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Molecular and Biochemical Evidence for the Involvement of the Asp-333–His-523 Pair in the Catalytic Mechanism of Soluble Epoxide Hydrolase*

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In order to investigate the involvement of amino acids in the catalytic mechanism of the soluble epoxide hydrolase, different mutants of the murine enzyme were produced using the baculovirus expression system. Our results are consistent with the involvement of Asp-333 and His-523 in a catalytic mechanism similar to that of other α/β hydrolase fold enzymes. Mutation of His-263 to asparagine led to the loss of approximately half the specific activity compared to wild-type enzyme. When His-332 was replaced by asparagine, 96.7% of the specific activity was lost and mutation of the conserved His-523 to glutamine led to a more dramatic loss of 99.9% of the specific activity. No activity was detectable after the replacement of Asp-333 by serine. However, more than 20% of the wild-type activity was retained in an Asp-333 → Asn mutant produced in *Spodoptera frugiperda* cells. We purified, by affinity chromatography, the wild-type and the Asp-333 → Asn mutant enzymes produced in *Trichoplusia ni* cells. We labeled these enzymes by incubating them with the epoxide containing radiolabeled substrate juvenile hormone III (JH III). The purified Asp-333 → Asn mutant bound 6% of the substrate compared to the wild-type soluble epoxide hydrolase. The mutant also showed 8% of the specific activity of the wild-type. Preincubation of the purified Asp-333 → Asn mutant at 37 °C (pH 8), however, led to a complete recovery of activity and to a change of isoelectric point (pI), both of which are consistent with hydrolysis of Asn-333 to aspartic acid. This intramolecular hydrolysis of asparagine to aspartic acid may explain the activity observed in this mutant. Wild-type enzyme that had been radiolabeled with the substrate was digested with trypsin. Using reverse phase-high pressure liquid chromatography, we isolated four radiolabeled peptides of similar polarity. These peptides were not radiolabeled if the enzyme was preincubated with a selective competitive inhibitor of soluble epoxide hydrolase 4-fluorochalcone oxide. This strongly suggested that these peptides contained a catalytic amino acid. Each peptide was characterized with N-terminal amino acid sequencing and electrospray mass spectrometry. All four radiolabeled peptides contained overlapping sequences. The only aspartic acid present in all four peptides and conserved in all epoxide hydrolases was Asp-333. These peptides resulted from cleavage at different trypsin sites and the mass of each was consistent with the covalent linkage of Asp-333 to the substrate.

Epoxide hydrolases (EC 3.3.2.3) are enzymes that convert epoxides to diols via addition of water. They have been found in all mammalian species tested and found in most organs. Based on subcellular localization and substrate specificity, five distinct enzyme groups have been defined: 1) microsomal epoxide hydrolase, 2) soluble epoxide hydrolase (sEH),[1] 3) cholesterol epoxide hydrolase, 4) leukotriene A_4 epoxide hydrolase, and 5) hepoxilin epoxide hydrolase. In mammals the majority of research has focused on microsomal epoxide hydrolase and sEH. Induction of hepatic microsomal epoxide hydrolase in rats and mice by a variety of foreign compounds suggests an involvement in xenobiotic metabolism (1–7). The broad spectrum of substrates utilized by sEH also suggests a detoxifying function (8). sEH might also participate in the metabolism of endogenous substrates since sEH hydrolyzes epoxides of fatty acids more rapidly than many other substrates (9–11).

Microsomal epoxide hydrolase has been cloned from rat, human, and rabbit (12–14). More recently sEH has been cloned from rat, mouse, and human (15–17), and also from plants: potato (*Solanum tuberosum*) (18) and mouse-eared cress (*Arabidobsis thaliana*) (19). Knowledge of these sequences enabled authors to perform sequence homology analyses (20–22). They noted that certain regions of the epoxide hydrolases showed significant sequence similarity to a bacterial haloalkane dehalogenase HLD1 (23). HLD1 and by inference epoxide hydrolases belong to a group of enzymes known as the α/β hydrolase fold family (24). α/β hydrolase fold enzymes are hydrolytic enzymes; all have a catalytic triad consisting of a nucleophile, a histidine, and an acid. Until recently, the most accepted hypothesis concerning the mechanism of action of EH was a general base catalysis involving a histidine that was thought to form a hydroxyl anion by attracting a proton from a molecule of water. The anion would then attack the oxirane. Recently, catalytic amino acids of HLD1 have been identified by x-ray crystallography (25). Catalysis by HLD1 proceeds by a two-step mechanism, where the substrate is covalently bound to the enzyme via an ester bond with Asp-124. The ester bond is then hydrolyzed by a molecule of water activated by the His-289–

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[1] The abbreviations used are: sEH, soluble epoxide hydrolase; 4-FCO, 4-fluorochalcone oxide; RP-HPLC, reverse phase-high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; JH III, juvenile hormone III; BSA, bovine serum albumin; IEF, isoelectric focusing.

