Mechanism of Soluble Epoxide Hydrolase

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

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†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.

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18O-Labeled epoxides of trans-1,3-diphenylpropene oxide (tDPPO) and cis-9,10-oxypolyunsaturated fatty acids 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 H218O resulted in the isolation and purification of a tryptic fragment containing Asp-333. Electrospray mass spectrometry of this fragment conclusively illustrated the incorporation of 18O. 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-hydroxy-ester-enzyme intermediate. Hydrolysis of the acyl-enzyme occurs by the addition of an activated water molecule 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) 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). The postulated acylated amino acid in mEH occurs by the 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 H218O (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), 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 H218O 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 (DHIA, from the bacterium Xanthobacter autotrophicus), which, among other regions of homology, possesses a conserved histidine and aspartic acid tandem (23–25). DHIA 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 glycols are discussed.

MATERIALS AND METHODS

General Procedures—1H-NMR spectra were obtained on a QE-300 MHz spectrometer (General Electric Co.) with deuteriochloroform. 2K
(CDCl₃) 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 (5 μm), a 3.9 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—16O and 18O cis-9,10-epoxystearic acid and trans-1,3-diphenylpropene oxide (tDPPO) were synthesized as described (27). The 16O/18O cis-9,10-epoxystearic acid were prepared by fractional distillation of the 16O and 18O cis-9,10-epoxystearic acid with diazomethane (28). Bis(trimethylsilyl) trifluoroacetamide (BSTFA), N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), immobilized protease Sg, and BCA reagent for protein concentration determination were obtained from Pierce. H₂¹⁶O (95% 16O) and all other chemicals were purchased from Aldrich. Solvents used were reagent grade and were freshly distilled before use. Synthesis of trans-1,3-diphenyl-1,2-propanediol was achieved by cis-hydroxylation of trans-1,3-diphenylpropene with catalytic asymmetric tetroxide and N-methylmorpholine oxide (29).

Enzyme Preparation and Specific Activity Measurement—Recombinant human 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 [H³H]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 μm 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 μM sEH or 100 μl sodium phosphate buffer alone. Synthesis of cis-9,10-epoxystearic acid and tDPPO was prepared by methyl- and dono-trolic acid (100 mM, pH 7.4) containing 100 μM sEH. Con-}

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 solution was added to neutralize the reaction mixture. Basic hydrolysis of 16O/18O-labeled tDPPO was effected by the addition of 2 μl of each 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.

GC/MS Analysis of Hydrolysis Products of tDPPO and cis-9,10-Epoxystearic Acid—GC/MS analysis 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 (5 μm), a 3.9 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.

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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 μl 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). The flow rate was set at 100 μl/min, 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 H2O and incubated with excess tDPPO to achieve multiple turnovers.

Purification and Concentration of Tryptic Fragments Obtained from H2O- and H218O-treated sEH—The tryptic digests from the H2O- and H218O-treated sEH were subjected to reverse phase HPLC analysis. The tryptic digests were eluted with the following acetonitrile/H2O gradient: 0–2 min at 100% A, changed to 75% B from 2 to 112 min, up to 100% B (A and B were 0.06% trifluoroacetic acid, H2O and 0.052% trifluoroacetic acid, 80% acetonitrile, H2O, 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 H2O- and H218O-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 the hydrolyzed amino acids was removed from the pellet, and the solution was frozen and lyophilized. The residue was derivatized as described previously (27). [18O]Methyl ester of eluted sample was analyzed by GC/MS. The flow rate was set at 100 μl/min, 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 detector temperature was programmed from 150°C (1-min hold) to 275°C at 10°C/min.

This chromatographic conditions have been found to give complete separation of tert-butylidemethylsilyl (TBDMS) derivatives of all 20 amino acids. Single ion monitoring of each GC/MS run was performed to detect the abundant [M – C5H11] (m/z = 57)1 fragments characteristic of the TBDMS derivatives for unlabeled Asp (m/z 418), [18O]Asp (m/z 420), [18O]Glut (m/z 422), unlabeled Glu (m/z 432), [18O]Glu (m/z 434), and [18O]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 (S2 1 type) versus A2 (S2n2 type) (32). As can be seen in Fig. 1a, acid hydrolysis of tDPPO led to the expected equal amounts of erythro- and threo-diols (tR = 6.92 and tR = 8.08 min) due to the trigonal planar geometry of the resultant benzyl carbonation transition state, which allows attack of water to occur from either face of the carbonation. Osmium tetroxide cis-dihydroxylation of trans-1,3-diphenylpropene results in formation of the three-diol, exclusively (Fig. 1b, tR = 8.15 min). Therefore, the erythro-diol was identified as the peak at 6.92 min. Fig. 1c illustrates the incubation of sEH with tDPPO after 5 min. As the epoxide (tR = 18.47 min) is turned over by the enzyme only the erythro-peak (tR = 6.57 min) is formed, and after 40 min (Fig. 1d) all the epoxide has been converted to 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 three-9,10-dihydroxystearic acid was the sole enzymatic product.

