implicated as the catalytic residue by sequence homology to other EHs, and the α/β fold hydrolase family of enzymes. Based on

the latter data, it seems very plausible that Asp-333 is the catalytic amino acid responsible for the initial step of hydrolysis. However, T26 contains a glutamic acid (Glu-348), which is not conserved but could conceivably be responsible for catalytic activity.

The complete digestion of T26 (from both $H_2{}^{16}O$ and $H_2{}^{18}O$ treated sEH) was achieved with a nonspecific protease (immobilized protease Sg). After derivatization of the hydrolyzed amino acid residues with MTBSTFA the samples were analyzed by GC/MS. Since there is only one aspartic acid in T26, and since the aspartic acid derivative obtained from the hydrolysis of the [$H_2{}^{18}O$]T26 exhibited an increased mass (Table II), it seems unequivocal that the aspartic acid in T26 is responsible for the nucleophilic attack onto tDPPO. Furthermore, it was shown that Glu-348 (the only other carboxylate containing amino acid in T26) did not incorporate any ^{18}O oxygen. The attack of Asp-333 initiates enzymatic activity, leading to the formation of an α-hydroxyester-enzyme intermediate. Hydrolysis of this acyl-enzyme is accomplished by the addition of an activated water to the carbonyl carbon of the ester bond, after which the resultant tetrahedral intermediate collapses, yielding the active enzyme and the diol product. The second step of the latter mechanism resembles the mechanism of most serine esterases and proteases.

A push-pull mechanism, where the epoxide oxygen is activated by protonation or hydrogen bonding that would weaken the C–O epoxide bond seems likely since the carboxylate anion (Asp-333) is the attacking nucleophile. The latter hypothesis fits well with our knowledge of carboxylate chemistry. Chemically, carboxylates are not considered strong nucleophiles that could easily open epoxide rings. However, the epoxide carbon can be chemically activated toward nucleophilic attack by weak nucleophiles such as carboxylate anions through the coordination of the epoxide oxygen with a lewis acid (37). Within the catalytic cavity of sEH, the epoxide oxygen can be activated by various amino acids through either proton donation or hydrogen bonding. The x-ray crystallographic solution of DhlA cocrystallized with substrate clearly demonstrated that two tryptophan residues are responsible for polarization of the halide-leaving group (36). Genetic sequence homology between DhlA and sEH investigated by Arand *et al.* (23) has shown that one of the two tryptophans in DhlA is conserved in sEH. This latter postulated tryptophan (Trp-334) could activate the epoxide by hydrogen bonding with the oxirane oxygen, but it is not necessarily the only mechanism for epoxide activation by sEH. A push-pull mechanism has also been suggested previously based on the general structure of sEH inhibitors (34, 38, 39). The general structure of sEH inhibitors contains a carbonyl or hydroxy α to the epoxide. It has been suggested that the presence of the α-hydroxy/carbonyl functionality interferes with the acidic activation of the epoxide by hydrogen bonding with the proton donor (17). However, in light of a two-step mechanism of action a detailed study is necessary to elucidate the inhibitory mechanism.

In conclusion, we report that we have been able to conclusively show that sEH effects hydrolysis of epoxides via an A2 type, back side nucleophilic attack of Asp-333. An isolatable acyl-enzyme intermediate is formed, which is hydrolyzed by an activated water, resulting in the regeneration of the active enzyme and the release of the diol product.

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