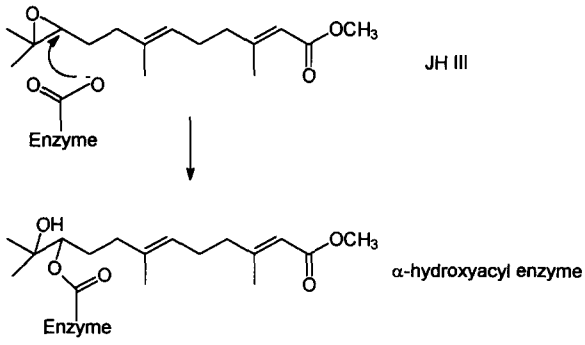

FIG. 1. **Formation of an α-hydroxyacyl intermediate between JH III and sEH.**

Asp-260 pair in the active site. It is noteworthy that the amino acids which form the catalytic triad are conserved in all sEH enzymes so far cloned (22). Therefore the hypothesis for a similar mechanism involving an ester intermediate in the mechanism of action of EH seems more likely than the general base catalysis hypothesis. This new hypothesis received additional support recently. Low turnover experiments with $H_2{}^{18}O$ provide evidence for this hypothesis with the microsomal epoxide hydrolase of rat liver (20). Although *trans*-stilbene oxide is the most commonly used substrate with sEH, we recently chose the epoxide containing substrate juvenile hormone (JH III) in order to test the hypothesis of a two step mechanism involving an ester intermediate in the catalysis by sEH (26). Its characteristics increased our chance to demonstrate the presence of an ester intermediate during catalysis. This substrate has a high affinity for sEH (27); moreover, like other trisubstituted epoxides, it is turned over more slowly than di- and monosubstituted epoxides (27). After a brief incubation of radiolabeled JH III with murine sEH, we isolated a covalently bound substrate enzyme intermediate. Chemical analysis of this complex revealed that it was an α-hydroxyacyl enzyme (Fig. 1). We also showed that the binding of the substrate to the enzyme was inhibited by a selective competitive inhibitor of sEH, 4-fluorochalcone oxide.

The goal of the work described in this paper was to study the mechanism of action of sEH. With knowledge of the amino acids involved in the catalytic mechanism of HLD1, we used site-directed mutagenesis to evaluate the catalytic importance of different amino acids of recombinant murine sEH. Wild-type sEH was radiolabeled with a substrate (JH III) and digested with trypsin, allowing us to isolate radiolabeled peptides from this protein. These peptides are thought to correspond to the hydroxyacyl intermediate. Determination of their partial sequence and molecular mass allowed us to position these peptides within the protein sequence and thus identify a putative catalytic nucleophile in the protein.

MATERIALS AND METHODS

Compounds—Juvenile hormone III (JH III) (481 GBq/mmol) was purchased from DuPont. Non radiolabeled juvenile hormone III was purchased from Sigma. The selective competitive sEH inhibitor 4-fluorochalcone oxide (4-FCO) was prepared as described previously (28).

Enzyme Preparation and Activity Measurement—An expression system composed of baculoviruses and cells from *Trichoplusia ni* (Tn5B1-4) or *Spodoptera frugiperda* (SF21) previously described for the production of human sEH (17), was used in order to produce the murine enzyme (16). The expressed murine sEH was purified from cell lysate by affinity chromatography (29).

Construction of the Mutants—Sequence numbers are based on the sEH sequence (GenBank®/EMBL Data Bank accession number L05781 (1994)). Mutants of murine sEH were prepared using sEH in the transfer plasmid pAcUW21 according to the method of Kunkel (30). The following mutants and oligonucleotides were used: (His-237 → Asn)-5′-ATGACGTCAGCAATGGATATGTGAC-3′; (His-263 → Asn)-5′-CTATGCCTTTGCAATGGGTTTCCTG-3′; (His-332 → Asn)-5′-GTGTTCATTGGCAATGACTGGGCTG-3′; (Asp-333 → Asn)-5′CAGTGTTCATTGGTCATAACTGGGCTGGTG-3′; (Asp-333 → Ser)-5′CAGTGTTCATTGGTCATAGCTGGGCTGGTGTG-3′; (His-523 → Gln)-5′-CATTGAAGACTGCGGCCAGTGGACACAGAT-3′.

Underlined residues have been modified to generate the corresponding mutations and to add or delete restriction sites in order to select for mutants by restriction digest. Restriction site changes were designed to give the same amino acid in the mutant protein as is found in the wild-type protein. All mutants were confirmed by restriction digest and dideoxy sequencing across the mutation site.

Measurement of Specific Activity—Specific activity of the purified enzymes was measured using ^{3}H-labeled *trans*-stilbene oxide as described previously (8). The same assay protocol was followed using baculovirus-infected whole SF21 cells in order to compare the specific activity of the unpurified wild-type and mutant enzymes. The assays were generally run for 10 min but were extended to 1 h for the Asp-333 → Ser and the His-523 → Gln mutants. Total protein concentrations for all assays were made constant by the addition of baculovirus-infected cells expressing a control (LacZ) protein. Protein concentrations were determined with BCA reagent from Pierce using bovine serum albumin as a standard.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (31) using 4% stacking and 10% resolving gels. Immunoblotting was performed according to Burnette (32) using 0.2-μm pore size nitrocellulose in a Bio-Rad Trans-Blot cell. After overnight transfer (35 V, room temperature), blots were blocked by shaking for 15 min in 1% BSA, then twice for 15 min each in blotting buffer (10 mM KH_2PO_4, 14 mM NaCl, 0.02% NaN_3, 0.25% Tween 20, pH 7.4). Primary antiserum to sEH diluted 5000-fold was incubated with the blot for 2 h with shaking at room temperature. Bound IgG was detected as described by Blake *et al.* (33), using alkaline phosphatase-labeled goat anti-rabbit IgG (1:2000 in blotting buffer, Fisher).

Isoelectric focusing (IEF) was performed in an LKB Multiphor electrophoresis apparatus with precast polyacrylamide gels (Ampholine PAGplate, pH 4.0-6.5; Pharmacia Biotech Inc.) using the conditions recommended by the manufacturer. Gels were cooled to 3 °C and focused for 1 h prior to loading. Aliquots of 15 μg of purified protein were loaded in each lane. Focusing was carried out at pH 4.0-6.5 at 2000 V, 25 watts, 25 mA for 2.5 h. Gels were stained with Coomassie Blue, and pH gradients were calibrated using IEF marker samples (Pharmacia). In some cases the gel lanes were sliced into 1-mm sections and incubated overnight in 200 μl of sodium phosphate buffer (100 mM, pH 7.4), and the supernatant assayed for epoxide hydrolase activity.