Regioselectivity of sEH with tDPPO and cis-9,10-Epoxy

Stearic Acid—Next, we turned our attention to the regioselectivity of epoxide opening with sEH. The oxygen-labeled [18O]tDPPO and [18O]cis-9,10-epoxystearic acid were synthesized as described previously (27). cis-9,10-Epoxypropeptide was synthesized from the reaction of excess di-amino acid and [18O]cis-9,10-epoxystearic acid. Each 18O-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 [18O]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 [18O]tDPPO was practically identical to the sEH opening of [18O]tDPPO with regard to the position of the 18O oxygen. The attack of hydroxyl anion occurred at the benzylic position (97.0% ± 1.0%). Acidic hydrolysis of [18O]tDPPO was even more selective, exhibiting 100% attack of the benzylic position. This result is not surprising due to the stability of the benzylic carbonation.

[18O]Epoxypropeptide acid also shows some regioselectivity of epoxide opening with sEH. Acidic hydrolysis of [18O]cis-9,10-

epoxypropeptide acid results in a 1:1 distribution of carbon 9- and carbon-10-labeled diol, a testament to the chemical equivalence.
of the two epoxide carbons. However, as depicted in Fig. 2, 68.5% of the enzymatic attack occurs at carbon 10. The 18O label distribution following enzymatic hydrolysis of [18O]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-epoxystearate as substrates. The sEH was lyophilized just prior to analysis, and it was rehydrated in H218O. 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 H218O) at 0 °C with hydrated enzyme (in H218O) and subsequent incubation at 37 °C, the results obtained conclusively supported a nucleophilic attack of the enzyme upon tDPPO, which was followed by the hydrolysis of the resultant acyl-enzyme intermediate. Acidic activation of the epoxide oxygen, which could enhance catalytic activity, is consistent with both mechanisms.
μM and 26200 nmdl/min/mg protein, respectively. 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-epoxyeic 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 18O insertion within tDPPO is at the benzyl carbon. The amount of 18O label incorporated in the product was monitored by GC/MS for each experiment. As can be seen in Fig. 4, equal or greater molar equivalent of sEH (i.e. 1 theoretical turnover) led to minimal 18O incorporation from the H218O solvent. The same pattern was observed with cis-9,10-epoxyeic acid (Fig. 4). These data suggest 18O incorporation from enzyme onto the epoxide leading to the formation of an acyl-enzyme intermediate.

18O Labeling of Catalytic Amino Acid and Isolation of Labeled Tryptic Fragment—The cephalic amino acid responsible for the initial attack of sEH onto epoxides was labeled with 16O and 18O by multiple turnovers of sEH incubated with excess tDPPO in H216O and H218O. 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 H216O and H218O 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 of sEH). 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 H216O and H218O incubated enzymes, along with the theoretical masses expected for each fragment. Peak I 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 donot exhibit any increase in mass between sEH turned over in H216O versus sEH turned over in H218O. Conversely, peaks IV, V, VI, IXa, and IXb exhibit an average of 3.6 Da increase due to the incorporation of two 18O oxygen atoms for the multiply turned over sEH in H218O. Peak IVA is 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 18O incorporation.

Isolation of 18O-Labeled Asp-333—The labeled and unlabeled tryptic fragments ([H216O]T26 and [H218O]T26) obtained as described above by multiple turnover of sEH with tDPPO in H216O and H218O 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-buty group ([M – 57] = A+). Table II lists the percentages of unlabeled Asp, [18O]Asp, and [18O2]Asp obtained for Asp-333 from the complete hydrolysis of [H216O]T26 and [H218O]T26, along with free aspartic acid that was derivatized as control. The control Asp exhibits 14.6% [A + 2]1 (equivalent to [18O2]Asp) and 0.5% [A + 4]1 (equivalent to [18O2]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]1 and [A + 4]1 values for control Asp and control Glu can be assumed as normal background (control Asp and Glu do not have any 18O incorporation). As can be seen from Table II, Asp-333 obtained from [H216O]T26 exhibits percentages closely matching the values obtained for control Asp. However, Asp-333 obtained from [H218O]T26 clearly has incorporated 18O as indicated by the large increase in the [A + 2]1 and [A + 4]1 percentages. The ratios of A+, [A + 2]1, and [A + 4]1 observed for control Glu and Glu-348 obtained from [H216O]T26 and [H218O]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-
mechanism of Soluble Epoxide Hydrolase Catalysis