Labeling of the Enzyme—A 10^{-2} M ethanolic solution of the substrate juvenile hormone III (C10 ^{3}H JH III) with a specific radioactivity of 33 × 10^{-2} Bq/pmol was prepared by diluting labeled with unlabeled juvenile hormone III. One μl of this solution was added with a Hamilton repeating dispenser to 100 μl of sodium phosphate buffer (100 mM, pH 7.4) containing either 10 μg of sEH (160 pmol) or bovine serum albumin in a polypropylene microcentrifuge tube on ice. The incubation solution was mixed using a Vortex mixer for 3 s at room temperature and the reaction was stopped by precipitating the protein with addition of 11 μl of 100% trichloroacetic acid solution. The precipitated protein was then pelleted by centrifuging the microcentrifuge tube (4 min at 16,000 × *g*) at 4 °C. The pellet was washed twice with 100 μl of cold acetone, resuspended in 100 μl of double distilled water containing 1% acetic acid, and then reprecipitated and washed as described above. After the final wash the pellet was dissolved in 5% (w/v) SDS (50 μl), and 1.4 ml of scintillation liquid was added to measure the radioactivity incorporated into the protein, all counts were corrected for differential quenching with a Wallac 1409 Liquid Scintillation Counter. To measure the effect of the selective competitive inhibitor 4-FCO on the labeling by juvenile hormone III, the same procedure was performed after preincubating the enzyme with 100 μM 4-FCO on ice for 5 min.

Tryptic Digest—The enzyme (100 μg) was labeled and precipitated as described above. Then, 25 μl of 8 M urea, 500 mM sodium phosphate buffer (pH 7.4) was added to the dry protein in the polypropylene microcentrifuge tube which remained at room temperature for 10 min. Following addition of 45 mM dithiothreitol (5 μl) and vortexing, the mixture was incubated at 50 °C for 15 min. After a 15-min incubation at room temperature, 60 μl of distilled water and 5 μg of trypsin (in 5 μl of water) were added. After vortexing, digestion was allowed to proceed for 2 h at 37 °C and was stopped by freezing or by injecting directly onto a RP-HPLC column. Increasing the time of digestion or the amount of trypsin did not increase the yield of the digest or change the size or relative patterns of the HPLC profile.

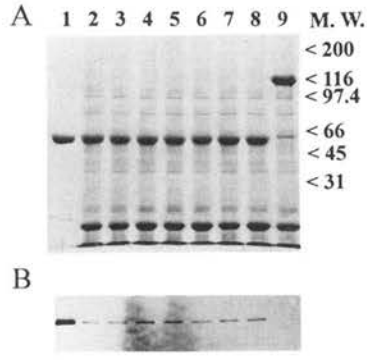

FIG. 2. **Expression of wild-type and mutant sEH in baculovirus-infected SF21 cells.** For SDS-PAGE (*A*), all lanes contain 20 μg of cellular protein, and for Western blot (*B*), all lanes contain 50 ng of protein. sEH is affinity-purified murine sEH (*lane 1*). Cellular proteins are from SF21 cells expressing the: His-237 → Asn mutant (*lane 2*), His-263 → Asn mutant (*lane 3*), His-332 → Asn mutant (*lane 4*), Asp-333 → Asn mutant (*lane 5*), Asp-333 → Ser mutant (*lane 6*), His-523 → Gln mutant (*lane 7*), wild-type (*lane 8*), and LacZ (*lane 9*).

Purification of the Radiolabeled Peptides—A tryptic digest of the radiolabeled enzyme was subjected to RP-HPLC analysis. A Perkin-Elmer Diode-Array system was used with a Vydac C4 (4.6×250 mm, 5 μm) column. Fragments were eluted with a gradient as follows: 100% A from 0 to 2 min, increased to 75% B in 110 min, then to 100% B in 3 min, followed by 15 min at 100% B (A and B were H_2O/trifluoroacetic acid (99.94:0.06, v/v) and H_2O/acetonitrile/trifluoroacetic acid (19.95:80:0.05; v/v/v), respectively). The flow rate was 0.5 ml/min. Peptides were monitored by following the UV absorbance at 215 nm and 280 nm. A Gilson model 203 fraction collector was used to collect 500-μl fractions, 150 μl of each fraction was transferred to a scintillation vial, and all counts were corrected for differential quenching with a Wallac model 1409 liquid scintillation counter.

Radiolabeled peptides were collected and pooled from eight tryptic digests (800 μg total) in order to determine their amino acid sequence and molecular mass. The pooled fractions containing each peptide were concentrated with a Savant Speed-Vac concentrator prior to analysis. Automated Edman degradation with precolumn PTH derivatization was used for peptide sequencing. An Applied Biosystems gas-phase sequenator (Applied Biosystems, Foster City, CA) coupled to an Applied Biosystems model 120A PTH analyzer and M900 data system was employed. Prior to each separate sequence analysis, the chromatographic system was calibrated with PTH-derivative standards.

Mass Spectrometry—Electrospray mass spectra of peptides were generated using a VG/Fisons Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altricham, United Kingdom) using acetonitrile/H_2O (50:50, v/v) as the mobile phase. An Isco 500-μl 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 200-2000 Da/e at a rate of 20 s/scan from 20 scans were combined using the VG MGA acquisition mode. Molecular mass were determined by using the maximum entropy deconvolution algorithm (MaxEnt™) to transform the range of 500-1500 Da/e to give a true mass scale spectrum. Mixed polyethylene glycols were used for mass calibration.

RESULTS

Expression and Activity of the Different Mutants—In order to test the hypothesis of a sEH catalysis similar to the HLD1 catalysis where a His-Asp pair has been shown to be involved, we made different mutants of the enzyme. Mutants were prepared by site-directed mutagenesis, and proteins were produced using baculovirus and SF21 cells as an expression system. SDS-PAGE and Western blotting of wild-type and mutant sEHs demonstrated equivalent expression of all enzymes and retention of immunoreactivity despite the mutations (Fig. 2). We measured sEH activity of the mutants and compared these activities to that of the wild-type enzyme. To minimize any effects which the mutations may have had on protein stability during cell lysis and purification, enzyme activity was measured in whole cells 4 days after infection. This time of harvest yielded the maximum expression of sEH. The same trends of catalytic activity were observed with lysed cells. As shown in Table I, each mutation had different effects. While mutation of the His-237 to an asparagine did not alter the activity of the enzyme, the same mutation at His-263 led to a loss of approximately half the activity compared to wild-type. When His-332 was replaced by an asparagine, 3.3% of the activity was retained, and after conversion of the conserved His-523 to glutamine only 0.1% of the activity remained. No activity was detectable after the replacement of Asp-333 with a serine. However, if this amino acid was changed to an asparagine more than 20% of the activity was retained.

TABLE I
Comparison of wild-type and mutant sEH activities

All assays were performed on baculovirus-infected, whole SF-21 cells. Total protein concentration for all assays was made constant by addition of baculovirus-infected cells expressing the LacZ protein. Assays were generally run for 10 min but were extended to 1 h for the Asp-333 → Ser and His-523 → Gln mutants. Only activity values showing a linear dependence on cellular protein concentration were used. All values are from at least two independent experiments, each performed in triplicate.

Enzyme	Specific activity	Activity of wild-type
	nmol/min/mg	%
Wild-type	138.3 ± 7.3	100
His-237 → Asn	162.1 ± 5.2	117.2
His-263 → Asn	72.3 ± 5.4	52.3
His-332 → Asn	4.6 ± 0.9	3.3
Asp-333 → Asn	29.7 ± 2.1	21.5
Asp-333 → Ser	Nondetectable	
His-523 → Gln	0.1 ± 0.01	0.1

Labeling and Specific Activity of the Asp-333 Mutant and the Wild-type sEH—Asp-124 from HLD1, which corresponds to Asp-333 in sEH, is implicated in the formation of ester bond in the enzyme-substrate intermediate (25). Furthermore, our previous results showed that JH III binds covalently to the wild-type enzyme (26). We investigated the possible role of Asp-333 in the formation of such an intermediate. The Asp-333 → Asn and the wild-type sEHs were purified to apparent homogeneity by affinity chromatography from our expression system using Tn5B1–4 cells instead of SF21 because of higher expression levels. We compared the abilities of these two enzymes to bind the substrate JH III. Bovine serum albumin (BSA) was also incubated with radiolabeled JH III as a control. As shown in Fig. 3, both sEH proteins were radiolabeled. However, after correcting for the radioactivity in the BSA pellet due to nonspecific binding, we found that the amount of JH III bound to the Asp-333 → Asn mutant was only 6% of the JH III that bound to the wild-type sEH. We also compared the specific activity of these two enzymes, the mutant having only 8% of the specific activity of the wild-type: 186 ± 33 and 2300 ± 160 nmol/min/mg for mutant and wild-type, respectively. These results suggest that the specific activity of the enzyme correlates with its ability to covalently bind the substrate.

The Asp-333 → Ser mutant had no activity, whereas the Asp-333 → Asn mutant had 8% or up to 20% wild-type activity when produced in Tn5B1–4 or in SF-21 cells, respectively. A possible explanation for this observation is that the Asn-333 in the mutant is being intramolecularly hydrolyzed back to the Asp-333 by activated H_2O. To test this latter hypothesis, we incubated the wild-type and the Asp-333 → Asn mutant proteins at 37 °C at pH 8. After different times of preincubation, their specific activities were measured. The mutant recovered activity as a function of preincubation time (Fig. 4); after 48 h, the recovery of activity was complete compared to the wild-type. In addition to recovering catalytic activity, the mutant

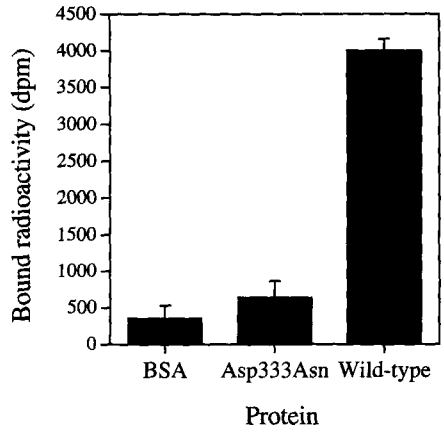

FIG. 3. **Labeling of wild-type and Asp-333 → Asn mutant sEHs by the radiolabeled substrate JH III.** The proteins (10 μg) were incubated with the radiolabeled JH III. After 3 s of incubation, the protein was precipitated. The pellets were washed, dissolved in 5% SDS (w/v), and transferred to counting vials in order to monitor radioactivity bound to the protein. The same protocol was followed using BSA as a control. Each value is the mean of two independent experiments performed in triplicate. Assuming one catalytic site per monomeric subunit (62,527 Da), this procedure resulted in the complete labeling of the protein molecules by substrate.