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 | R. T. | Theoretical M | H$_2^{18}$O M | H$_2^{16}$O M | Asp$^a$ | Glu$^b$ |
|------|-----------------|-------|---------------|----------------|---------------|---------|---------|
| 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        | Mixture       |         |         |
| IV   | (T26)           | 93    | 3218.8        | 3218.3         | 3221.8        | 1       | 1       |
| Va   | (T25-T26)       | 100   | 4183.0        | 4182.5         | 4185.7        | 2       | 2       |
| Vb   | (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       |
| IXa  | (T24-T25-T26)   | 120   | 6843.6        | 6848.3         | 6847.2        | 3       | 6       |
| IXb  | (T24-T25-T26-T27) | 120 | 6738.9        | 6738.4         | 6742.6        | 3       | 6       |

$^a$ Tryptic fragment T26 isolated from digestion of sEH that had been previously subjected to multiple turnovers with tDPPO in H$_2^{18}$O or H$_2^{16}$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 nucelophilic 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 SN2 reaction. It is generally accepted that sEH catalyzes the 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-diols from the trans-epoxide substrate. A completely acid-catalyzed mechanism would probably result in a mixture of three- 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-diols (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 cis-[18O] epoxides of the latter substrates were synthesized as described previously. The chemical (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 [18O]tDPPO (97%). 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 [18O]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 between two chemically similar epoxide carbons. The enzymatic hydrolysis of cis-[18O]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 cis-[18O]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 nucelophilic 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 (į-hydroxyster-enzyme) (36). Also, single turnover experiments with mEH incubated in H2 18O have shown that the oxygen introduced within the epoxide is not 18O-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 H2 16O 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 16O would be incorporated within the product. Therefore, after a single turnover of sEH with tDPPO the diol product should be labeled with 13O. 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 16O oxygens it is clear that the diol product obtained by the first turnover would contain only 16O 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 16O:18O labeled amino acid after one turnover. As multiple turnovers occur, the catalytic amino acid would be completely labeled with 18O 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 18O within the diol product for equimolar or greater sEH:substrate refutes the direct incorporation of 18O from H2 18O 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 kcat such as tDPPO. Initially our results were inconclusive since we added concentrated substrate to the enzyme buffer solution. Based on our initial results it was 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-16O oxygen observed in the product that had been obtained by hydrolysis of substrate in H2 18O. With the recent investigation into the homology of epoxide hydrolyase 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 18O with excess substrate hydrolyzed by sEH in H2 16O (via multiple enzyme turnovers that would incorporate 116O within the catalytic amino acid). Tryptic digestion of sEH labeled with H2 16O and H2 18O 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 H2 16O and H2 18O 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 H2 16O- and H2 18O-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, Vα, Vβ, IXα, and IXβ for the H2 16O-treated sEH as compared with the H2 18O-treated sEH, which corresponds to almost two isotopic oxygens substituted per fragment. The molecular mass of the fragment isolated as peak IV from the H2 16O-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 H2 18O-treated sEH increased 3.5 Da, therefore implicating T26 as the fragment containing the catalytic amino acid. Peaks IV, Vα, Vβ, IXα, and IXβ, 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 18O 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 18O-labeled peptides than for the unlabeled peptides. This suggests the presence of a small amount of peptide that contains only a single 18O, and this would account for the molecular weights being slightly less than expected for incorporation of two 18O units. It should be noted that the isotopic purity of the [18O]water used for experiments was 95% at best.

The presence of two 18O 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 than 8 Da or more in peaks IV, Vα, Vβ, IXα, and IXβ support the hypothesis that there is minimal exchange of H2 18O 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 H2 18O for 1 h yielded the exact same results as the normal single turnover experiment, discounting any catalytic exchange of 18O 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 nonselective 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 I), it seems unequivocal that the aspartic acid in T26 is responsible for the nucleophilic attack onto the dPPO. 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 $\alpha$-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 DHA crystallized with substrate clearly demonstrated that two tryptophan residues are responsible for polarization of the halide-leaving group (36). Genetic sequence homology between DHA and sEH investigated by Arand et al. (23) has shown that one of the two tryptophans in DHA 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 $\alpha$ to the epoxide. It has been suggested that the presence of the $\alpha$-hydroxycarbonyl 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.