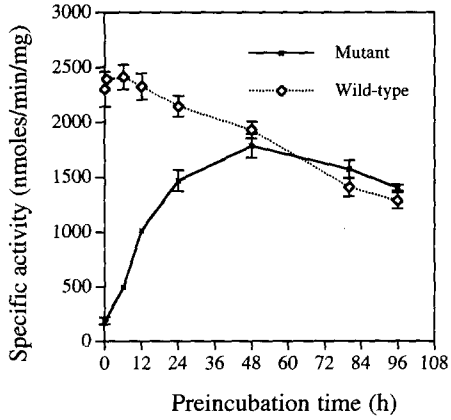

FIG. 4. **Effect of preincubating the wild-type and the Asp-333 → Asn mutant sEHs on the specific activity.** Purified enzymes were preincubated at 37 °C in 100 mM Tris buffer (pH 8) containing bovine serum albumin (1 mg/ml) and EDTA (3.3 mM). Enzyme concentrations in the preincubation were 16.5 and 38.7 μg/ml for wild-type and mutant, respectively. All values are the mean of triplicates with bars representing the standard deviation. Bars do not appear when standard deviations are too small.

was able to covalently bind JH III at the same level as the wild-type (not shown). Furthermore, analytical IEF demonstrated that the pI of the mutant was altered from a major band at 5.48 and a minor band at 5.44 before preincubation to a major 5.44 band after preincubation, which is the pI that was determined for the wild-type. IEF gels were also cut into slices, proteins were eluted by incubation overnight in buffer and the supernatant assayed for activity. Before preincubation of the Asp-333 → Asn mutant, the major 5.48 band had no catalytic activity, while the 5.44 band was catalytically active. After preincubation the 5.44 band had catalytic activity indistinguishable from the wild-type enzyme.

Labeling and Tryptic Digest of the Wild Type—In order to localize the binding site of JH III, we digested the radiolabeled enzyme with trypsin and isolated radiolabeled peptides. Wild-type enzyme (100 μg) was incubated with 100 μM of radiolabeled JH III, then precipitated and digested with trypsin (see "Materials and Methods"). The tryptic digest was resolved by RP-HPLC (Fig. 5*A*). The elution profile shown in Fig. 5*B* was

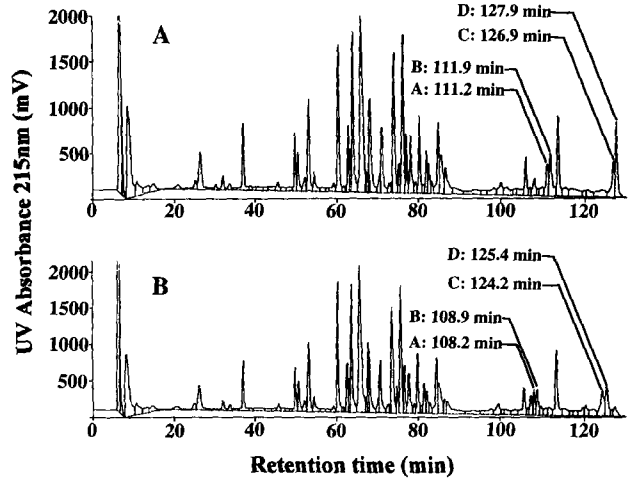

FIG. 5. **RP-HPLC elution profiles of peptides generated by tryptic digest of the murine wild-type sEH.** *A*, enzyme (100 μg) was incubated with radiolabeled JH III, then precipitated and digested with trypsin. Fragments were eluted with a gradient as follows: 100% A from 0 to 2 min, increased to 75% of B over 110 min, an additional increase to 100% B over 3 min, following by 15 min at 100% B (A and B were H_2O/trifluoroacetic acid (99.94:0.06, v/v) and H_2O/acetonitrile/trifluoroacetic acid (19.95:80:0.05, v/v/v), respectively). The flow rate was 0.5 ml/min. The same procedure was used to obtain profile (*B*), but the enzyme was preincubated with 4-FCO before addition of the substrate. *A*, *B*, *C*, and *D* are the peptides radiolabeled in presence of JH III only (*A*) or non radiolabeled in the presence of 4-FCO and JH III (*B*). Assuming one catalytic site per monomeric subunit (62,527 Da), 45% of the original protein was recovered labeled following the above procedure.

obtained by following the same procedure, but prior to incubation with JH III, the enzyme was incubated with 100 μM selective competitive inhibitor 4-FCO in order to inhibit the interaction of JH III with the protein. As shown in Fig. 6, when the enzyme was incubated with JH III and not protected by the 4-FCO, monitoring of radioactivity revealed two peaks. The first peak corresponds to peptides A (retention time = 111.2 min) and B (retention time =111.9 min), and the second peak corresponds to peptides C (retention time = 126.9 min) and D (retention time = 127.9 min) (Fig. 5*A*). Only two radioactive peaks were obtained because 500-μl fractions were collected for counting. Under these conditions, peptides A and B were not completely separated, and both of them are in the same radioactive peak between 110 and 114 min (Fig. 6). Similarly, peptides C and D also form only one radioactive peak between 126 and 128 min (Fig. 6). Incubation of the enzyme with 4-FCO, prior to labeling with JH III led to a shift of the retention time of the four peptides (108.2, 108.9, 124.2, and 125.4 min for A, B, C, and D, respectively) (Fig. 5*B*). This was the result of a decrease in hydrophobicity, because the hydrophobic substrate JH III did not bind the enzyme. Monitoring of the radioactivity revealed that these peptides were not radiolabeled. We noticed a third radioactive peak just above background at 80 min. However, incubation with 4-FCO did not alter the amount of radioactivity in this peak. Interestingly, the authentic diol of JH III has a retention time of 80 min in these conditions (data not shown). This third peak is probably due to the presence of JH III diol resulting from hydrolysis, in the acidic HPLC solvent, of trace amounts of JH III nonspecifically trapped in the precipitated protein.

Characterization of the Radiolabeled Peptides—The four radiolabeled peptides were collected in order to determine their location within the sEH protein. To insure purity, only the central portion of each UV dense peak was collected. Each of the four peaks showed high radioactivity. Each peptide was subjected to automated Edman degradation and electrospray

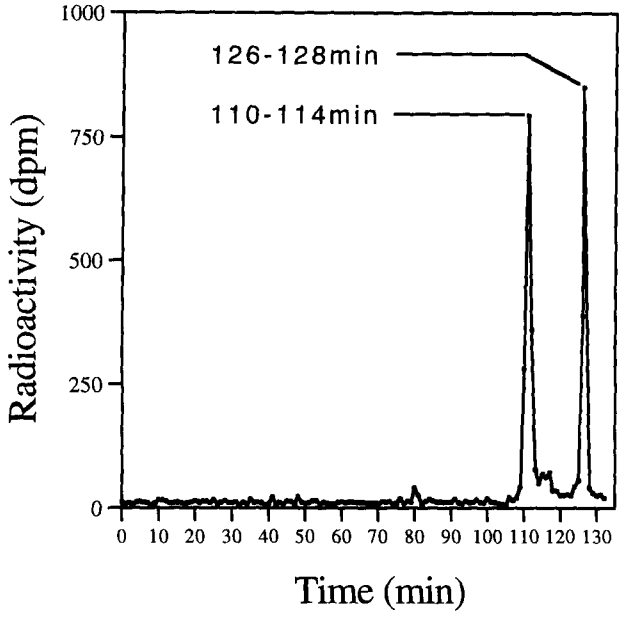

FIG. 6. **Radiochromatogram of the tryptic digest of the murine wild-type sEH incubated with JH III.** Fractions (500 μl) were collected from the RP-HPLC analysis of Fig. 5*A*. Aliquot (150 μl) of each fraction was transferred to a scintillation vial. All counts were corrected for differential quenching.

mass spectrometry to determine its amino acid sequence and molecular mass (*m*). Results of these experiments are shown in Table II. Comparison of the peptide sequences with the known sequence of the protein (16) reveals identity of all four sequences with one region of the protein. Two of these peptides, A and B, have the same N-terminal amino acid sequence, which results from a cleavage by trypsin after Lys-313. The other two peptides, C and D, also share an N-terminal amino acid sequence resulting from cleavage after Lys-292. The C terminus of each peptide was defined using the *m* determined by electrospray mass spectrometry. The *m* of peptide A is 4703 Da, which corresponds to the fragment Glu-314–Arg-351 (expected *m* 4704 Da). Peptide B has a *m* of 4449 Da, which is identical to that expected for Glu-314–Arg-349. The expected *m* of the fragment Gly-293–Arg-351 is 7005 Da, the same value as the *m* experimentally determined for peptide C. Finally, the *m* of peptide D (6750 Da) and the calculated *m* of the fragment Gly-293–Arg-349 (6746 Da) are also very similar. Therefore, these peptides are the result of a partial tryptic digest, which cleaves at four sites within this region of the protein (Fig. 7). Furthermore, the mass of each peptide is consistent with the covalent linkage to the JH III.

DISCUSSION

The purpose of this study was to examine the mechanism of sEH catalysis and, in particular, to identify catalytic amino acids. Based on sequence homology, epoxide hydrolases have been classified as members of the α/β hydrolase fold family (20–22). A common feature of the members of this family is the presence of a catalytic triad consisting of a nucleophile, a histidine and an acid (24). Recently, x-ray crystallography revealed that catalysis by HLD1, which belongs to this family, proceeds by a two-step mechanism. The substrate is covalently bound to the enzyme via an ester bond with Asp-124, then this ester bond is hydrolyzed by a molecule of water activated by the pair His-289–Asp-260 (25). We tested the hypothesis that a similar mechanism exists for sEH. Using site-directed mutagenesis and recombinant enzymes, we investigated the possible involvement of conserved residues in the catalytic mechanism of murine sEH. Of the nine histidine residues present in the protein, four are unlikely to be involved in the catalysis since they are either not conserved (position 204 and 419) (22) or are predicted to be in a strong α helix (position 146) or in a strong β strand (position 249).[2] Furthermore, the decrease of activity after mutation of His-516 of the recombinant rat sEH was not consistent with the involvement of this amino acid in the catalysis.[3] Therefore, we focused our attention on the histidine residues in positions 237, 263, 332, and 523. All of these residues are good candidates for direct involvement in the catalytic event. Indeed, the fact that they are all next to a glycine may increase their accessibility. The His-263 → Asn mutant retained 52% of wild-type activity, suggesting that His-263 is not involved in catalysis. However, this residue is conserved in all EHs (22), and its mutation led to a significant loss of specific activity. This suggests that His-263 may be important for other properties of the protein (*i.e.* conformation). Since mutation of His-523 led to the most dramatic reduction in catalytic activity, our results suggest that it is the general base involved in water activation. We will test the hypothesis that His-332 is involved in maintaining an active conformation orienting the active water or in activation of the epoxide moiety. The loss of activity of the His-332 mutant may be the result of a conformational change in the vicinity of Asp-333 due to the replacement of the histidine by an asparagine. Furthermore, this His-332 is not conserved in all the different EHs (22). On the other hand, His-523 is absolutely conserved in all EHs (22). This latter residue is, therefore, a more likely candidate for participation in the activation of H_2O in the EH catalytic mechanism. We are in the process of confirming this hypothesis by modifying the enzyme with diethylpyrocarbonate, which is selective for histidine residues.

We recently used JH III, which is a substrate with a high affinity and a low turnover, to isolate a hydroxyacyl enzyme intermediate of the murine sEH (26). Asp-124 is the residue that binds to the substrate during catalysis by HLD1 (25). It corresponds to Asp-333 of the murine sEH and is conserved in all EHs. Two mutants of Asp-333 were produced. Replacement of this residue by a serine completely abolished activity. This is in agreement with the involvement of Asp-333 in the catalysis. However, the Asp-333 → Asn mutant had 20% of wild-type activity when purified from SF21 cells and 8% of wild-type activity when purified from Tn5B1–4. Interestingly, this latter mutant bound 6% of the JH III that the wild-type bound. Deamidation of asparagine residues to aspartic acid residues has been shown in many cases (34). Such an event could explain the low but measurable activity of the Asp-333 → Asn and the fact that it still binds the substrate. Different processing of the expressed protein in the two cells lines might explain the different levels of activity resulting from different rates of deamidation. Preincubation of the Asp-333 → Asn mutant at 37 °C (pH 8) led to a complete recovery of enzyme activity and the ability to bind covalently to JH III. Furthermore, preincubation changed the pI of the mutant from 5.48 to 5.44, which is the pI of the wild-type enzyme. This is consistent with the results expected if an asparagine is altered to an aspartic acid. Interestingly, the mechanism of deamidation may occur via a mechanism similar to the one that has been shown for catalysis by HLD1. Indeed, it is possible to envisage a mechanism where histidine activates a molecule of water, which will then attack the amide and hydrolyze it to aspartic acid.

The fact that we were able to purify the Asp-333 → Asn mutant by affinity chromatography implies that the mutation

[2] B. Haymore, personal communication.
[3] M. Arand, personal communication.

TABLE II

N-terminal sequence and molecular mass of the radiolabeled peptides from the tryptic digest of the murine wild-type sEH

Radiolabeled peptides generated from the tryptic digest of the labeled murine sEH were purified by RP-HPLC. They were sequenced by automated Edman degradation, and their molecular mass values were determined by electrospray mass spectrometry. Xaa, not determined. Values in parentheses are the theoretical molecular mass. The molecular mass (measured and expected) include the JH III, which is bound to the peptides. The expected sequences are from the sequence of the cloned enzyme.

Peptide	Partial sequence	Molecular mass
		Da
A	H_2N-Xaa-Xaa-Xaa-Thr-Xaa-Leu-Asp-Lys-Leu-Gly-Ile-Pro	4704 (4703)
B	H_2N-Xaa-Met-Val-Thr-Phe-Leu-Asp-Lys-Leu-Gly-Ile-Pro	4449 (4449)
Expected A and B	H_2N-Glu-Met-Val-Thr-Phe-Leu-Asp-Lys-Leu-Gly-Ile-Pro 314	
C	H_2N-Xaa-Tyr-Gly-Xaa-Ser-Ser-Ala-Pro-Pro-Glu-Ile	7005 (7005)
D	H_2N-Xaa-Tyr-Xaa-Xaa-Ser-Xaa-Xaa-Pro-Pro-Glu-Ile-Xaa-Xaa-Tyr-Ala-Met	6750 (6746)
Expected C and D	H_2N-Gly-Tyr-Gly-Asp-Ser-Ser-Ser-Pro-Pro-Glu-Ile-Glu-Glu-Tyr-Ala-Met 293	

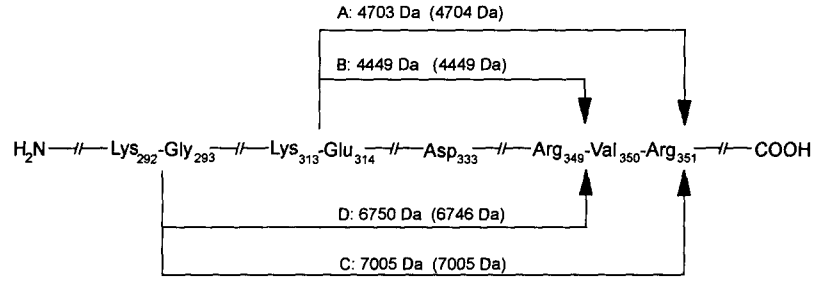

FIG. 7. **Trypsin cleavage sites in the region of Asp-333.** *A*, *B*, *C*, and *D* are the peptides resulting from the tryptic digest at the different sites. The experimentally determined molecular mass of each peptide is indicated and followed in parentheses by the expected molecular mass.

did not alter the integrity of the protein. Our affinity chromatography system is based on the hypothetical binding of the immobilized ligand to a hydrophobic pocket near the catalytic site but does not involve the catalytic amino acids. Therefore, it is unlikely that the loss of activity is the result of a change in conformation. These data also suggest that the Asp-333 → Asn mutation blocks catalysis without totally preventing substrate binding.

We isolated and characterized four peptides from the wild-type enzyme. These four peptides are the result of a tryptic digest at different sites. They overlap and cover the same region of the protein. These peptides contain the residue involved in the ester bond with the substrate since they were each radiolabeled when the enzyme was incubated with substrate. Furthermore, the binding of the substrate and the radiolabeling of the peptides was inhibited in the presence of the specific inhibitor 4-FCO. The presence of Asp-333 in all of them is further indication of its participation in the catalysis. The other aspartic acid residue present in all four peptides (Asp-320) is only conserved in the human sEH (22).

In conclusion, our results are consistent with the hypothesis that catalysis by sEH occurs via a similar mechanism to catalysis by HLD1, where the substrate and enzyme are covalently bound. Interestingly, it has been shown recently, that the irreversible inhibition of human immunodeficiency virus type 2 protease by 1,2-epoxy-3-(*p*-nitrophenoxy)propane occurs via a covalent binding between the protein and the inhibitor. This bond involves an aspartic acid from the protein and the epoxyde moiety of the inhibitor (35). We also now have strong evidence for the involvement of the Asp-333–His-523 pair in catalysis by sEH. This pair corresponds to the pair Asp-124–His-289, which has been shown to be catalytic in HLD1. Moreover, a similar His–Asp pair of residues is conserved in all the enzymes belonging to the α/β hydrolase fold family (22). Consequently, if it is possible to generalize this mechanism to other members of the family, it could provide a useful tool for radiolabeling enzymes for purification. Our results might be more important in elucidating the physiological role of EHs. Despite the fact that these enzymes have been well characterized (8), their functions remain unclear. An understanding of the mechanism of catalysis allows us to design more effective substrates and/or inhibitors, which will help assess the role of these enzymes *in vivo*.

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REFERENCES

1. Oesch, F. (1973) *Xenobiotica* **3,** 305–340
2. Guenthner, T. M., and Oesch, F. (1981) in *Polycyclic Hydrocarbon and Cancer* (Gelboin, H. V., and Tso, P. P., eds) pp. 183–212, Academic Press, New York
3. Guengerich, F. P. (1982) *Rev. Biochem. Toxicol.* **4,** 5–30
4. Seidegard, J., and DePierre, J. W. (1983) *Biochim. Biophys. Acta* **695,** 251–270
5. Lake, B. G., Kozlen, S., Evans, J. G., Gray, T. B., Young, P. J., and Gangolli, S. D. (1987) *Toxicology* **44,** 213–228
6. Meijer, J., and DePierre, J. W. (1987) *Chem.-Biol. Int.* **62,** 249–269
7. Meijer, J., and DePierre, J. W. (1988) *Chem.-Biol. Int.* **64,** 207–249
8. Wixtrom, R. N., and Hammock, B. D. (1985) in *Biochemical Pharmacology and Toxicology* (Zakim, D., and Vessey, D. A., eds) Vol. 1, pp. 1–93, John Wiley & Sons, New York
9. Halarnkar, P. P., Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1989) *Arch. Biochem. Biophys.* **272,** 226–236
10. Halarnkar, P. P., Nourooz-Zadeh, J. T., Kuwano, E., Jones, A. D., and Hammock, B. D. (1992) *Arch. Biochem. Biophys.* **294,** 586–593
11. Nourooz-Zadeh, J. T., Uematsu, B., Borhan, B., Kurth, M. J., and Hammock, B. D. (1992) *Arch. Biochem. Biophys.* **294,** 675–685
12. Falany, C. N., McQuiddy, P., and Kasper, C. B. (1987) *J. Biol. Chem.* **262,** 5924–5930
13. Skoda, R. C., Demierre, A., McBride, O. W., Gonzalez, F. J., and Meyer U. A. (1988) *J. Biol. Chem.* **263,** 1549–1554
14. Hassett, C., Turnblom, S. M., DeAngeles, A., and Omiecinski, C. J. (1989) *Arch. Biochem. Biophys.* **271,** 380–389
15. Knehr, M., Thomas, H., Arand, M., Gebel, T., Zeller, H.-D., and Oesch, F. (1993) *J. Biol. Chem.* **268,** 17623–17627
16. Grant, D. F., Storms, D. H., and Hammock, B. D. (1993) *J. Biol. Chem.* **268,** 17628–17633
17. Beetham, J. K., Tian, T., and Hammock, B. D. (1993) *Arch. Biochem. Biophys.* **305,** 197–201
18. Stapleton, A., Beetham, J. K., Pinot, F., Garbarino, J. E., Rockhold, D. E., Hammock, B. D., and Belknap, W. R. (1994) *Plant J.* **6,** 251–258
19. Kiyosue, T., Beetham, J. K., Pinot, F., Hammock, B. D., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994) *Plant J.* **6,** 259–269
20. Lacourciere, G. M., and Armstrong, R. N. (1993) *J. Am. Chem. Soc.* **115,** 10466–10467
21. Arand, M., Grant, D. F., Beetham, J. K., Friedberg, T., Oesch, F., and Hammock, B. D. (1994) *FEBS Lett.* **338,** 251–256
22. Beetham, J. K., Grant, D. F., Arand, M., Garbarino, J. E., Kiyosue, T., Pinot, F., Oesch, F., Belknap, W. R., Shinozaki, K., and Hammock, B. D. (1995) *DNA Cell Biol.* **14,** 61–71
23. Janssen, D. B., Pries, F., van der Ploeg, J., Kazemier, B., Tersptra P., and Withold, B. (1989) *J. Bacteriol.* **171,** 6791–6799
24. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) *Protein Eng.* **5,** 197–211

25. Verschueren, K. H. G., Seljee, F., Rozeboom, H., Kalk, K. H., and Dijkstra, B. W. (1993) *Nature* **363,** 693–698
26. Hammock, B. D., Pinot, F., Beetham, J. K., Grant, D. F., Arand, M. E., and Oesch, F. (1994) *Biochem. Biophys. Res. Commun.* **198,** 850–856
27. Mumby, S. M., and Hammock, B. D. (1979) *Pest. Biochem. Physiol.* **11,** 275–284
28. Mullin, C. A., and Hammock B. D. (1982) *Arch. Biochem. Biophys.* **216,** 423–439
29. Wixtrom, R. N., Silva, M. H., and Hammock, B. D. (1988) *Anal. Biochem.* **169,** 71–80
30. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
31. Laemmi, U. K. (1970) *Nature* **227,** 680–685
32. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203
33. Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem.* **136,** 175–179
34. Wright, T. H. (1991) *Crit. Rev. Biochem. Mol. Biol.* **26,** 1–52
35. Salto, R., Babé, L. M., Li, J., Rosé, J. R., Yu, Z., Burlingame, A., De Voss, J. J., Sui, Z., Ortiz de Montellano, P., and Craik, C. S. (1994) *J. Biol. Chem.* **269,** 10691–10698